GENETIC AND BIOLOGICAL DIVERSITY IN TEL-AML1 POSITIVE ALL

ISBN 90 5677 016 0

The studies described in this thesis were financially supported by grants from the Sophia Foundation for Medical Research (SSWO grant 309) and the Pediatric Oncology Foundation Rotterdam, The Netherlands.

Financial support from the Pediatric Oncology Foundation Rotterdam and Pharmachemie BV is gratefully acknowledged.

Layout: Optima Grafische Communicatie, Rotterdam (www.ogc.nl)
Cover: Hidde Renzen
Printed by Optima Grafische Communicatie, Rotterdam

GENETIC AND BIOLOGICAL DIVERSITY IN TEL-AML1 POSITIVE ALL

Genetische en biologische variatie in TEL-AML1 positieve ALL

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op 4 november 2005 om 11.00 uur

door

Wendy Antoinetta Gerarda Stams

geboren te Oisterwijk

PROMOTIECOMMISSIE

Promotor:	Prof.dr. R. Pieters
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Een voorwoord . . . . . .

Ik kijk naast me
Daar zit hij, onze Hidde
Een gezond ogende krullenbol
Met grote ondeugende ogen

Zijn evenwicht is soms wat wankel
Fietsen zonder zijwielen moet nog even wachten
Tekenen is bijna een straf
En kralen rijgen is ook geen favoriet

Maar hij is de beste in 'Pro Evolution Soccer 4'
(een voetbalspel op de playstation)
En ook op de computer is hij een ster

Ach ja . . . . . .

Je houdt er wat van over
Van 109 weken chemo
En wij ook van 34 weken dexa

Maar alles (nou ja, bijna alles) is geoorloofd voor dat ene doel
BETER WORDEN !

We hebben de eerste stop gehaald
Het gaat nu goed
Wij genieten nu

Anne Marie Renzen – Kuijs

Ps. Hidde kreeg in september 2002 ALL, hij was toen 2 jaar en 10 maanden. Sinds november 2004 is hij klaar met zijn behandeling. Hidde is de jongste van 4 jongens. Het gaat nu goed met hem.

CONTENTS

Chapter 1

General introduction

Bone marrow is the soft tissue within the bones where hematopoietic stem cells reside. Hematopoietic stem cells are immature blood cells within the bone marrow, which continuously divide to form new cells. Some of the new cells remain unchanged as stem cells and have a lifelong capacity for self-renewal. These pluripotent stem cells give rise to progenitor stem cells (also called lineage-committed stem cells) that have a limited capacity for self-renewal. Progenitor stem cells are committed to form only one lineage of blood cells: oxygen-carrying red blood cells (erythrocytes), infection-fighting white blood cells (leukocytes), or blood-clotting cells (platelets or thrombocytes). The bone marrow is made up of blood cells at different stages of maturation. When cells further differentiate, they are released from the bone marrow into the peripheral blood circulation. The process of proliferation and differentiation is regulated by cellular interaction, the micro-environment of the bone marrow, several regulatory glycoproteins and hematopoietic growth factors. Normally, blood cells are produced in an orderly, controlled way as the body needs them.

Leukemia, which literally means "white blood" in Greek, is a form of cancer that begins in the progenitor cells of the bone marrow. It is characterized by the accumulation of immature, abnormal white blood cells in blood and bone marrow that cannot perform their usual function of fighting against infections. When the leukemic cells begin to fill the marrow, production of normal platelets, red and white cells decrease. The decrease of normal hematopoietic cells co-incides with the appearance of clinical symptoms. Low red cell counts (anemia) cause fatigue and pale skin. Low platelet counts (thrombocytopenia) may result in bleeding and bruising. If mature white cells are reduced, the body has little or no defence against infectious threats by bacteria and viruses. Some other symptoms are malaise, fever, bone aches, anorexia, abdominal discomfort (due to hepatosplenomegaly), and headaches. According to the clinical presentation, leukemias are divided in acute and chronic leukemias. Leukemia can arise in either of the two main types of white blood cells: lymphoid cells or myeloid cells. When leukemia affects lymphoid cells, it is called lymphocytic or lymphoblastic leukemia. When myeloid cells are affected, the disease is called myeloid or myelogenous leukemia.

1.1 ACUTE LYMPHOBLASTIC LEUKEMIA

In children, leukemia accounts for one third of all cancer cases. About 80% is acute lymphoblastic (ALL), 15-20% is acute myeloid leukemia (AML) and the remaining cases are juvenile chronic myeloid leukemia and related leukemia types. The peak incidence of childhood leukemia is between 2 and 6 years of age. In adults, haematological

malignancies are greatly outnumbered by solid tumors and represent only about 2% of all malignancies.

Molecular (cytogenetic) analysis of the common genetic abnormalities in leukemic cells has contributed to our understanding of the pathogenesis and prognosis of ALL in children[1,2]. Although the frequency of genetic subtypes differs between children and adults (Figure 1), the general mechanisms underlying the induction of ALL are

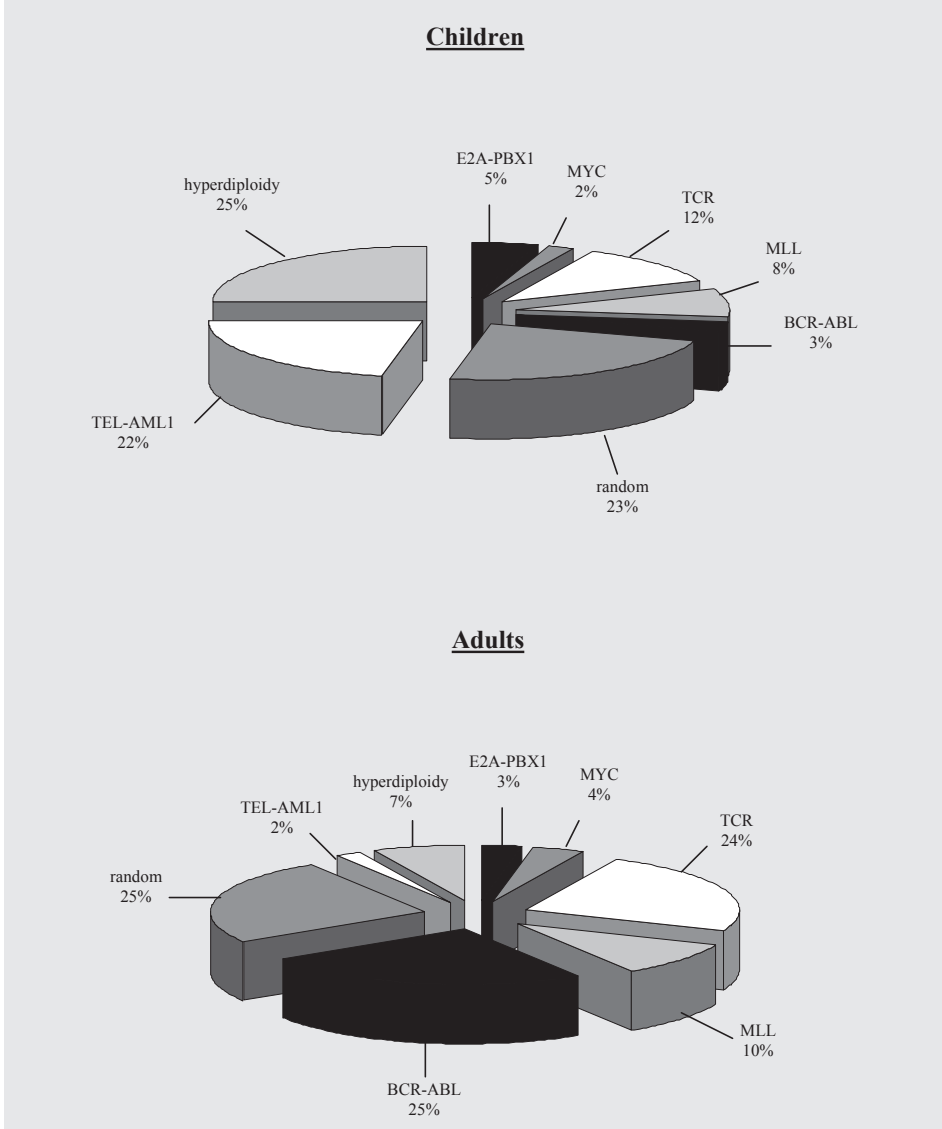

Figure 1. Estimated frequencies of specific genotypes of ALL in children and adults[1].
The TCR rearrangements are found in T-lineage ALL. All other rearrangements account for B-lineage ALL.

presumably similar. In both adults and children, translocations of genes are found that cause aberrant expression of proto-oncogenes and create fusion genes that encode active kinases and altered transcription factors. These genetic alterations contribute to the leukemic transformation of hematopoietic stem cells or their committed progenitors by changing cellular functions. They alter important regulatory processes by maintaining or enhancing an unlimited capacity for self-renewal, abolishing the control of normal proliferation, blocking differentiation, and promoting resistance to death signals (apoptosis).

1.2 t(12;21) POSITIVE ALL

The most common chromosomal translocation observed in pediatric ALL is the t(12;21)(p13;q22) translocation restricted to precursor B-cell leukemia. This translocation is found in approximately 25% of precursor B-ALL[1,3]. The t(12;21) involves fusion of the *TEL* (also named *ETV6*) gene at chromosome 12p13 with the *AML1* (also named *CBFA2* or *RUNX1*) gene at chromosome 21q22 (Figure 2). This translocation fuses the 5' terminus of the *TEL* gene (residues 1-336) in frame with almost the entire coding sequence (residues 21-480) of the *AML1* gene. The breakpoints occur in intron 5 of *TEL* and intron 1 of *AML1*. Another frequent translocation variant results in fusion between intron 5 of *TEL* and intron 2 of *AML1*. The t(12;21) results in the chimeric fusion gene *TEL-AML1* on the der(21)t(12;21) and its reciprocal fusion gene *AML1-TEL* on the der(12)t(12;21). Approximately 70% of the t(12;21) positive patients also show a deletion of the untranslocated *TEL* gene[4-9]. Trisomy 21 and duplication of the der(12)t(12;21) are also described in t(12;21) positive patients, and the latter appears to be more common in relapse cases[10-13]. However, the incidence and possible prognos-

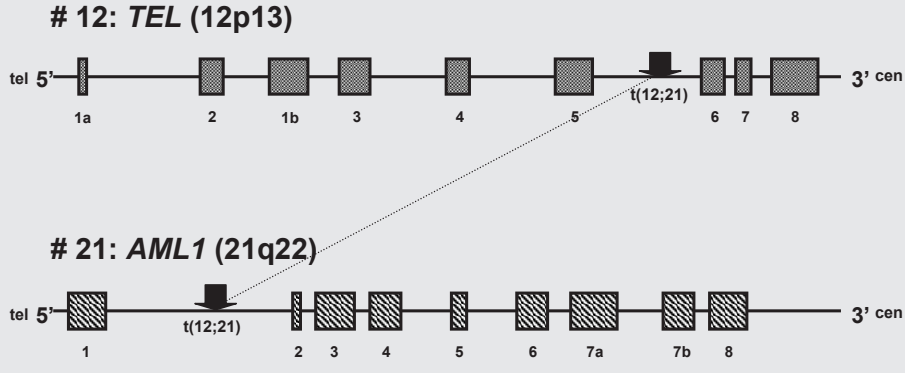

Figure 2. Overview of the breakpoints in the *TEL* and *AML1* gene involved in t(12;21) positive ALL

tic changes of such genetic variation was unknown and led to our study described in chapter 2.

The t(12;21) is most frequent in children between 1 and 12 years of age, with a peak incidence between 2 and 5 years and is absent in infants (children below 1 year of age)[14,15]. In adult ALL the incidence of t(12;21) is < 3%[16-23], and the majority of adults with the t(12;21) are under 20 years of age. This indicates that t(12;21) positive ALL is mainly restricted to pediatric precursor B-ALL.

1.2.1 Etiology of t(12;21) positive ALL

Like several other subgroups of pediatric ALL, t(12;21) positive ALL is initiated in utero[24]. Studies in mouse models showed however that the presence of *TEL-AML1* by itself is insufficient for leukemogenesis[25], but the fusion gene may result in leukemia when additional mutations are present[26]. In concordance with these observations, a variable and protracted occurrence of t(12;21) positive ALL in twin studies indicated that additional postnatal events are necessary for full malignant transformation[27-31]. Since the second non-translocated *TEL* allele is often deleted in t(12;21) positive ALL patients (up to 70%), this deletion is generally considered as the second hit in leukemogenesis[4-9,20,29,32-34].

DNA rearrangements of the immunoglobulin heavy chain (IgH) and T-cell receptor (TCR) genes are markers for the differentiation stage of leukemic cells and are considered clonotypic markers in precursor B-ALL cells[35]. This process is closely connected to the cell cycle[36]. Analysis of IgH and TCR molecular markers illuminates the order of molecular pathogenetic events during leukemogenesis. Several studies have shown that the t(12;21) occurs in an immature B-cell prior to rearrangements of the IgH and TCR loci and a final transformation event (second hit) occurs after these rearrangements[37-40]. In B-cells, IgH is rearranged first, joining a D_H to a J_H segment on both alleles, followed by a V_H to DJ_H rearrangement on one of the two alleles. TEL-AML1 may affect this somatic recombination of IgH and TCR by proliferation retardation thereby providing optimal conditions for V(D)J recombination[39]. This is shown by the fact that TEL-AML1 positive ALL cells have a higher number of IgH/TCR rearrangements with a more immature immunophenotype compared to TEL-AML1 negative ALL cells[39]. Thus, continuing rearrangement processes lead to a variety of immune receptor rearrangements in individual cells of the TEL-AML1 positive pre-leukemic clone, resulting in a heterogeneous population[39,41,42]. A secondary event presumably terminates this in utero initiated pre-leukemic phase and drives one of these cells to overt leukemia.

1.2.2 Prognosis of t(12;21) positive ALL

The success rate in the treatment of ALL has increased steadily since the 1960s. The 5-years event free survival (EFS) is about 80% for children with ALL[1]. Conflicting data on

the prognostic relevance of t(12;21) positive ALL have been reported ranging from 60% to 100% survival[43-47]. Initially, studies reported favourable outcome of t(12;21) positive ALL patients compared to t(12;21) negative ALL patients[43]. Other studies could not confirm this prognostic relevance including a study in Dutch DCOG-treated patients with t(12;21) positive ALL[34,48]. The outcome of the treatment of ALL patients is dependent on the pharmacokinetics, the cellular drug resistance and the outgrowth potential of leukemic cells that survive therapy. Cellular drug resistance can be measured at the patient level, i.e. the reduction in peripheral blood leukemic cells after systemic exposure to a drug (which also includes a pharmacokinetic component) and at the cellular level, i.e. after in vitro exposure to drugs using drug cytotoxicity assays (e.g. MTT-assay). In vitro drug resistance profiles are independently associated with treatment outcome, and treatment risk groups show specific resistance profiles[49]. The conflicting data in t(12;21) positive ALL may be explained by differences in the amount of L-Asparaginase that is being used in these treatment protocols as t(12;21) positive ALL is in vitro highly sensitive to L-Asparaginase[50,51] and L-Asparaginase sensitive patients have been shown to have a more favourable outcome[52]. Beside L-Asparaginase, t(12;21) positive ALL patients are also more sensitive in vitro to doxorubicin, etoposide and dexamethasone[53,54]. Therefore, the relative sensitivity to selected drugs may explain the favourable outcome observed in t(12;21) positive patients treated with relative intensive treatment protocols. In correspondence with this explanation are studies showing that the outcome of t(12;21) positive ALL patients improved with more intensive therapy[55-57].

1.3 GENES INVOLVED IN t(12;21) POSITIVE ALL

1.3.1 The *TEL* gene

The *TEL* gene (Figure 3) is located in the 12p13 chromosomal band and is a member of the ETS-domain transcription family[58-60]. ETS proteins control the expression of genes that are critical for a number of biological processes, including cellular proliferation, differentiation, development, transformation, and apoptosis[61]. Beside protein-DNA interactions, the ETS domain is also involved in protein-protein interactions. TEL also shares another conserved domain known as the pointed domain (PNT) with a large subgroup of ETS family members. The pointed domain has been shown to function in homo-oligomerization[62,63], heterodimerization[64], transcriptional repression[65] and nuclear export of TEL[66]. TEL belongs to the small class of ETS transcriptional repressors whose activity is controlled by mitogenic and/or cell cycle-dependent signals[67]. These ETS proteins are targets of the MAPK signal pathway[68]. The large central domain of TEL (encoded by exon 5) includes a repression domain[67,69]. An additional repression domain was identified which encompasses the ETS domain and the 50 amino-acids im-

mediately upstream of the ETS domain[67]. TEL-mediated repression is dependent upon the recruitment of co-repressors and histone deacetylases[70]. TEL is required for the homing of hematopoietic progenitor cells to the bone marrow[71] and interferes with the activity of the FLI-1 oncoprotein[72], the matrix metalloproteinase 3 gene Stromelysin-1[73] and interferes with the apoptotic pathway through the anti-apoptotic BCL-2 family member Bcl-X_L[74].

Chromosomal rearrangements that disrupt *TEL* lead to a variety of myeloid and lymphoid leukemias[75] and solid tumors, and the proteins fused to TEL can be divided into two broad categories: protein tyrosine kinases and transcription factors[76]. Translocations of the *TEL* gene to protein tyrosine kinases occurs for example in t(5;12) (TEL-PDGFRβ) found in chronic myelomonocytic leukaemia[77,78], in t(9;12)(q34;p13) (TEL-ABL) found in AML, atypical chronic myeloid leukaemia (CML) and ALL[78,79], and in t(9;12)(p24;p13) (TEL-JAK2) found in ALL and CML[62,80]. In these cases, aberrant regulation of the kinase activity of the fusion proteins contributes to leukemogenesis. Fusions of TEL with transcription factors can either retain or eliminate the DNA bind-

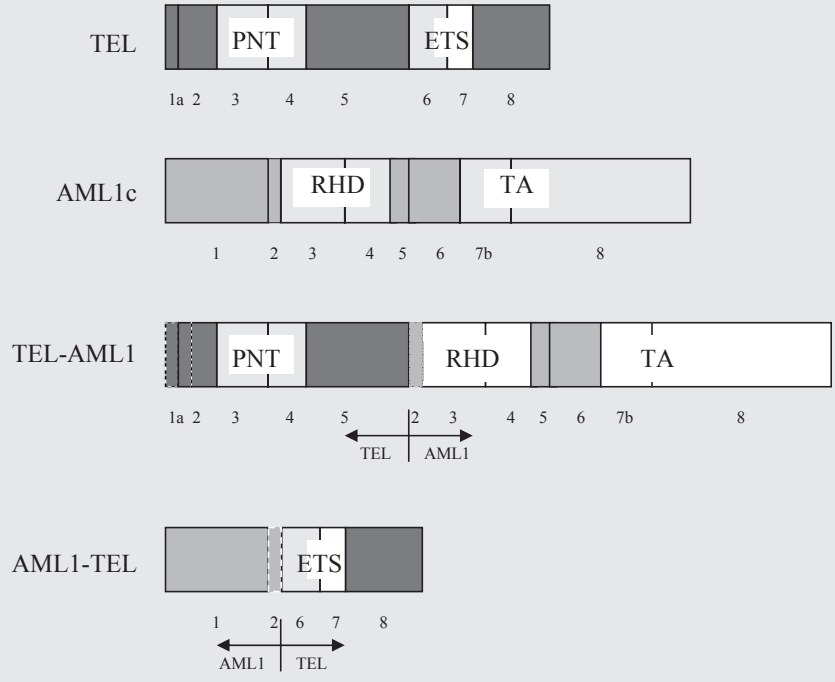

Figure 3. The TEL, AML1, TEL-AML1 and AML1-TEL fusion products with their functional domains.
PNT: pointed domain, dimerisation domain
ETS: protein-DNA or protein-protein interaction domain
RHD: runt homology domain, DNA-binding domain and dimerisation site for CBFβ subunit
TA: transactivation domain, transcriptional activation

ing activity of TEL. The t(12;22) (MN1-TEL) in myeloproliferative disorders retains the ETS domain and converts TEL from a repressor to an activator[81,82]. The t(12;21) (TEL-AML1) in ALL[14] looses the ETS domain and functions as a constitutive inhibitor of transcription of AML1-dependent gene targets[83]. In the majority of t(12;21) positive ALL, the second *TEL* gene is deleted[4-9,20,29,32,33]. Loss of heterozygosity of the *TEL* gene is also observed in other leukemias and some solid tumors[84-87], suggesting that *TEL* is a tumor suppressor gene.

TEL is an inhibitor of growth in a number of transformed cell types including Ras-transformed NIH 3T3 cells. TEL expression increases the level of apoptosis (via Bcl-X_L), rather than affecting cell cycle progression. In case of translocation of the *TEL* gene to a protein tyrosine kinase, loss of one copy of the *TEL* gene may facilitate the tyrosine kinase-mediated activation of STAT signalling and contribute to activation of Bcl-X_L[74]. In contrast to tyrosine kinase fusion proteins, TEL-AML1 itself is not capable to induce transformation, and cells require additional genetic abnormalities to become leukemogenic[25]. In this respect, loss of the second *TEL* gene is regarded as the second hit in leukemogenesis, as mentioned above.

1.3.2 The *AML1* gene

The *AML1* gene is located in the 21q22 chromosomal band and encodes a transcription factor that binds the enhancer core sequence, TGTGGT[88]. AML1 and the core binding factor beta (CBFβ) together form the core binding factor (CBF)[89-91]. The AML1 protein contains a highly evolutionary conserved domain of 128 amino acids called runt homology domain (RHD), responsible for both heterodimerization with the CBFβ subunit and for DNA binding (Figure 3)[92-94]. Transcriptional activation occurs through the C-terminal transactivation (TA) domain. AML1 is normally expressed in all hematopoietic lineages and regulates the transcription of myeloid and lymphoid lineage-specific genes, including cell surface receptors such as subunits of the T-cell antigen receptor and macrophage colony-stimulating factor receptor; myeloid-associated enzymes such as myeloperoxidase, neutrophil elastase and granzyme B and also cytokines such as interleukin-3 and granulocyte macrophage colony-stimulating factor[95-101]. Mice lacking AML1 or CBFβ have no fetal liver hematopoiesis, showing that the heterodimeric complex CBF is essential for definitive hematopoiesis of all lineages[102-104]. CBF is thought to target the expression of members of the important family of *HOX* genes, [105,106]. The HOX transcription factors bind to DNA and regulate genes involved in differentiation of both the embryonal and hematopoietic stem cells. In addition, *HOX* genes are also important for the self-renewal and proliferative capacity of hematopoietic stem cells[105,107].

AML1 is one of the most frequently deregulated genes in leukaemia, mainly through translocations. Translocations of the *AML1* gene occur in different leukaemia subtypes,

leading to the formation of fusion genes encoding for chimerical proteins including AML1-ETO in t(8;21) positive AML, TEL-AML1 in t(12;21) positive childhood ALL and less often AML1-MDS1 in t(3;21) positive myelodysplastic syndrome and CML, or other rare translocations[20,108,109]. AML1 is also targeted indirectly by inv(16), which fuses CBFβ to the smooth muscle myosin heavy chain gene[110]. More recently, other mechanisms of inactivation of AML1 in haematological malignancies were reported such as point mutations in AML and myelodysplastic syndrome[111-116] and gene amplification in ALL[117-122].

AML1 has the unusual property that both the wild-type and the translocated genes can affect cellular proliferation and pathways of cellular differentiation. Overexpression of the AML1 fusion protein encoded by inv(16) acts as a dominant repressor that inhibits cell cycle progression[123], suggesting a role for AML1 in the G_1 phase of the cell cycle. In addition, several studies indicated that AML1 expression regulates G_1 to S phase transition of the cell cycle[123-128]. Furthermore, AML1 levels are upregulated during the cell cycle[129]. The binding of AML1 to the enhancer core sequence recruits other transcription factors and co-activators to this region, and the resulting protein complex regulates transcription. This complex includes histone acetylases, which add acetyl groups to DNA-bound histones, thereby causing conformational changes in chromatin that enhance the transcription of target genes. On the other hand, AML1 can also associate with mSin3 and groucho/TLE proteins, which recruit histone deacetylases that induce the closure of chromatin and, hence, results into inhibition of transcription[130]. Since AML1 plays a pivotal role in transcription of target genes that are needed for proliferation and differentiation of normal hematopoietic cells, abnormalities in this gene may contribute to leukemogenesis in different ways.

It is suggested that the simple inactivation of one AML1 allele (haploinsufficiency) is sufficient to induce predisposition to acute leukaemia in familial thrombocytopenia[116]. In acquired haematological malignancies, haploinsufficiency of AML1 may also have a pathogenic role and mono-allelic alterations of the AML1 gene are indeed found[121]. Furthermore, some of the mutations act in a dominant negative manner and prevent an effect of the remaining wildtype AML1 in case of the mono-allelic alteration[111,130]. The dominant negative effect of the chimeric products of t(8;21) or t(3;21) is due to the fact that the mutated protein did not bind DNA but had enhanced capacity to bind CBFβ and, by sequestering it, inhibited action of wildtype AML1[131]. The chimeric product of t(12;21), TEL-AML1, can compete with wildtype AML1 for DNA binding, thus acting as a transcriptional repressor of AML1 target genes[83]. It has enhanced capacity to bind CBFβ and as a result also an enhanced DNA binding capacity. Altogether these data suggest that loss of normal AML1 function has enormous consequences for the biological behaviour of the cell.

1.3.3 The *TEL-AML1* fusion gene

The *TEL-AML1* fusion product contains the pointed domain of *TEL* and the runt homology domain and transactivation domain of *AML1* (Figure 3). The biological function of TEL-AML1 in leukemia remains unclear. The fusion protein can form homodimers and heterodimers with TEL and converts AML1 from functioning as a transcriptional activator to a transcriptional repressor[83,132]. The pointed domain and the central region of TEL as well as the runt homology domain and amino acids 216-290 within AML1, appear critical in mediating repression via binding of nuclear receptors such as SMRT, mSin3A, N-CoR and HDAC-3[65,69,133-135] (as outlined before). Like AML1, the abnormal TEL-AML1 fusion protein can bind to the enhancer core sequence. However, instead of activating transcription, it recruits histone deacetylases thereby inducing closure of the chromatin structure and, hence, inhibition of transcription[1,83,132,136]. These changes in the normal AML1-mediated transcriptional cascade alter both the self-renewal capacity and the differentiation capacity of hematopoietic cells[137,138]. Recently, TEL-AML1 was also found to prevent TEL-induced transcriptional repression by heterodimerisation with TEL indicating that TEL-AML1 exerts dominant-interfering effects on both AML1 and TEL[139].

1.3.4 The *AML1-TEL* fusion gene

The *AML1-TEL* fusion product contains exon 1 or exon 1 and 2 of *AML1* lacking a known functional domain and the last three exons of *TEL* containing the ETS domain. The biological function of the *AML1-TEL* fusion gene in leukemia also remains unclear. It is hypothesized that ETS proteins with transcriptional repression activity (like TEL) are primarily involved in the balance between cellular proliferation and differentiation in response to extracellular signals[70]. The isolated ETS domain of TEL binds to artificial DNA containing conventional ETS binding sites and regulates the transcription of targeted genes[140-142]. It can be hypothesized that AML1-TEL acts like an isolated ETS domain and competes with normal TEL for binding to target genes or acts like TEL in the absence of wild-type TEL.

1.4 SCOPE OF THIS THESIS

The t(12;21) is the most common translocation in pediatric ALL. The group of ALL patients with a t(12;21) is very heterogeneous since additional genetic abnormalities in the *TEL* and *AML1* gene involved in this particular translocation (for example a deletion of the untranslocated *TEL* gene or an extra copy of the *AML1* gene) are frequently described. As a group, t(12;21) positive ALL patients are highly sensitive to L-Asparaginase and often associated with a favourable prognosis. Still, several studies could not associate t(12;21) positive ALL with a favourable prognosis and even described this translocation to be present in 25% of relapse cases (same percentage as at initial diagnosis). So, to improve the treatment of these patients, we need more background on the genetic and biological abnormalities in these t(12;21) positive ALL cells. Therefore, the aim of the research described in this thesis was to identify genetic and biological features responsible for outcome and response to drugs in t(12;21) positive ALL.

A. Clinical relevance of genetic abnormalities in t(12;21) positive ALL

Conflicting data on the prognostic relevance and proportion of t(12;21) positive cases at relapse have been described. The favourable prognosis of t(12;21) positive ALL patients seems to depend on the intensity of the treatment protocol. A possible contribution for this can be the fact that t(12;21) positive ALL patients are in vitro highly sensitive to L-Asparaginase. However, within this group of patients large interindividual differences in in vitro sensititivity to L-asparaginase and other drugs were found. So, both the conflicting data on the prognostic relevance of the t(12;21) as well as the large heterogeneity in in vitro sensitivity of the t(12;21) positive ALL group, indicates that additional genetic changes might be responsible for the differences in outcome and drug sensitivity. Therefore, in **chapter 2** we studied the incidence of additional genetic changes in the *TEL* and *AML1* genes in t(12;21) positive pediatric ALL and their relationship with in vitro drug sensitivity and clinical outcome. In addition in **chapter 3**, we analyzed whether the expression levels of *TEL, AML1* and the fusion products *TEL-AML1* and *AML1-TEL* are associated with in vitro drug sensitivity and long-term clinical outcome in t(12;21) positive ALL.

B. Causes of sensitivity and resistance to L-Asparaginase in t(12;21) positive ALL

L-Asparaginase is an enzyme widely used in chemotherapeutic protocols for children with ALL. *In vitro* resistance to L-Asparaginase is correlated with a relative poor prognosis *in vivo.* The proposed mechanism of action of L-Asparaginase is the depletion of asparagine and glutamine in the blood leading to cellular efflux and depletion of these amino acids within cells. ALL cells are thought to be particularly sensitive to L-Asparaginase treatment because of a relative low capacity to synthesize sufficient asparagine

due to intrinsic lower asparagine synthetase levels. Resistance to L-Asparaginase is suggested to be caused by an elevated cellular level of asparagine synthetase and/or by the ability of resistant cells to rapidly induce the expression of the asparagine synthetase gene upon L-Asparaginase exposure. In **chapter 4** the relation between asparagine synthetase and L-Asparaginase sensitivity in t(12;21) positive pediatric ALL was studied. Asparagine synthetase expression (like AML1 mentioned above) is associated with cell cycle regulation. In **chapter 5** we comment on the hypothesis that t(12;21) positive cells are unable to progress into the S phase of cell cycle under nutrition stress caused by L-Asparaginase, despite the ability of asparagine synthetase upregulation. In **chapter 6** we describe the relation between asparagine synthetase and L-Asparaginase resistance in t(12;21) negative ALL, which differs from t(12;21) positive ALL. Furthermore, to gain more insight into the heterogeneity in L-Asparaginase cytotoxicity in t(12;21) positive ALL patients, we studied the expression profile (by micro-array analysis) of genes differentially expressed in t(12;21) positive ALL patients sensitive and resistant to L-Asparaginasein **chapter 7**.

C. Specificity of targeted drugs for t(12;21) positive ALL

The fusion protein TEL-AML1 converts AML1 from functioning as a transcriptional activator to a transcriptional repressor. Like AML1, the abnormal TEL-AML1 fusion protein can bind to enhancer core sequences, but instead of activating transcription through recruitment of co-activators and histone acetylases, it recruits co-repressors and histone deacetylases (HDACs), which induce closure of the chromatin structure and, hence, inhibition of transcription. These changes in the normal AML1-mediated transcriptional cascade alter both the self-renewal capacity and the differentiation capacity of hematopoietic cells. TEL-AML1 therefore, seems a promising target for treatment with HDAC inhibitors to reverse its transcriptional repression. In **chapter 8** we studied the effect of histone deacetylase (HDAC) inhibitors on B-cell differentiation induction and on L-Asparaginase cytotoxicity in t(12;21) positive and negative ALL.

Finally, the work presented in this thesis is summarized and discussed in **chapter 9** and **10**.

REFERENCES

1. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. N Engl J Med. 2004;350:1535-1548.
2. Gilliland DG, Tallman MS. Focus on acute leukemias. Cancer Cell. 2002;1:417-420.
3. Sawinska M, Ladon D. Mechanism, detection and clinical significance of the reciprocal translocation t(12;21)(p12;q22) in the children suffering from acute lymphoblastic leukaemia. Leuk Res. 2004;28:35-42.
4. Raynaud S, Cave H, Baens M, et al. The 12;21 translocation involving TEL and deletion of the other TEL allele: two frequently associated alterations found in childhood acute lymphoblastic leukemia. Blood. 1996;87:2891-2899.
5. Cave H, Cacheux V, Raynaud S, et al. ETV6 is the target of chromosome 12p deletions in t(12;21) childhood acute lymphocytic leukemia. Leukemia. 1997;11:1459-1464.
6. Romana SP, Le Coniat M, Poirel H, Marynen P, Bernard O, Berger R. Deletion of the short arm of chromosome 12 is a secondary event in acute lymphoblastic leukemia with t(12;21). Leukemia. 1996;10:167-170.
7. Busson-Le Coniat M, Poirel H, Leblanc T, Bernard OA, Berger R. Loss of the TEL/ETV6 gene by a second translocation in ALL patients with t(12;21). Leuk Res. 1999;23:895-899.
8. Kempski HM, Sturt NT. The TEL-AML1 fusion accompanied by loss of the untranslocated TEL allele in B-precursor acute lymphoblastic leukaemia of childhood. Leuk Lymphoma. 2000;40:39-47.
9. Raimondi SC, Shurtleff SA, Downing JR, et al. 12p abnormalities and the TEL gene (ETV6) in childhood acute lymphoblastic leukemia. Blood. 1997;90:4559-4566.
10. Fears S, Vignon C, Bohlander SK, Smith S, Rowley JD, Nucifora G. Correlation between the ETV6/CBFA2 (TEL/AML1) fusion gene and karyotypic abnormalities in children with B-cell precursor acute lymphoblastic leukemia. Genes Chromosomes Cancer. 1996;17:127-135.
11. Kobayashi H, Satake N, Maseki N, Sakashita A, Kaneko Y. The der(21)t(12;21) chromosome is always formed in a 12;21 translocation associated with childhood acute lymphoblastic leukaemia. Br J Haematol. 1996;94:105-111.
12. Loncarevic IF, Roitzheim B, Ritterbach J, et al. Trisomy 21 is a recurrent secondary aberration in childhood acute lymphoblastic leukemia with TEL/AML1 gene fusion. Genes Chromosomes Cancer. 1999;24:272-277.
13. Ma SK, Wan TS, Cheuk AT, et al. Characterization of additional genetic events in childhood acute lymphoblastic leukemia with TEL/AML1 gene fusion: a molecular cytogenetics study. Leukemia. 2001;15:1442-1447.
14. Shurtleff SA, Buijs A, Behm FG, et al. TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis. Leukemia. 1995;9:1985-1989.
15. Borkhardt A, Cazzaniga G, Viehmann S, et al. Incidence and clinical relevance of TEL/AML1 fusion genes in children with acute lymphoblastic leukemia enrolled in the German and Italian multicenter therapy trials. Associazione Italiana Ematologia Oncologia Pediatrica and the Berlin-Frankfurt-Munster Study Group. Blood. 1997;90:571-577.
16. Cayuela JM, Baruchel A, Orange C, et al. TEL-AML1 fusion RNA as a new target to detect minimal residual disease in pediatric B-cell precursor acute lymphoblastic leukemia. Blood. 1996;88:302-308.

17. Raynaud S, Mauvieux L, Cayuela JM, et al. TEL/AML1 fusion gene is a rare event in adult acute lymphoblastic leukemia. Leukemia. 1996;10:1529-1530.
18. Aguiar RC, Sohal J, van Rhee F, et al. TEL-AML1 fusion in acute lymphoblastic leukaemia of adults. M.R.C. Adult Leukaemia Working Party. Br J Haematol. 1996;95:673-677.
19. Kwong YL, Wong KF. Low frequency of TEL/AML1 in adult acute lymphoblastic leukemia. Cancer Genet Cytogenet. 1997;98:137-138.
20. Romana SP, Poirel H, Leconiat M, et al. High frequency of t(12;21) in childhood B-lineage acute lymphoblastic leukemia. Blood. 1995;86:4263-4269.
21. Shih LY, Chou TB, Liang DC, et al. Lack of TEL-AML1 fusion transcript resulting from a cryptic t(12;21) in adult B lineage acute lymphoblastic leukemia in Taiwan. Leukemia. 1996;10:1456-1458.
22. Garcia-Sanz R, Alaejos I, Orfao A, et al. Low frequency of the TEL/AML1 fusion gene in acute lymphoblastic leukaemia in Spain. Br J Haematol. 1999;107:667-669.
23. Jabber Al-Obaidi MS, Martineau M, Bennett CF, et al. ETV6/AML1 fusion by FISH in adult acute lymphoblastic leukemia. Leukemia. 2002;16:669-674.
24. Greaves M. Molecular genetics, natural history and the demise of childhood leukaemia. Eur J Cancer. 1999;35:173-185.
25. Andreasson P, Schwaller J, Anastasiadou E, Aster J, Gilliland DG. The expression of ETV6/CBFA2 (TEL/AML1) is not sufficient for the transformation of hematopoietic cell lines in vitro or the induction of hematologic disease in vivo. Cancer Genet Cytogenet. 2001;130:93-104.
26. Bernardin F, Yang Y, Cleaves R, et al. TEL-AML1, expressed from t(12;21) in human acute lymphocytic leukemia, induces acute leukemia in mice. Cancer Res. 2002;62:3904-3908.
27. Ford AM, Bennett CA, Price CM, Bruin MC, Van Wering ER, Greaves M. Fetal origins of the TEL-AML1 fusion gene in identical twins with leukemia. Proc Natl Acad Sci U S A. 1998;95:4584-4588.
28. Wiemels JL, Cazzaniga G, Daniotti M, et al. Prenatal origin of acute lymphoblastic leukaemia in children. Lancet. 1999;354:1499-1503.
29. Maia AT, Ford AM, Jalali GR, et al. Molecular tracking of leukemogenesis in a triplet pregnancy. Blood. 2001;98:478-482.
30. Wiemels JL, Ford AM, Van Wering ER, Postma A, Greaves M. Protracted and variable latency of acute lymphoblastic leukemia after TEL-AML1 gene fusion in utero. Blood. 1999;94:1057-1062.
31. Maia AT, Koechling J, Corbett R, Metzler M, Wiemels JL, Greaves M. Protracted postnatal natural histories in childhood leukemia. Genes Chromosomes Cancer. 2004;39:335-340.
32. Cave H, Gerard B, Martin E, et al. Loss of heterozygosity in the chromosomal region 12p12-13 is very common in childhood acute lymphoblastic leukemia and permits the precise localization of a tumor-suppressor gene distinct from p27KIP1. Blood. 1995;86:3869-3875.
33. Golub TR, Barker GF, Stegmaier K, Gilliland DG. Involvement of the TEL gene in hematologic malignancy by diverse molecular genetic mechanisms. Curr Top Microbiol Immunol. 1996;211:279-288.
34. Stams WA, Beverloo HB, den Boer ML, et al. Incidence of additional genetic changes in the TEL and AML1 genes in t(12;21) positive pediatric ALL and their relation with drug sensitivity and clinical outcome. submitted.
35. Pongers-Willemse MJ, Seriu T, Stolz F, et al. Primers and protocols for standardized detection of minimal residual disease in acute lymphoblastic leukemia using immunoglobulin

and T cell receptor gene rearrangements and TAL1 deletions as PCR targets: report of the BIOMED-1 CONCERTED ACTION: investigation of minimal residual disease in acute leukemia. Leukemia. 1999;13:110-118.
36. Lin WC, Desiderio S. V(D)J recombination and the cell cycle. Immunol Today. 1995;16:279-289.
37. Tsuzuki S, Seto M, Greaves M, Enver T. Modeling first-hit functions of the t(12;21) TEL-AML1 translocation in mice. Proc Natl Acad Sci U S A. 2004;101:8443-8448.
38. Pine SR, Wiemels JL, Jayabose S, Sandoval C. TEL-AML1 fusion precedes differentiation to pre-B cells in childhood acute lymphoblastic leukemia. Leuk Res. 2003;27:155-164.
39. Hubner S, Cazzaniga G, Flohr T, et al. High incidence and unique features of antigen receptor gene rearrangements in TEL-AML1-positive leukemias. Leukemia. 2004;18:84-91.
40. Hotfilder M, Rottgers S, Rosemann A, Jurgens H, Harbott J, Vormoor J. Immature CD34+CD19- progenitor/stem cells in TEL/AML1-positive acute lymphoblastic leukemia are genetically and functionally normal. Blood. 2002;100:640-646.
41. Peham M, Konrad M, Harbott J, Konig M, Haas OA, Panzer-Grumayer ER. Clonal variation of the immunogenotype in relapsed ETV6/RUNX1-positive acute lymphoblastic leukemia indicates subclone formation during early stages of leukemia development. Genes Chromosomes Cancer. 2004;39:156-160.
42. Konrad M, Metzler M, Panzer S, et al. Late relapses evolve from slow-responding subclones in t(12;21)-positive acute lymphoblastic leukemia: evidence for the persistence of a preleukemic clone. Blood. 2003;101:3635-3640.
43. Loh ML, Rubnitz JE. TEL/AML1-positive pediatric leukemia: prognostic significance and therapeutic approaches. Curr Opin Hematol. 2002;9:345-352.
44. Veiga LB, Coser VM, Cavalli LR, et al. High frequency of t(12;21)(p13;q22) in children with acute lymphoblastic leukemia and known clinical outcome in southern Brazil. Leuk Res. 2004;28:1033-1038.
45. Zen PR, Lima MC, Coser VM, et al. Prevalence of TEL/AML1 fusion gene in Brazilian pediatric patients with acute lymphoblastic leukemia. Cancer Genet Cytogenet. 2004;151:68-72.
46. Sazawal S, Bhatia K, Gutierrez MI, Saxena R, Arya LS, Bhargava M. Paucity of TEL-AML 1 translocation, by multiplex RT-PCR, in B-lineage acute lymphoblastic leukemia (ALL) in Indian patients. Am J Hematol. 2004;76:80-82.
47. Kanerva J, Saarinen-Pihkala UM, Niini T, et al. Favorable outcome in 20-year follow-up of children with very-low-risk ALL and minimal standard therapy, with special reference to TEL-AML1 fusion. Pediatr Blood Cancer. 2004;42:30-35.
48. Hubeek I, Ramakers-van Woerden NL, Pieters R, et al. TEL/AML1 fusion is not a prognostic factor in Dutch childhood acute lymphoblastic leukaemia. Br J Haematol. 2001;113:254-255.
49. Den Boer ML, Harms DO, Pieters R, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol. 2003;21:3262-3268.
50. Ramakers-van Woerden NL, Pieters R, Loonen AH, et al. TEL/AML1 gene fusion is related to in vitro drug sensitivity for L-asparaginase in childhood acute lymphoblastic leukemia. Blood. 2000;96:1094-1099.

51. Stams WA, den Boer ML, Beverloo HB, et al. Sensitivity to L-asparaginase is not associated with expression levels of asparagine synthetase in t(12;21)+ pediatric ALL. Blood. 2003;101:2743-2747.
52. Kaspers GJ, Veerman AJ, Pieters R, et al. In vitro cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. Blood. 1997;90:2723-2729.
53. Frost BM, Forestier E, Gustafsson G, et al. Translocation t(12;21) is related to in vitro cellular drug sensitivity to doxorubicin and etoposide in childhood acute lymphoblastic leukemia. Blood. 2004;104:2452-2457.
54. Krishna Narla R, Navara C, Sarquis M, Uckun FM. Chemosensitivity of TEL-AML1 fusion transcript positive acute lymphoblastic leukemia cells. Leuk Lymphoma. 2001;41:615-623.
55. Takahashi Y, Horibe K, Kiyoi H, et al. Prognostic significance of TEL/AML1 fusion transcript in childhood B-precursor acute lymphoblastic leukemia. J Pediatr Hematol Oncol. 1998;20:190-195.
56. Maloney K, McGavran L, Murphy J, et al. TEL-AML1 fusion identifies a subset of children with standard risk acute lymphoblastic leukemia who have an excellent prognosis when treated with therapy that includes a single delayed intensification. Leukemia. 1999;13:1708-1712.
57. Avigad S, Kuperstein G, Zilberstein J, et al. TEL-AML1 fusion transcript designates a favorable outcome with an intensified protocol in childhood acute lymphoblastic leukemia. Leukemia. 1999;13:481-483.
58. Sharrocks AD, Brown AL, Ling Y, Yates PR. The ETS-domain transcription factor family. Int J Biochem Cell Biol. 1997;29:1371-1387.
59. Graves BJ, Petersen JM. Specificity within the ets family of transcription factors. Adv Cancer Res. 1998;75:1-55.
60. Sharrocks AD. The ETS-domain transcription factor family. Nat Rev Mol Cell Biol. 2001;2:827-837.
61. Sementchenko VI, Watson DK. Ets target genes: past, present and future. Oncogene. 2000;19:6533-6548.
62. Lacronique V, Boureux A, Valle VD, et al. A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. Science. 1997;278:1309-1312.
63. Kim CA, Phillips ML, Kim W, et al. Polymerization of the SAM domain of TEL in leukemogenesis and transcriptional repression. Embo J. 2001;20:4173-4182.
64. Baker DA, Mille-Baker B, Wainwright SM, Ish-Horowicz D, Dibb NJ. Mae mediates MAP kinase phosphorylation of Ets transcription factors in Drosophila. Nature. 2001;411:330-334.
65. Fenrick R, Amann JM, Lutterbach B, et al. Both TEL and AML-1 contribute repression domains to the t(12;21) fusion protein. Mol Cell Biol. 1999;19:6566-6574.
66. Wood LD, Irvin BJ, Nucifora G, Luce KS, Hiebert SW. Small ubiquitin-like modifier conjugation regulates nuclear export of TEL, a putative tumor suppressor. Proc Natl Acad Sci U S A. 2003;100:3257-3262.
67. Lopez RG, Carron C, Oury C, Gardellin P, Bernard O, Ghysdael J. TEL is a sequence-specific transcriptional repressor. J Biol Chem. 1999;274:30132-30138.
68. Wasylyk B, Hagman J, Gutierrez-Hartmann A. Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. Trends Biochem Sci. 1998;23:213-216.

69. Chakrabarti SR, Nucifora G. The leukemia-associated gene TEL encodes a transcription repressor which associates with SMRT and mSin3A. Biochem Biophys Res Commun. 1999;264:871-877.
70. Mavrothalassitis G, Ghysdael J. Proteins of the ETS family with transcriptional repressor activity. Oncogene. 2000;19:6524-6532.
71. Wang LC, Swat W, Fujiwara Y, et al. The TEL/ETV6 gene is required specifically for hematopoiesis in the bone marrow. Genes Dev. 1998;12:2392-2402.
72. Kwiatkowski BA, Bastian LS, Bauer TR, Jr., Tsai S, Zielinska-Kwiatkowska AG, Hickstein DD. The ets family member Tel binds to the Fli-1 oncoprotein and inhibits its transcriptional activity. J Biol Chem. 1998;273:17525-17530.
73. Fenrick R, Wang L, Nip J, et al. TEL, a putative tumor suppressor, modulates cell growth and cell morphology of ras-transformed cells while repressing the transcription of stromelysin-1. Mol Cell Biol. 2000;20:5828-5839.
74. Irvin BJ, Wood LD, Wang L, et al. TEL, a putative tumor suppressor, induces apoptosis and represses transcription of Bcl-XL. J Biol Chem. 2003;278:46378-46386.
75. Wlodarska I, La Starza R, Baens M, et al. Fluorescence in situ hybridization characterization of new translocations involving TEL (ETV6) in a wide spectrum of hematologic malignancies. Blood. 1998;91:1399-1406.
76. Rubnitz JE, Pui CH, Downing JR. The role of TEL fusion genes in pediatric leukemias. Leukemia. 1999;13:6-13.
77. Golub TR, Barker GF, Lovett M, Gilliland DG. Fusion of PDGF receptor beta to a novel ets-like gene, tel, in chronic myelomonocytic leukemia with t(5;12) chromosomal translocation. Cell. 1994;77:307-316.
78. Golub TR, Goga A, Barker GF, et al. Oligomerization of the ABL tyrosine kinase by the Ets protein TEL in human leukemia. Mol Cell Biol. 1996;16:4107-4116.
79. Papadopoulos P, Ridge SA, Boucher CA, Stocking C, Wiedemann LM. The novel activation of ABL by fusion to an ets-related gene, TEL. Cancer Res. 1995;55:34-38.
80. Peeters P, Raynaud SD, Cools J, et al. Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9;12) in a lymphoid and t(9;15;12) in a myeloid leukemia. Blood. 1997;90:2535-2540.
81. Buijs A, Sherr S, van Baal S, et al. Translocation (12;22) (p13;q11) in myeloproliferative disorders results in fusion of the ETS-like TEL gene on 12p13 to the MN1 gene on 22q11. Oncogene. 1995;10:1511-1519.
82. Buijs A, van Rompaey L, Molijn AC, et al. The MN1-TEL fusion protein, encoded by the translocation (12;22)(p13;q11) in myeloid leukemia, is a transcription factor with transforming activity. Mol Cell Biol. 2000;20:9281-9293.
83. Hiebert SW, Sun W, Davis JN, et al. The t(12;21) translocation converts AML-1B from an activator to a repressor of transcription. Mol Cell Biol. 1996;16:1349-1355.
84. Hatta Y, Takeuchi S, Yokota J, Koeffler HP. Ovarian cancer has frequent loss of heterozygosity at chromosome 12p12.3-13.1 (region of TEL and Kip1 loci) and chromosome 12q23-ter: evidence for two new tumour-suppressor genes. Br J Cancer. 1997;75:1256-1262.
85. Sato Y, Suto Y, Pietenpol J, et al. TEL and KIP1 define the smallest region of deletions on 12p13 in hematopoietic malignancies. Blood. 1995;86:1525-1533.
86. Spirin KS, Simpson JF, Takeuchi S, Kawamata N, Miller CW, Koeffler HP. p27/Kip1 mutation found in breast cancer. Cancer Res. 1996;56:2400-2404.

87. Stegmaier K, Pendse S, Barker GF, et al. Frequent loss of heterozygosity at the TEL gene locus in acute lymphoblastic leukemia of childhood. Blood. 1995;86:38-44.
88. Meyers S, Downing JR, Hiebert SW. Identification of AML-1 and the (8;21) translocation protein (AML-1/ETO) as sequence-specific DNA-binding proteins: the runt homology domain is required for DNA binding and protein-protein interactions. Mol Cell Biol. 1993;13:6336-6345.
89. Miyoshi H, Shimizu K, Kozu T, Maseki N, Kaneko Y, Ohki M. t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. Proc Natl Acad Sci U S A. 1991;88:10431-10434.
90. Levanon D, Negreanu V, Bernstein Y, Bar-Am I, Avivi L, Groner Y. AML1, AML2, and AML3, the human members of the runt domain gene-family: cDNA structure, expression, and chromosomal localization. Genomics. 1994;23:425-432.
91. Speck NA, Stacy T, Wang Q, et al. Core-binding factor: a central player in hematopoiesis and leukemia. Cancer Res. 1999;59:1789s-1793s.
92. Ogawa E, Maruyama M, Kagoshima H, et al. PEBP2/PEA2 represents a family of transcription factors homologous to the products of the Drosophila runt gene and the human AML1 gene. Proc Natl Acad Sci U S A. 1993;90:6859-6863.
93. Downing JR. AML1/CBFbeta transcription complex: its role in normal hematopoiesis and leukemia. Leukemia. 2001;15:664-665.
94. Wang Q, Stacy T, Miller JD, et al. The CBFbeta subunit is essential for CBFalpha2 (AML1) function in vivo. Cell. 1996;87:697-708.
95. Tanaka T, Tanaka K, Ogawa S, et al. An acute myeloid leukemia gene, AML1, regulates hemopoietic myeloid cell differentiation and transcriptional activation antagonistically by two alternative spliced forms. Embo J. 1995;14:341-350.
96. Lutterbach B, Hiebert SW. Role of the transcription factor AML-1 in acute leukemia and hematopoietic differentiation. Gene. 2000;245:223-235.
97. Rhoades KL, Hetherington CJ, Rowley JD, et al. Synergistic up-regulation of the myeloid-specific promoter for the macrophage colony-stimulating factor receptor by AML1 and the t(8;21) fusion protein may contribute to leukemogenesis. Proc Natl Acad Sci U S A. 1996;93:11895-11900.
98. Uchida H, Zhang J, Nimer SD. AML1A and AML1B can transactivate the human IL-3 promoter. J Immunol. 1997;158:2251-2258.
99. Britos-Bray M, Friedman AD. Core binding factor cannot synergistically activate the myeloperoxidase proximal enhancer in immature myeloid cells without c-Myb. Mol Cell Biol. 1997;17:5127-5135.
100. Wargnier A, Legros-Maida S, Bosselut R, et al. Identification of human granzyme B promoter regulatory elements interacting with activated T-cell-specific proteins: implication of Ikaros and CBF binding sites in promoter activation. Proc Natl Acad Sci U S A. 1995;92:6930-6934.
101. Frank R, Zhang J, Uchida H, Meyers S, Hiebert SW, Nimer SD. The AML1/ETO fusion protein blocks transactivation of the GM-CSF promoter by AML1B. Oncogene. 1995;11:2667-2674.
102. Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH, Speck NA. Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc Natl Acad Sci U S A. 1996;93:3444-3449.

103. Sasaki K, Yagi H, Bronson RT, et al. Absence of fetal liver hematopoiesis in mice deficient in transcriptional coactivator core binding factor beta. Proc Natl Acad Sci U S A. 1996;93:12359-12363.
104. Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell. 1996;84:321-330.
105. Buske C, Humphries RK. Homeobox genes in leukemogenesis. Int J Hematol. 2000;71:301-308.
106. Canon J, Banerjee U. Runt and Lozenge function in Drosophila development. Semin Cell Dev Biol. 2000;11:327-336.
107. Ferrando AA, Look AT. Gene expression profiling in T-cell acute lymphoblastic leukemia. Semin Hematol. 2003;40:274-280.
108. Nucifora G, Rowley JD. AML1 and the 8;21 and 3;21 translocations in acute and chronic myeloid leukemia. Blood. 1995;86:1-14.
109. Roulston D, Espinosa R, 3rd, Nucifora G, Larson RA, Le Beau MM, Rowley JD. CBFA2(AML1) translocations with novel partner chromosomes in myeloid leukemias: association with prior therapy. Blood. 1998;92:2879-2885.
110. Liu P, Tarle SA, Hajra A, et al. Fusion between transcription factor CBF beta/PEBP2 beta and a myosin heavy chain in acute myeloid leukemia. Science. 1993;261:1041-1044.
111. Osato M, Asou N, Abdalla E, et al. Biallelic and heterozygous point mutations in the runt domain of the AML1/PEBP2alphaB gene associated with myeloblastic leukemias. Blood. 1999;93:1817-1824.
112. Preudhomme C, Warot-Loze D, Roumier C, et al. High incidence of biallelic point mutations in the Runt domain of the AML1/PEBP2 alpha B gene in Mo acute myeloid leukemia and in myeloid malignancies with acquired trisomy 21. Blood. 2000;96:2862-2869.
113. Imai Y, Kurokawa M, Izutsu K, et al. Mutations of the AML1 gene in myelodysplastic syndrome and their functional implications in leukemogenesis. Blood. 2000;96:3154-3160.
114. Steer EJ, Goldman JM, Cross NC. Mutations of the transcription factor AML1/CBFA2 are uncommon in blastic transformation of chronic myeloid leukaemia. Leukemia. 2001;15:476-477.
115. Langabeer SE, Gale RE, Rollinson SJ, Morgan GJ, Linch DC. Mutations of the AML1 gene in acute myeloid leukemia of FAB types M0 and M7. Genes Chromosomes Cancer. 2002;34:24-32.
116. Song WJ, Sullivan MG, Legare RD, et al. Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. Nat Genet. 1999;23:166-175.
117. Niini T, Kanerva J, Vettenranta K, Saarinen-Pihkala UM, Knuutila S. AML1 gene amplification: a novel finding in childhood acute lymphoblastic leukemia. Haematologica. 2000;85:362-366.
118. Dal Cin P, Atkins L, Ford C, et al. Amplification of AML1 in childhood acute lymphoblastic leukemias. Genes Chromosomes Cancer. 2001;30:407-409.
119. Streubel B, Valent P, Lechner K, Fonatsch C. Amplification of the AML1(CBFA2) gene on ring chromosomes in a patient with acute myeloid leukemia and a constitutional ring chromosome 21. Cancer Genet Cytogenet. 2001;124:42-46.

120. Busson-Le Coniat M, Nguyen Khac F, Daniel MT, Bernard OA, Berger R. Chromosome 21 abnormalities with AML1 amplification in acute lymphoblastic leukemia. Genes Chromosomes Cancer. 2001;32:244-249.
121. Roumier C, Fenaux P, Lafage M, Imbert M, Eclache V, Preudhomme C. New mechanisms of AML1 gene alteration in hematological malignancies. Leukemia. 2003;17:9-16.
122. Harewood L, Robinson H, Harris R, et al. Amplification of AML1 on a duplicated chromosome 21 in acute lymphoblastic leukemia: a study of 20 cases. Leukemia. 2003;17:547-553.
123. Cao W, Britos-Bray M, Claxton DF, et al. CBF beta-SMMHC, expressed in M4Eo AML, reduced CBF DNA-binding and inhibited the G1 to S cell cycle transition at the restriction point in myeloid and lymphoid cells. Oncogene. 1997;15:1315-1327.
124. Bernardin F, Friedman AD. AML1 stimulates G1 to S progression via its transactivation domain. Oncogene. 2002;21:3247-3252.
125. Friedman AD. Leukemogenesis by CBF oncoproteins. Leukemia. 1999;13:1932-1942.
126. Strom DK, Nip J, Westendorf JJ, et al. Expression of the AML-1 oncogene shortens the G(1) phase of the cell cycle. J Biol Chem. 2000;275:3438-3445.
127. Cao W, Adya N, Britos-Bray M, Liu PP, Friedman AD. The core binding factor (CBF) alpha interaction domain and the smooth muscle myosin heavy chain (SMMHC) segment of CBFbeta-SMMHC are both required to slow cell proliferation. J Biol Chem. 1998;273:31534-31540.
128. Lou J, Cao W, Bernardin F, Ayyanathan K, Rauscher IF, Friedman AD. Exogenous cdk4 overcomes reduced cdk4 RNA and inhibition of G1 progression in hematopoietic cells expressing a dominant-negative CBF - a model for overcoming inhibition of proliferation by CBF oncoproteins. Oncogene. 2000;19:2695-2703.
129. Bernardin-Fried F, Kummalue T, Leijen S, Collector MI, Ravid K, Friedman AD. AML1/RUNX1 increases during G1 to S cell cycle progression independent of cytokine-dependent phosphorylation and induces cyclin D3 gene expression. J Biol Chem. 2004;279:15678-15687.
130. Imai Y, Kurokawa M, Tanaka K, et al. TLE, the human homolog of groucho, interacts with AML1 and acts as a repressor of AML1-induced transactivation. Biochem Biophys Res Commun. 1998;252:582-589.
131. Westendorf JJ, Yamamoto CM, Lenny N, Downing JR, Selsted ME, Hiebert SW. The t(8;21) fusion product, AML-1-ETO, associates with C/EBP-alpha, inhibits C/EBP-alpha-dependent transcription, and blocks granulocytic differentiation. Mol Cell Biol. 1998;18:322-333.
132. Chakrabarti SR, Sood R, Nandi S, Nucifora G. Posttranslational modification of TEL and TEL/AML1 by SUMO-1 and cell-cycle-dependent assembly into nuclear bodies. Proc Natl Acad Sci U S A. 2000;97:13281-13285.
133. Guidez F, Petrie K, Ford AM, et al. Recruitment of the nuclear receptor corepressor N-CoR by the TEL moiety of the childhood leukemia-associated TEL-AML1 oncoprotein. Blood. 2000;96:2557-2561.
134. Wang L, Hiebert SW. TEL contacts multiple co-repressors and specifically associates with histone deacetylase-3. Oncogene. 2001;20:3716-3725.
135. Uchida H, Downing JR, Miyazaki Y, Frank R, Zhang J, Nimer SD. Three distinct domains in TEL-AML1 are required for transcriptional repression of the IL-3 promoter. Oncogene. 1999;18:1015-1022.
136. Zelent A, Greaves M, Enver T. Role of the TEL-AML1 fusion gene in the molecular pathogenesis of childhood acute lymphoblastic leukaemia. Oncogene. 2004;23:4275-4283.

137. Speck NA, Gilliland DG. Core-binding factors in haematopoiesis and leukaemia. Nat Rev Cancer. 2002;2:502-513.
138. Downing JR. The core-binding factor leukemias: lessons learned from murine models. Curr Opin Genet Dev. 2003;13:48-54.
139. Gunji H, Waga K, Nakamura F, et al. TEL/AML1 shows dominant-negative effects over TEL as well as AML1. Biochem Biophys Res Commun. 2004;322:623-630.
140. Poirel H, Oury C, Carron C, et al. The TEL gene products: nuclear phosphoproteins with DNA binding properties. Oncogene. 1997;14:349-357.
141. Poirel H, Lopez RG, Lacronique V, et al. Characterization of a novel ETS gene, TELB, encoding a protein structurally and functionally related to TEL. Oncogene. 2000;19:4802-4806.
142. Potter MD, Buijs A, Kreider B, van Rompaey L, Grosveld GC. Identification and characterization of a new human ETS-family transcription factor, TEL2, that is expressed in hematopoietic tissues and can associate with TEL1/ETV6. Blood. 2000;95:3341-3348.

Part A

Clinical relevance of genetic abnormalities in t(12;21) positive ALL

Chapter 2

Incidence of additional genetic changes in the *TEL* and *AML1* genes in t(12;21) positive pediatric ALL and their relation with drug sensitivity and clinical outcome

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Submitted

ABSTRACT

t(12;21)(p13;q22), resulting in *TEL-AML1* gene fusion, is detected in ~25% of childhood common/pre B-ALL. Clinical heterogeneity within t(12;21) positive ALL indicates that additional genetic changes might be responsible for differences in drug sensitivity and clinical outcome. We studied the relation between additional genetic changes in *TEL(ETV6)* and *AML1(RUNX1)* (FISH), drug sensitivity (MTT assay) and clinical outcome in 143 t(12;21) positive ALL patients. Additional genetic changes were present in 83%, and consisted of (partial) deletion of the second *TEL* gene (70%), an extra *AML1* gene (23%) or an extra der(21)t(12;21) (10%). More than one additional change was observed in 20%. Patients without additional genetic changes (4-yrs pDFS 53% ± 17%) or with an extra der(21)t(12;21) (4-yrs pDFS 60% ± 22%) have an unfavourable prognosis compared to patients with other additional genetic changes in *TEL* or *AML1* (4-yrs pDFS 79% ± 6%; $p = 0.03$) which was mainly due to the occurrence of early relapses within 2.5 years after first diagnosis. Prednisolone resistance is related to an extra der(21)t(12;21) ($p = 0.004$) and an unfavourable prognosis ($p = 0.02$). Multivariate analysis including age, WBC and genetic abnormalities in *TEL* and/or *AML1* showed that only in vitro resistance to prednisolone is an independent prognostic factor in t(12;21) positive ALL.

INTRODUCTION

The t(12;21)(p13;q22) occurs in ~25% of childhood acute lymphoblastic leukemia (ALL), and is restricted to precursor B-cell lineage leukemia. The t(12;21) involves fusion of the *TEL(ETV6)* gene at chromosome 12p13 with the *AML1(RUNX1)* gene at chromosome 21q22. This translocation fuses the 5' terminus of the *TEL* gene (residues 1-336) in frame with almost the entire coding sequence (residues 21-480) of the *AML1* gene. The breakpoint most often occurs in intron 5 of *TEL* and intron 1 of *AML1*. A frequent translocation variant results in fusion between intron 5 of *TEL* and intron 2 of *AML1*. Both *TEL* and *AML1* are frequent targets of chromosomal translocations in a variety of myeloid and lymphoid leukemias[1,2]. *TEL* contains an N-terminal pointed (PNT) dimerization domain which mediates homodimerization[3,4]. The C-terminal DNA-binding domain homologous to all Ets proteins recognizes a purine-rich GGAA/T core motif within promoters and enhancers of various genes[5]. TEL has a role in both angiogenesis and hematopoiesis[6]. *AML1* encodes a transcription factor that binds the enhancer core sequence TGT/cGGT through its N-terminal Runt homology domain (RHD)[7]. The DNA-binding affinity of AML1 is increased by heterodimerization through the RHD with the Core Binding Factor (CBF) β protein, forming the CBF. Transcriptional activation occurs through the C-terminal transactivation (TA) domain. CBF is essential for definitive hematopoiesis of all lineages[1]. The translocation fusion product *TEL-AML1* contains the PNT domain of *TEL* and the RHD and TA domain of *AML1*.

Several studies have investigated the prognostic value of t(12;21) positive ALL[8]. In general, t(12;21) positive ALL is associated with a good prognosis. However, conflicting data on the percentage of patients entering relapse and the proportion of t(12;21) positive cases at relapse have been reported[8]. Overall, the reported prognostic relevance of t(12;21) seems to depend on the intensity of the treatment protocol. A possible explanation for this finding can be ascribed to the fact that t(12;21) positive ALL cells are *in vitro* more sensitive to L-Asparaginase compared to t(12;21) negative ALL cells[9,10]. However, within the t(12;21) positive ALL group, large interindividual differences in cellular *in vitro* sensitivity to L-Asparaginase were found. The clinical heterogeneity in response to therapy as well as the large heterogeneity in *in vitro* sensitivity to L-Asparaginase, suggests that additional genetic changes might be important for the differences in drug sensitivity and clinical outcome. Approximately 70% of t(12;21) positive ALL cases also show loss of the second *TEL* allele[11,12]. Trisomy 21 and duplication of the der(21)t(12;21) are also found in t(12;21) positive patients, with the latter apparently being more common in relapsed cases[13-16]. However, the number of patients screened in these studies is limited. The prognostic significance of additional genetic abnormalities in *TEL* and *AML1* is therefore unknown. We retrospectively studied the incidence of

additional genetic changes in the *TEL* and *AML1* genes and their relationship with drug sensitivity and clinical outcome in 143 t(12;21) positive children with ALL.

MATERIALS AND METHODS

Patient samples

Bone marrow and/or peripheral blood samples from 343 untreated children with common/pre B-ALL at initial diagnosis were collected at the Erasmus MC - Sophia Children's hospital, the Dutch Childhood Oncology Group (DCOG) (ALL-7, 8 and 9 treatment protocol) and the German COALL study group (COALL-92 and 97 treatment protocol). Within 24 hours after sampling, mononuclear cells were isolated and contaminating non-leukemic cells were eliminated as described earlier[17]. All resulting samples contained ≥ 90% leukemic cells, as determined morphologically on May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins.

FISH analysis

272 Erasmus MC - Sophia Children's Hospital and DCOG common/pre B-ALL patients were retrospectively analyzed for the presence of t(12;21) by FISH, whereas the t(12;21) positivity of 71 COALL patients was already known by RT-PCR. The FISH protocol was based on that described previously[18]. The t(12;21) positivity was determined on cryopreserved cytospins with dual colored FISH using a digoxigenin labeled cosmid from intron 1 to exon 2 of *TEL* (50F4), together with a biotinylated cosmid for the first 5 exons of *AML1* (C0664) (a kind gift of Dr. N. Sacchi, University of Milan, Italy[19]) (Figure 1.). Probe 50F4 was detected with Texas Red labelled α digoxigenin and probe C0664 with avidin-FITC. In t(12;21) positive patients a yellow fusion spot will be seen denoting the der(21)t(12;21), one green signal for the normal *AML1* on chromosome 21 and one red signal for the normal *TEL* on chromosome 12. Two green spots next to the yellow fusion spot indicate an extra copy of *AML1*, whereas the absence of a single red signal indicates a deletion of *TEL*. To determine the size of the deletion of the second *TEL* allele in t(12;21) positive patients, additional dual-colored FISH experiments were performed using *TEL* cosmid probes located at the 5' (179A6-biotinylated) and 3' (54D5-digoxigenin labeled) part of the *TEL* gene (Figure 1). If the complete non-translocated *TEL* gene is deleted, only one yellow fusion spot spanning exon 1a to 5 of *TEL* on the der(21)t(12;21) and a red signal spanning exon 6 to 8 of *TEL* on der(12)t(12;21) will be present. If the non-translocated *TEL* gene is only partially deleted, two yellow fusion spots are visible, one derived from der(21)t(12;21) and the other from the non-translocated *TEL* gene. In addition, a red signal from the der(12)t(12;21) will be present. The partial deletion of *TEL* was indicated by a loss of the 50F4 signal

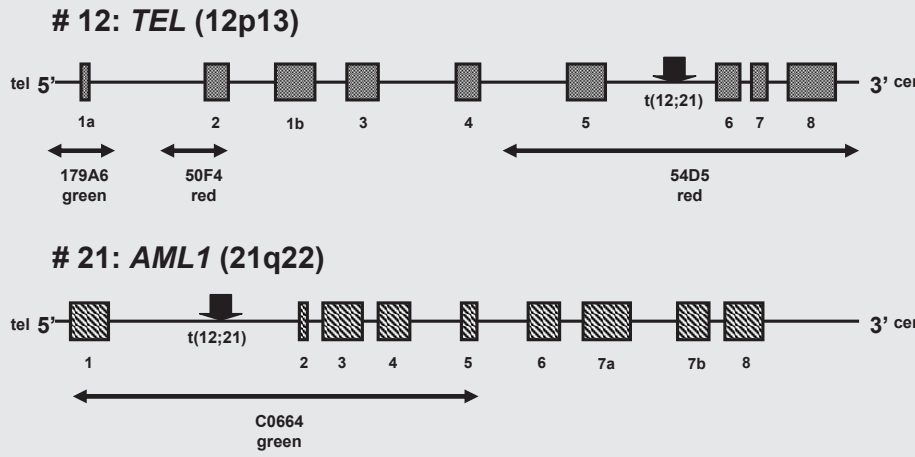


Figure 1. Overview of FISH probes used to identify genetic abnormalities in *TEL* and *AML1* genes in t(12;21) positive ALL
Vertical arrows indicate the breakpoint region for t(12;21) on chromosome 12 and 21. Horizontal arrows indicate the localisation of the 4 probes used to analyze additional genetic changes in *TEL* and/or *AML1* in t(12;21) positive ALL.

whereas the 179A6 and 54D5 fusion signal remained visible. All *TEL* cosmids were a kind gift of Prof. Dr. P. Marynen, Human Genetics, University of Leuven, Belgium[20]. In all instances two independent observers each examined 100-300 interphase nuclei.

In vitro L-Asparaginase, prednisolone and vincristine cytotoxicity assay

In vitro L-Asparaginase, prednisolone and vincristine cytotoxicity was determined using the MTT assay as described previously[17]. Briefly, 100 µl aliquots of cell suspension (1.6×10^5 cells; >90% leukemic cells) were exposed to six duplicate concentrations of L-Asparaginase (Paronal, Christiaens, Breda, The Netherlands) ranging from 0.0032 – 10 IU/ml, prednisolone (prednisoloni natrii phosphas, Bufa BV, Uitgeest, The Netherlands) ranging from 0.08 – 250 µg/ml and vincristine (vincristinesulphate, TEVA Pharma BV, Mijdrecht, The Netherlands) ranging from 0.049 – 50 µg/ml. Control cells were cultured without L-Asparaginase, prednisolone or vincristine. 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-diphenyltetrazoliumbromide (MTT, 5 mg/ml; Sigma Aldrich, Zwijndrecht, The Netherlands) was added. During a 6-hour incubation, the yellow MTT tetrazolium salt is reduced to purple-blue formazan crystals by viable cells only. Samples with ≥70% leukemic cells in the control wells after 4 days of culture and an optical density higher than 0.050 arbitrary units (adjusted for blank values) were used to calculate the concentration of drug lethal to 50% of the cells (LC_{50}).

Statistics

In all patient samples one major aberrant clone representing more than 80% of all cells was observed. The size of subclones, if present, was small, and accounted together with normal cells only for less than 20% of the total population. In this study, we therefore divided the samples in 3 groups according to the major clone present in patients, i.e. (1) without additional genetic changes in *TEL* or *AML1*, (2) with an extra der(21)t(12;21), or (3) with other additional genetic changes such as a deletion of *TEL* and/or an extra copy of *AML1*. Probability of disease-free survival (pDFS) was calculated from the date of diagnosis to the date of relapse (event) or last contact (censored). The pDFS curves were calculated according the Kaplan-Meier method and analyzed by the log-rank test. Multivariate analysis with stratification for study-group was performed with the Cox proportional-hazard regression model. Differences in the distribution of variables between 2 groups of patients were analyzed using the Mann-Whitney U or χ^2 test. Differences in the distribution of variables between more than 2 groups were analyzed using the Kruskal-Wallis test. Statistical tests were performed at a two-tailed significance level of 0.05.

RESULTS

143 t(12;21) positive cases were analyzed for additional genetic changes in *TEL* and *AML1* by extensive FISH analysis. 83% (118/143) of the patients showed one or more additional genetic changes (Figure 2). These additional genetic changes could be di-

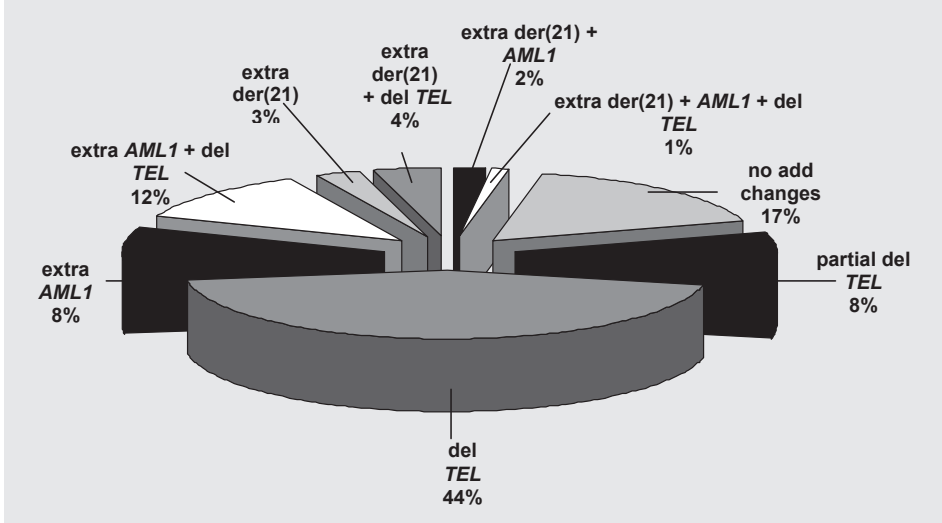
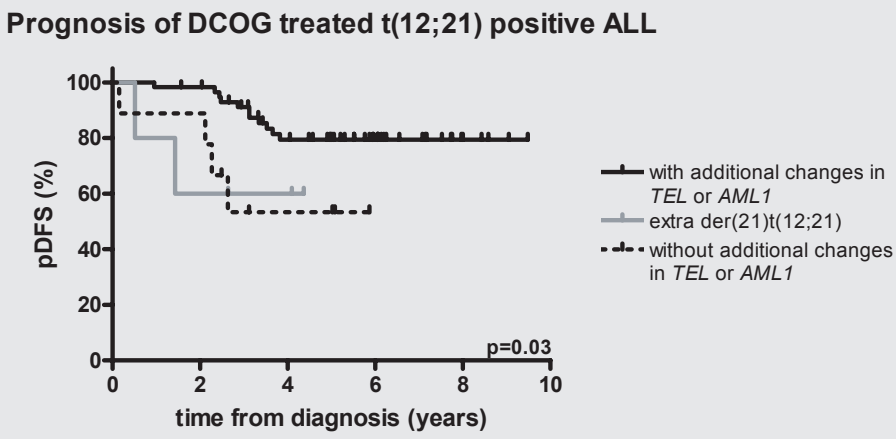


Figure 2. Incidence of additional genetic changes in *TEL* and *AML1* genes in major clone of t(12;21) positive ALL.

A.



B.

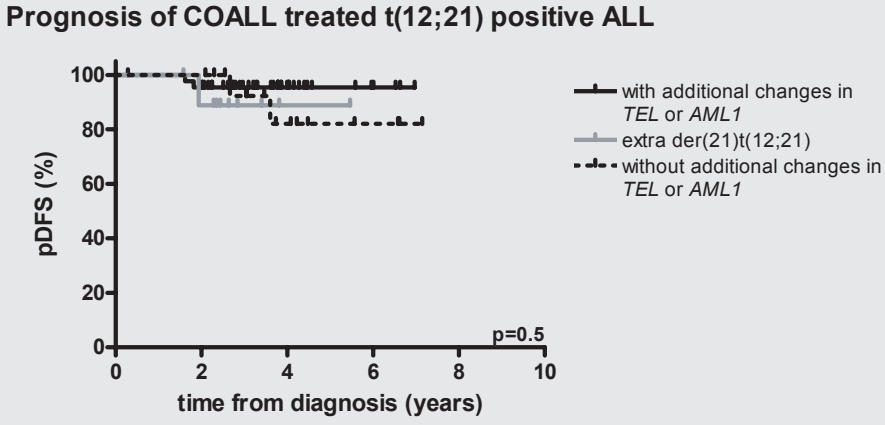


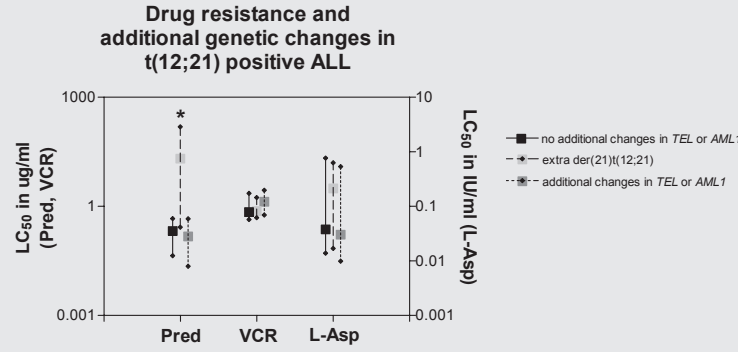
Figure 3. Disease-free survival analysis of additional genetic changes in *TEL* and *AML1* genes in t(12;21) positive ALL
A. Probability of disease-free survival (pDFS) in DCOG-treated children with t(12;21) positive ALL according to presence or absence of additional genetic changes in *TEL* or *AML1* genes. Black solid line, patients with additional genetic changes, i.e. deletion of *TEL* or extra copy of *AML1* (4-yrs pDFS 79% ± 6%; n=58, 10 events). Grey solid line, patients with an extra der(21)t(12;21) (4-yrs pDFS 60% ± 22%; n=5, 2 events). Dotted line, patients without additional genetic changes in *TEL* or *AML1* (4-yrs pDFS 53% ± 17%; n=9, 4 events; p =0.03).
B. pDFS in COALL-treated children with t(12;21) positive ALL. Black solid line, patients with additional genetic changes in *TEL* or *AML1* (4-yrs pDFS 95% ± 6%; n=45, 2 events). Grey solid line, patients with an extra der(21)t(12;21) (4-yrs pDFS 89% ± 17%; n=10, 1 event). Dotted line, patients without additional genetic changes in *TEL* or *AML1* (4-yrs pDFS 82% ± 17%; n=16, 2 events; p=0.5).

vided into a complete deletion of the second *TEL* allele (62%; 88/143), a partial deletion of the second *TEL* allele (8%; 12/143), an extra copy of *AML1* (23%; 33/143) and an extra der(21)t(12;21) indicating an extra fusion product (10%; 15/143). Twenty percent (28/143) of all t(12;21) positive ALL patients had more than one additional genetic abnormality in *TEL* and *AML1*.

The 143 t(12;21) positive patients consisted of 72 Dutch DCOG patients and 71 German COALL patients. Patient characteristics did not differ between the two treatment groups except for the median follow-up time of patients at risk and number of relapses: DCOG median follow-up time was 6.0 years (range 1.57-12.76) with 16 relapses compared to the COALL median follow-up time of 2.9 years (range 0.28-7.14) with 5 relapses ($p < 0.0001$). Kaplan-Meier curves of patients without additional changes in *TEL* or *AML1*, of patients with an extra der(21)t(12;21), or patients with additional changes in *TEL* or *AML1* other than an extra der(21)t(12;21) are shown in Figure 3. Absence of additional genetic changes in *TEL* or *AML1* and an extra der(21)t(12;21) showed to be of significant prognostic value only in the DCOG group (Figure 3A). The absence of additional genetic changes in *TEL* or *AML1* (4-yrs pDFS of 53% ± 17%) or the presence of an extra der(21)t(12;21) (4-yrs pDFS of 60% ± 22%) are linked to a poorer prognosis compared with t(12;21) positive patients with additional genetic changes in *TEL* or *AML1* (4-yrs pDFS of 79% ± 6%) in the DCOG group (Figure 3A; $p = 0.03$). Moreover, in the patients without additional genetic changes or with an extra der(21)t(12;21) all six events (i.e. non-response or relapse) occurred early, i.e. within 2.5 yrs after first diagnosis, compared to the patients with additional genetic changes (4 out of 11 events < 2.5 yrs; $\chi^2 = 6.49$, $p = 0.04$). In the COALL group, none of the additional genetic changes was statistically significantly associated with prognosis although the same relative trend in the curves is seen in COALL compared to DCOG patients (Figure 3B); 4-yrs pDFS 82% ± 17% for patients without additional genetic changes in *TEL* or *AML1*, 4-yrs pDFS 89% ± 17% for patients with an extra der(21)t(12;21) and 4-yrs pDFS 95% ± 6% for patients with additional genetic changes in *TEL* or *AML1*. Univariate analysis using a study group-stratified Cox proportional hazards model, which took into account the origin of our patients (DCOG and COALL), indicated that the absence of additional genetic changes in *TEL* or *AML1* (Hazard rate 3.3, 95% CI 1.2-9.0) and an extra der(21)t(12;21) (Hazard rate 3.3, 95% CI 0.9-11.7) are associated with an unfavourable outcome compared to the reference group of patients with additional genetic changes in *TEL* and *AML1* ($p = 0.009$).

Within t(12;21) positive patients, *in vitro* resistance to L-Asparaginase and vincristine was not associated with an unfavourable outcome (data not shown). In addition, *in vitro* cytotoxicity of these drugs did not significantly differ in t(12;21) positive patients with or without additional genetic abnormalities in *TEL* or *AML1* (Figure 4A). However, we found t(12;21) positive ALL patients with an extra der(21)t(12;21) to be 123-fold more resistant to prednisolone compared to patients without an extra der(21)t(12;21) ($p = 0.004$; Figure 4A). *In vitro* prednisolone resistance was significantly related to a unfavourable prognosis within t(12;21) positive DCOG patients ($p = 0.02$; Figure 4B) and the same trend (but non-significant) was found among COALL patients (Figure 4C). Univariate analysis of the total group (taking into account the study group) revealed

A.



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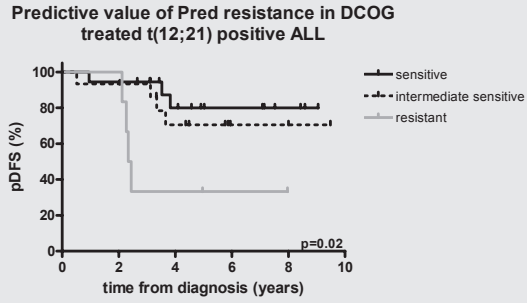

C.

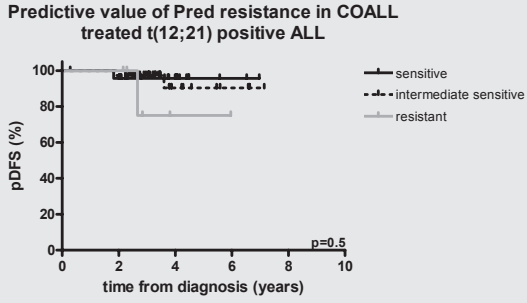

Figure 4. Drug resistance in t(12;21) positive ALL

A. Relationship between cellular resistance for prednisolone (Pred), vincristine (VCR) and L-Asparaginase (L-Asp) and absence of additional genetic changes in *TEL* and *AML1*, presence of an extra der(21)t(12;21) or additional changes in *TEL* or *AML1* in t(12;21) positive ALL. Prednisolone and vincristine, LC_{50} values in µg/ml, left Y-axis; L-Asparaginase LC_{50} values in IU/ml, right Y-axis. Square = median LC_{50} value, upper and lower diamond represent the 75th and 25th percentile of the LC_{50} value, respectively. *p=0.004, 123-fold.

B. Probability of disease-free survival (pDFS) in t(12;21) positive DCOG ALL patients sensitive, intermediate sensitive or resistant to prednisolone. Black solid line, patients sensitive to prednisolone (4-yrs pDFS 80% ± 14%, n=18, 3 events). Dotted line, patients intermediate sensitive to prednisolone (4-yrs pDFS 59% ± 24%, n=15, 4 events). Grey solid line, patients resistant to prednisolone (4-yrs DFS 33% ±19%, n=6, 4 events), p=0.02. The division in the indicated prednisolone subgroups is based on previously reported cut-off points[21,22].

C. pDFS in t(12;21) positive COALL ALL patients sensitive, intermediate sensitive or resistant to prednisolone. Black solid line, patients sensitive to prednisolone (4-yrs pDFS 95% ± 7%, n=23, 1 event). Dotted line, patients intermediate sensitive to prednisolone (4-yrs pDFS 88% ± 12%, n=38, 2 events). Grey solid line, patients resistant to prednisolone (4-yrs DFS 75% ± 22%, n=6, 1 event), p=0.5.

that prednisolone resistance was a poor prognostic variable ($p = 0.008$; study-group stratified). Multivariate analysis including age, WBC and additional genetic changes revealed that only prednisolone resistance ($p = 0.02$) is a significantly independent poor prognostic factor in t(12;21) positive ALL (Table 1; study group stratified).

DISCUSSION

In our present study, the incidence of additional genetic changes in the *TEL* and *AML1* genes at initial diagnosis in 143 t(12;21) positive ALL is 83%. A (partial) deletion of the non-translocated *TEL* allele (70%) occurred most frequently followed by the presence of an extra copy of *AML1* (23%) and an extra der(21)t(12;21) (10%). Moreover, 20% of the t(12;21) positive patients had more than one additional genetic change. This incidence is in concordance with reports of additional genetic abnormalities in lower numbers of patients[11-16].

In t(12;21) positive ALL, an extra der(21)t(12;21) has been reported to be more frequently present in relapsed cases[13-16]. In the present study, we show that even in a small number of patients, the presence of this additional abnormality at diagnosis is linked to an unfavourable long-term clinical outcome. Furthermore, we observed that absence of additional genetic changes in *TEL* or *AML1* is related to a poor prognosis in t(12;21) positive ALL. These observations were significant in DCOG treated patients, whereas a similar trend was seen for COALL treated cases (Figure 3) albeit not significant due to the relatively short follow-up and, hence, a smaller number of events that had been occurred. In correspondence with these data is the observation that deletions of the short arm of chromosome 12 (including the *TEL* gene) are a favourable feature in childhood ALL[21]. These data are in contrast to the study by Attarbaschi et al., in which a deletion of *TEL* in t(12;21) positive ALL was linked with an unfavourable prognosis[22]. A possible explanation may be the differences in definition of an event (event is defined a relapse in our study whereas it is defined as a failure to achieve remission, relapse at any site, death during continuous remission or the development of a secondary malignancy in the study of Attarbaschi et al.) and in the treatment protocol that was used (DCOG ALL-7/8/9 and COALL-92/97 in this study and ALL-BFM-A 1990/1995 in the Attarbaschi study). Our data further indicated that an extra der(21)t(12;21) was linked to prednisolone resistance. Multivariate analysis indicated that an extra der(21)t(12;21) as well as absence of additional changes in *TEL* and *AML1* had no independent predictive value in contrast to prednisolone resistance which was independently associated with an unfavourable outcome in t(12;21) positive ALL.

Conflicting data[8] on the prognostic value of t(12;21) might be due to differences in use of L-Asparaginase in the treatment protocols as t(12;21) positive ALL is highly

Table 1. Multivariate analysis in t(12;21) positive ALL patients stratified by study group*

Variable	Unfavourable feature	N	Hazard ratio	95% CI	P-value
Age at diagnosis (yrs)	>10	15	1.8	0.37-8.68	0.47
WBC at diagnosis (*10^9cells/L)	>25	32	1.4	0.43-4.38	0.59
Additional genetic changes** (deletion of *TEL* or extra copy of *AML1*)	absent	21	3.2	0.88-11.39	0.08
Extra der(21)t(12;21)**	present	13	1.7	0.34-8.56	0.51
Pred resistance (LC_{50} in µg/ml)	≥ 150	13	4.2	1.31-13.50	0.02

*In 110 out of 143 t(12;21) positive patients, all variables of the model were determined. Multivariate analysis was performed taking into account the study group of each patient (=study group stratified multivariate analysis). No significant interactions (p>0.2) were found between study group and presence or absence of additional genetic changes in *TEL* or *AML1*, presence or absence of an extra der(21)t(12;21) and prednisolone (Pred) resistance, validating the simultaneous use of both study cohorts in one stratified multivariate analysis.
**Hazard ratio with 95% CI and p-value of patients with an extra der(21)t(12;21) or without additional genetic changes in *TEL* or *AML1* (both unfavourable features) compared with patients with additional genetic changes in *TEL* or *AML1* (favourable feature).

sensitive to L-Asparaginase[9,10] and L-Asparaginase sensitive patients have a more favourable outcome[23]. Both DCOG and COALL study groups use similar E-coli L-Asparaginase preparations, although the cumulative dosage of L-Asparaginase used in the COALL treatment protocol is higher. It is possible that a general intensification of therapy, not only by L-Asparaginase but also by other drugs, might contribute to the fact that in some recent protocols patients with t(12;21) positive ALL have a favourable outcome[24]. This might also explain the difference in survival of t(12;21) positive ALL patients between the DCOG and COALL group (5-yrs event-free survival of 73% and 80%, respectively)[25,26], since COALL patients are treated with a more intensive protocol compared to the DCOG patients. The presence of t(12;21) in COALL97 treated ALL patients has no prognostic value in the low risk arm of the protocol. However, t(12;21) positive patients have a favourable prognosis in the high risk arm of this protocol compared with t(12;21) negative ALL (Prof. Dr. G.E. Janka-Schaub; personal communication).

A relapse in t(12;21) positive ALL is thought to occur after completion of therapy, i.e. > 2.5 years after first diagnosis[8]. As shown in Figure 3, the majority of relapses in our series of t(12;21) positive ALL occured after 2.5 years of diagnosis. However, absence of additional genetic changes in *TEL* or *AML1*, or presence of an extra der(21)t(12;21) in t(12;21) positive patients identified patients at higher risk for early events (Figure 3); all events in these small subgroups were within 2.5 years after first diagnosis. Early relapses may occur as a result of regrowth of leukemic cells despite chemotherapy or insufficient clearance from the bone marrow during the first months of therapy[27]. Several studies strongly suggest that the initiating event of the t(12;21) rearrangement occurs in utero and subsequent events occur in early childhood facilitating the development of leukemia[28,29]. Continuing differentiation processes lead to a variety of immune receptor rearrangements in individual cells of the t(12;21) positive pre-leukemic clone, resulting

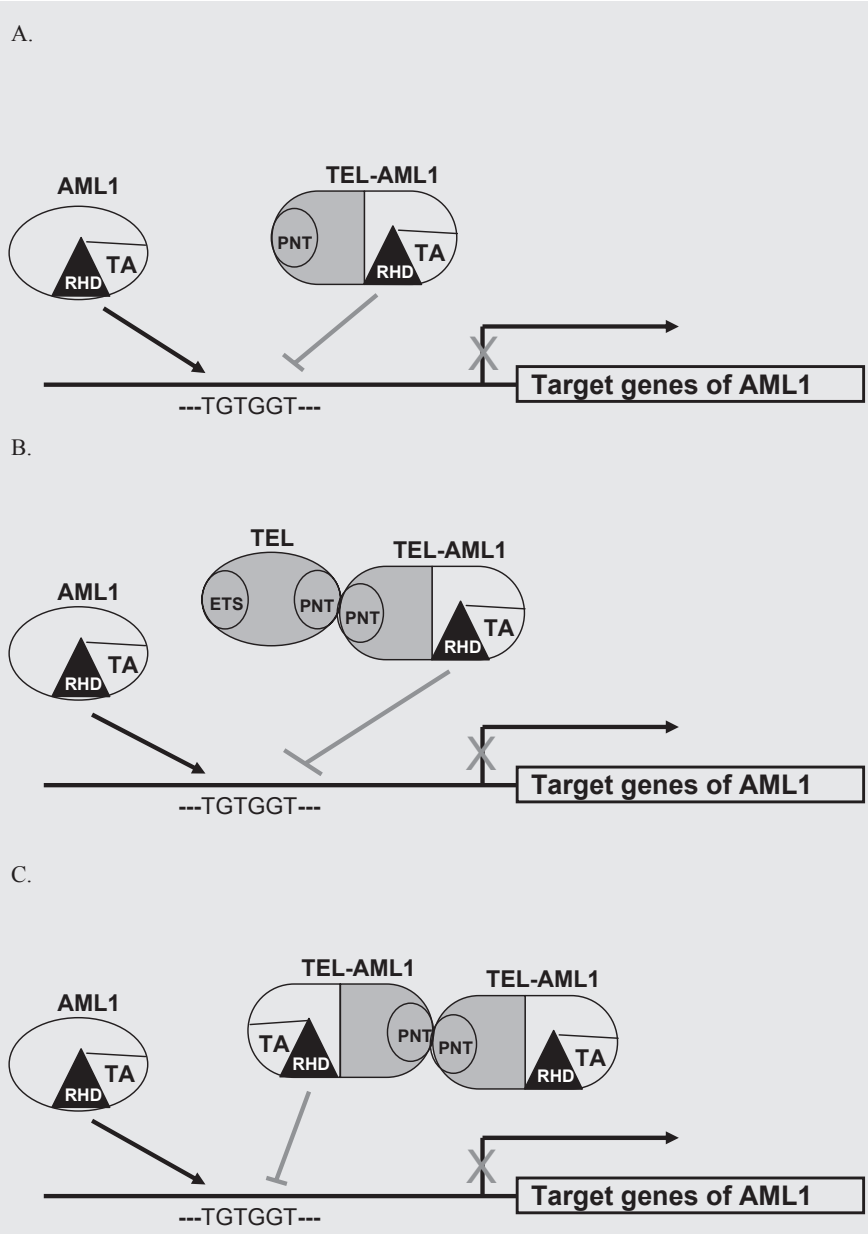

Figure 5. Model for the role of additional abnormalities in *TEL* and *AML1* for binding to targeted genes by *AML1* and/or *TEL-AML1*.
A. Competition for the DNA-binding site on promoters of AML1 target genes between wild-type AML1 (transcriptional activation) and the fusion-product TEL-AML1 (transcriptional repression). **B**. When the non-translocated *TEL* allele is not deleted, a heterodimer between TEL-AML1 and wild-type TEL can be formed. This heterodimer increases the DNA-binding capacity of TEL-AML1 and the competition with wild-type AML1, which leads to more repression of AML1 target genes. **C**. TEL-AML1 can also form a homodimer that increases its DNA-binding capacity leading to more repression of AML1 responsive genes.
RHD, Runt homology domain (DNA-binding domain) of AML1; TA, transactivation domain of AML1; PNT, dimerization domain of TEL; ETS, DNA-binding domain of TEL; TGTGGT, core binding sequence on promoter region of AML1 target genes.

in a heterogenous population of t(12;21) positive cells at the time of clinical presentation of the disease. Rapid and sustained disappearance of the dominant leukemia clones and a slow response of the smaller clones upon first chemotherapy has been observed in t(12;21) positive ALL patients[30,31]. However, no correlation between the immunogenotype pattern or clonal change in t(12;21) positive ALL and the duration of first remission has been observed[32]. It is even suggested that the commonly used combination therapy fails to eradicate the underlying pre-leukemic clone allowing a second independent transformation event after treatment, giving rise to a new leukemia masquerading as relapse[33]. So, the early as well as the late relapses in t(12;21) positive ALL may result from the slow-responding subclones, as well as genetic alteration of a pre-leukemic cell to a new, overt leukemia. It may be speculated that patients without any additional genetic abnormalities in *TEL* or *AML1*, or with an extra der(21)t(12;21) are more sensitive to new hits in other genes (unrelated to *TEL* or *AML1* genes), giving rise to an early relapse.

In concordance with a current model proposing an order of molecular events needed for leukemogenesis[34,35], the expression of TEL-AML1 alone is not sufficient for the induction of hematomalignancies in transgenic mice[36,37]. As the second *TEL* allele is most frequently deleted in t(12;21) positive ALL, it is speculated that wild-type *TEL* acts as a tumor suppressor gene and its deletion in t(12;21) positive ALL is the second hit required for leukemogenesis. However, 30% of the t(12;21) positive ALL patients do not show a deletion of *TEL* indicating that another genetic abnormality is necessary for leukemogenesis. *TEL* itself is known to cooperate with other transcriptional factors in transcriptional regulation of many genes[6]. The chimeric fusion protein TEL-AML1 retains the N-terminal pointed (PNT) domain of TEL and the Runt homology domain (RHD) and transactivation (TA) domain of AML1. The TEL-AML1 protein structure seems an essential element in leukemogenesis, because of the high affinity of these domains for transcription-corepressors such as N-CoR, mSin3 and HDAC3 and the ability of this complex to influence the transcriptional activity of other genes[38,39]. The fusion product TEL-AML1 may compete with AML1 for binding to enhancer core sequences TGTcGGT via the RHD (Figure 5A); the balance between both proteins determines whether AML1-responsive genes are transcribed[40]. The following hypothesis might explain why t(12;21) positive ALL without additional genetic changes in *TEL* or *AML1* or with an extra der(21)t(12;21) may be linked to a poor prognosis. When the non-translocated *TEL* gene is intact, a heterodimer between TEL-AML1 and wild-type TEL can be formed. This heterodimer increases the DNA-binding capacity of TEL-AML1 and shifts the balance into more repression of AML1 target genes (Figure 5B). An extra copy of der(21)t(12;21) may result into more TEL-AML1 homodimers that may compete more effectively with normal AML1-targeted genes than TEL-AML1 monomers (Figure 5C). Both situations (absence of additional genetic changes in *TEL* or *AML1*

and presence of an extra der(21)t(12;21)) may lead to repression of AML1 target genes. Since patients with an extra der(21)t(12;21) are also more resistant to prednisolone, it may be suggested that TEL-AML1 also competes with the glucocorticoid-receptor complex for transcriptional activation of glucocorticoid-responsive genes. Hence our present study postulates that TEL-AML1 itself is associated with an adverse effect on the transcription of targeted genes, whereas the additional genetic changes in *TEL* or *AML1* (deletion or extra copy, respectively) may affect the function of TEL-AML1 resulting in a more favourable phenotype. The high frequency of early relapses in patients with an extra der(21)t(12;21) and patients without additional genetic changes in *TEL* or *AML1* is in line with this new model.

In conclusion, 83% of patients with t(12;21) positive ALL have additional genetic changes in the *TEL* and *AML1* genes. An extra der(21)t(12;21) and the absence of additional genetic abnormalities in these genes are associated with a poorer outcome in t(12;21) positive ALL, which was mainly explained by the fact that these patients suffer from early relapses within 2.5 years after first diagnosis. An extra copy of der(21)t(12;21) was linked to >100-fold prednisolone resistance compared to the remaining group. Multivariate analysis including known risk factors and genetic abnormalities in *TEL* and/or *AML1* showed that only in vitro resistance to prednisolone is an independent prognostic factor in t(12;21) positive ALL.

ACKNOWLEDGEMENTS

We wish to express our gratitude to the members of the DCOG and the German COALL study group for their support to this study by providing leukemic samples.

REFERENCES

1. Hart SM, Foroni L. Core binding factor genes and human leukemia. Haematologica. 2002;87:1307-1323.
2. Golub TR. TEL gene rearrangements in myeloid malignancy. Hematol Oncol Clin North Am. 1997;11:1207-1220.
3. McLean TW, Ringold S, Neuberg D, et al. TEL/AML-1 dimerizes and is associated with a favorable outcome in childhood acute lymphoblastic leukemia. Blood. 1996;88:4252-4258.
4. Jousset C, Carron C, Boureux A, et al. A domain of TEL conserved in a subset of ETS proteins defines a specific oligomerization interface essential to the mitogenic properties of the TEL-PDGFR beta oncoprotein. EMBO J. 1997;16:69-82.
5. Graves BJ, Petersen JM. Specificity within the ets family of transcription factors. Adv Cancer Res. 1998;75:1-55.
6. Sawinska M, Ladon D. Mechanism, detection and clinical significance of the reciprocal translocation t(12;21)(p12;q22) in the children suffering from acute lymphoblastic leukaemia. Leuk Res. 2004;28:35-42.
7. Meyers S, Downing JR, Hiebert SW. Identification of AML-1 and the (8;21) translocation protein (AML-1/ETO) as sequence-specific DNA-binding proteins: the runt homology domain is required for DNA binding and protein-protein interactions. Mol Cell Biol. 1993;13:6336-6345.
8. Loh ML, Rubnitz JE. TEL/AML1-positive pediatric leukemia: prognostic significance and therapeutic approaches. Curr Opin Hematol. 2002;9:345-352.
9. Ramakers-van Woerden NL, Pieters R, Loonen AH, et al. TEL/AML1 gene fusion is related to in vitro drug sensitivity for L-asparaginase in childhood acute lymphoblastic leukemia. Blood. 2000;96:1094-1099.
10. Stams WA, den Boer ML, Beverloo HB, et al. Sensitivity to L-asparaginase is not associated with expression levels of asparagine synthetase in t(12;21)+ pediatric ALL. Blood. 2003;101:2743-2747.
11. Raynaud S, Cave H, Baens M, et al. The 12;21 translocation involving TEL and deletion of the other TEL allele: two frequently associated alterations found in childhood acute lymphoblastic leukemia. Blood. 1996;87:2891-2899.
12. Cave H, Cacheux V, Raynaud S, et al. ETV6 is the target of chromosome 12p deletions in t(12;21) childhood acute lymphocytic leukemia. Leukemia. 1997;11:1459-1464.
13. Fears S, Vignon C, Bohlander SK, Smith S, Rowley JD, Nucifora G. Correlation between the ETV6/CBFA2 (TEL/AML1) fusion gene and karyotypic abnormalities in children with B-cell precursor acute lymphoblastic leukemia. Genes Chromosomes Cancer. 1996;17:127-135.
14. Kobayashi H, Satake N, Maseki N, Sakashita A, Kaneko Y. The der(21)t(12;21) chromosome is always formed in a 12;21 translocation associated with childhood acute lymphoblastic leukaemia. Br J Haematol. 1996;94:105-111.
15. Andreasson P, Johansson B, Strombeck B, Donner M, Mitelman F, Hoglund M. Childhood acute lymphoblastic leukaemia with ider(21)(q10)t(12;21)(p12;q22): a new recurrent abnormality showing ETV6/CBFA2 fusion. Br J Haematol. 1997;98:216-218.
16. Loncarevic IF, Roitzheim B, Ritterbach J, et al. Trisomy 21 is a recurrent secondary aberration in childhood acute lymphoblastic leukemia with TEL/AML1 gene fusion. Genes Chromosomes Cancer. 1999;24:272-277.

17. Den Boer ML, Harms DO, Pieters R, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol. 2003;21:3262-3268.
18. Hagemeijer A, Buijs A, Smit E, et al. Translocation of BCR to chromosome 9: a new cytogenetic variant detected by FISH in two Ph-negative, BCR-positive patients with chronic myeloid leukemia. Genes Chromosomes Cancer. 1993;8:237-245.
19. Sacchi N, Nisson PE, Watkins PC, Faustinella F, Wijsman J, Hagemeijer A. AML1 fusion transcripts in t(3;21) positive leukemia: evidence of molecular heterogeneity and usage of splicing sites frequently involved in the generation of normal AML1 transcripts. Genes Chromosomes Cancer. 1994;11:226-236.
20. Wlodarska I, Baens M, Peeters P, et al. Biallelic alterations of both ETV6 and CDKN1B genes in a t(12;21) childhood acute lymphoblastic leukemia case. Cancer Res. 1996;56:2655-2661.
21. Heerema NA, Sather HN, Sensel MG, et al. Prognostic significance of cytogenetic abnormalities of chromosome arm 12p in childhood acute lymphoblastic leukemia: a report from the Children's Cancer Group. Cancer. 2000;88:1945-1954.
22. Attarbaschi A, Mann G, Konig M, et al. Incidence and relevance of secondary chromosome abnormalities in childhood TEL/AML1+ acute lymphoblastic leukemia: an interphase FISH analysis. Leukemia. 2004;18:1611-1616.
23. Kaspers GJ, Veerman AJ, Pieters R, et al. In vitro cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. Blood. 1997;90:2723-2729.
24. Takahashi Y, Horibe K, Kiyoi H, et al. Prognostic significance of TEL/AML1 fusion transcript in childhood B-precursor acute lymphoblastic leukemia. J Pediatr Hematol Oncol. 1998;20:190-195.
25. Kamps WA, Bokkerink JP, Hakvoort-Cammel FG, et al. BFM-oriented treatment for children with acute lymphoblastic leukemia without cranial irradiation and treatment reduction for standard risk patients: results of DCLSG protocol ALL-8 (1991-1996). Leukemia. 2002;16:1099-1111.
26. Harms DO, Janka-Schaub GE. Co-operative study group for childhood acute lymphoblastic leukemia (COALL): long-term follow-up of trials 82, 85, 89 and 92. Leukemia. 2000;14:2234-2239.
27. Szczepanski T, Orfao A, van der Velden VH, San Miguel JF, van Dongen JJ. Minimal residual disease in leukaemia patients. Lancet Oncol. 2001;2:409-417.
28. Wiemels JL, Cazzaniga G, Daniotti M, et al. Prenatal origin of acute lymphoblastic leukaemia in children. Lancet. 1999;354:1499-1503.
29. McHale CM, Wiemels JL, Zhang L, et al. Prenatal origin of TEL-AML1-positive acute lymphoblastic leukemia in children born in California. Genes Chromosomes Cancer. 2003;37:36-43.
30. Konrad M, Metzler M, Panzer S, et al. Late relapses evolve from slow-responding subclones in t(12;21)-positive acute lymphoblastic leukemia: evidence for the persistence of a preleukemic clone. Blood. 2003;101:3635-3640.
31. Pine SR, Wiemels JL, Jayabose S, Sandoval C. TEL-AML1 fusion precedes differentiation to pre-B cells in childhood acute lymphoblastic leukemia. Leuk Res. 2003;27:155-164.
32. Peham M, Konrad M, Harbott J, Konig M, Haas OA, Panzer-Grumayer ER. Clonal variation of the immunogenotype in relapsed ETV6/RUNX1-positive acute lymphoblastic leukemia

indicates subclone formation during early stages of leukemia development. Genes Chromosomes Cancer. 2004;39:156-160.
33. Ford AM, Fasching K, Panzer-Grumayer ER, Koenig M, Haas OA, Greaves MF. Origins of "late" relapse in childhood acute lymphoblastic leukemia with TEL-AML1 fusion genes. Blood. 2001;98:558-564.
34. Greaves M. Molecular genetics, natural history and the demise of childhood leukaemia. Eur J Cancer. 1999;35:173-185.
35. Mori H, Colman SM, Xiao Z, et al. Chromosome translocations and covert leukemic clones are generated during normal fetal development. Proc Natl Acad Sci U S A. 2002;99:8242-8247.
36. Andreasson P, Schwaller J, Anastasiadou E, Aster J, Gilliland DG. The expression of ETV6/CBFA2 (TEL/AML1) is not sufficient for the transformation of hematopoietic cell lines in vitro or the induction of hematologic disease in vivo. Cancer Genet Cytogenet. 2001;130:93-104.
37. Bernardin F, Yang Y, Cleaves R, et al. TEL-AML1, expressed from t(12;21) in human acute lymphocytic leukemia, induces acute leukemia in mice. Cancer Res. 2002;62:3904-3908.
38. Fenrick R, Amann JM, Lutterbach B, et al. Both TEL and AML-1 contribute repression domains to the t(12;21) fusion protein. Mol Cell Biol. 1999;19:6566-6574.
39. Guidez F, Petrie K, Ford AM, et al. Recruitment of the nuclear receptor corepressor N-CoR by the TEL moiety of the childhood leukemia-associated TEL-AML1 oncoprotein. Blood. 2000;96:2557-2561.
40. Hiebert SW, Sun W, Davis JN, et al. The t(12;21) translocation converts AML-1B from an activator to a repressor of transcription. Mol Cell Biol. 1996;16:1349-1355.

Chapter 3

Expression levels of *TEL*, *AML1* and the fusion products *TEL-AML1* and *AML1-TEL* versus drug sensitivity and clinical outcome in t(12;21) positive pediatric ALL

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Clin Cancer Res 2005;11(8):2974-80

ABSTRACT

PURPOSE: t(12;21)(p13;q22), present in ~25% of pediatric precursor B-ALL, is highly sensitivity to L-Asparaginase and the prognosis depends on the intensity of the treatment protocol. This study analyzes the relationship between the mRNA expression of the genes and fusion products involved in t(12;21), *in vitro* sensitivity to prednisolone, vincristine and L-Asparaginase, and long-term clinical outcome in t(12;21) positive ALL patients.

EXPERIMENTAL DESIGN: Long-term clinical outcome in 45 t(12;21) positive ALL patients was related to mRNA expression of *TEL*, *AML1*, *TEL-AML1* and *AML1-TEL*, determined by real-time quantitative PCR, and the *in vitro* sensitivity to prednisolone, vincristine and L-Asparaginase, using MTT assays.

RESULTS: A significant ~3.5-fold lower *TEL* expression in t(12;21) positive compared to t(12;21) negative ALL samples ($p = 0.006$) and normal controls ($p = 0.004$) was found. Expression of *AML1* did not differ between t(12;21) positive and t(12;21) negative ALL. However, *AML1* expression in the leukemic cells was 2-fold higher compared to normal controls ($p = 0.02$). The *TEL-AML1* fusion product was expressed in all t(12;21) positive cases, whereas the reciprocal fusion product *AML1-TEL* was expressed in only 76%. High expression levels of *TEL-AML1* (hazard ratio (HR) 1.3, 95% confidence interval (CI): 1.10-1.57, $p = 0.003$), *AML1-TEL* (HR 4.9, 95% CI: 1.99-12.40, $p = 0.001$) and *AML1* (HR 1.1, 95% CI: 1.03-1.22, $p = 0.006$) were associated with a poor long-term clinical outcome within t(12;21) positive ALL. Cellular drug resistance towards prednisolone, vincristine and L-Asparaginase could not explain this predictive value. Multivariate analysis including age and WBC showed that only high *AML1-TEL* expression is an independent poor prognostic factor in t(12;21) positive childhood ALL.

CONCLUSION: High *AML1-TEL* expression is an independent poor prognostic factor in t(12;21) positive childhood ALL.

INTRODUCTION

The t(12;21)(p13;q22) occurs in ~25% of childhood acute lymphoblastic leukemia (ALL), and is restricted to precursor B-cell leukemia. The t(12;21) involves fusion of the *TEL (ETV6)* gene at 12p13 with the *AML1 (CBFA2/RUNX1)* gene at 21q22. The breakpoint most often occurs in intron 5 of *TEL* and intron 1 of *AML1*. A frequent translocation variant results in fusion between intron 5 of *TEL* and intron 2 of *AML1*. The *TEL* gene is a member of the ETS family of transcription factors and functions as a transcriptional repressor[1]. *AML1* encodes a transcription factor that acts as a transcription activator as well as a transcriptional repressor[2]. Both genes are frequent targets of chromosomal translocations in a variety of myeloid and lymphoid leukemias[3,4].

Since the discovery of t(12;21), several studies addressed the prognostic value of this particular translocation (reviewed by Loh and Lubnitz[5]). In general, t(12;21) positive ALL is associated with a favourable prognosis although conflicting results have been reported[5]. In the Dutch DCOG ALL-7 and ALL-8 treatment protocols, no prognostic value was found for t(12;21) positive ALL[6]. In addition, the t(12;21) positive ALL group does not seem to be a homogenous group, since ± 20% of the Dutch t(12;21) positive ALL patients relapsed[7]. Furthermore, additional genetic changes in *TEL* and *AML1* e.g. deletion of the non-translocated *TEL* gene, an additional copy of *AML1*, an extra der(21)t(12;21) or combinations of these genetic abnormalities in t(12;21) positive ALL are present in > 80% of patients[7]. We recently showed that the absence of additional genetic changes in *TEL* and *AML1* as well as the presence of an extra der(21)t(12;21) are associated with an un favourable prognosis within t(12;21) positive ALL, which is not independent from prednisolone resistance[7].

In the present study we analyzed whether the expression levels of *TEL, AML1* and the fusion products *TEL-AML1* and *AML1-TEL* are associated with drug sensitivity and long-term clinical outcome in t(12;21) positive ALL.

MATERIALS AND METHODS

Patient samples

Bone marrow and peripheral blood samples from untreated children with common/pre B-ALL at initial diagnosis were collected from the Erasmus MC - Sophia Children's Hospital, the Dutch Childhood Oncology Group (DCOG) and the German COALL study group. Bone marrow and/or peripheral blood samples from the Erasmus MC - Sophia children's hospital from children, who turned out to be non-leukemic, were included as controls. Within 24 hours after sampling, mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (density 1.077 g/ml; Nycomed

Pharma, Oslo, Norway). Contaminating non-leukemic cells in the ALL samples were removed by immunomagnetic beads as described earlier[8]. All resulting samples contained ≥ 90% leukemic cells, as determined morphologically on May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins. For RNA extraction, a minimum of 5×10^6 leukemic cells were lysed in Trizol reagent (Life Technologies, Gaithersburg, USA) and stored at -80°C. 25×10^3 leukemic cells were used for cytospin preparations and stored at -20°C.

FISH analysis

The presence of the t(12;21) was determined on cytospin preparations with dual colored FISH[9] using a digoxigenin labeled cosmid from intron 1 to exon 2 of *TEL* (50F4), together with a biotinylated cosmid for the first 5 exons of AML1 (CO664). FISH probes were kind gifts of Dr. N. Sacchi, University of Milan, Italy[10] (CO664) and Prof. Dr. P. Marynen, Human Genetics, University of Leuven, Belgium[11] (50F4). Probe 50F4 was visualized with Texas Red and probe CO664 with FITC. In t(12;21) positive patients a yellow fusion spot will be visible denoting the der(21)t(12;21), one green signal for the normal *AML1* on chromosome 21 and one red signal for the normal *TEL* on chromosome 12 if not deleted. In all instances two independent observers examined 100-300 interphase nuclei each.

In vitro L-Asparaginase, prednisolone and vincristine cytotoxicity assay

In vitro L-Asparaginase, prednisolone and vincristine cytotoxicity was determined using the MTT assay as described previously[12]. Briefly, 100 µl aliquots of cell suspension (1.6×10^5 cells) were cultured in round-bottomed 96 well microtitre plates in the presence of six concentrations of L-Asparaginase (Paronal, Christiaens B.V., Breda, The Netherlands) ranging from 0.0032 – 10 IU/ml, prednisolone (prednisoloni natrii phosphas, Bufa BV, Uitgeest, The Netherlands) ranging from 0.08 – 250 µg/ml and vincristine (vincristine sulfate, TEVA Pharma BV, Mijdrecht, The Netherlands) ranging from 0.049 – 50 µg/ml in duplicate. Control cells were cultured without L-Asparaginase, prednisolone or vincristine. 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-diphenyltetrazoliumbromide (MTT, 5 mg/ml; Sigma Aldrich, Zwijndrecht, The Netherlands) was added. During a 6-hour incubation, the yellow MTT tetrazolium salt is reduced to purple-blue formazan crystals by viable cells. Samples with ≥ 70% leukemic cells in the control wells and an optical density higher than 0.050 arbitrary units (adjusted for blank values) were used to calculate the concentration of drug lethal to 50% of the cells (LC_{50}).

Real-time quantitative PCR

The t(12;21) positive ALL patients with sufficient material available were selected to perform real-time quantitative PCR, but patients were selected without pre-existing knowledge about the clinical outcome of these patients. Total cellular RNA was extracted from a minimum of 5×10^6 (≥ 90% leukemic) cells using Trizol reagent (Life Technologies) according to the manufacturer's protocol, with minor modifications that improved the quality of RNA. cDNA was synthesized using random hexamers and oligo dT as published previously[13]. The mRNA expression levels of *TEL, AML1, TEL-AML1 and AML1-TEL* and as a reference the endogenous housekeeping gene encoding for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), were quantified using real-time PCR analysis (Taqman chemistry) on a ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster City, USA). Amplification of specific PCR products was detected using dual-fluorescent non-extendable probes labeled with 6-carboxyfluorescein (FAM) at the 5'-end and 6-carboxytetramethylrhodamine (TAMRA) at the 3'-end. The primers and probe combinations were designed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, CO, USA) and purchased from Eurogentec (Seraing, Belgium). The forward and reverse primer and probe combinations were respectively: 5'-ACC CTC TGA TCC TGA ACC-3', 5'-CCG TTG GGA TCC ACT ATC-3' and 5'-TCA TCG GGA AGA CCT GGC TT-3' for *TEL*; 5'-GAC AGC CCC AAC TTC C-3', 5'-CCA CTT CGA CCG ACA A-3' and 5'-CCT GCC CAT CGC TTT CA-3' for *AML1*; 5'-ACC CTC TGA TCC TGA ACC-3', 5'-CAT CCG TGG ACG TCT CT-3' and 5'-TCA TCG GGA AGA CCT GGC TT-3' for *TEL-AML1*; 5'-GAG TCC CAG AGG TAT CCA G-3', 5'-AAT CCC AAA GCA GTC TAC A-3' and 5'-TGA CCT GTC TTG GTT TTC GC- 3' for *AML1-TEL*; 5'-GTC GGA GTC AAC GGA TT-3', 5'-AAG CTT CCC GTT CTC AG-3' and 5'-TCA ACT ACA TGG TTT ACA TGT TCC AA-3' for *GAPDH*. All primers had a melting temperature (T_m; nearest neighbor method) of 65-66.5°C at salt concentration of 303 mM of Na^+-equivalent and 300 nM of primer concentration. Both internal probes had a T_m of 75 ± 1°C. All PCRs performed at comparable efficiencies of ≥ 95%. The real-time quantitative PCR was performed under the same conditions as described before[13]. The comparative cycle time (C_t) value is the target PCR C_t value normalized by subtracting the *GAPDH* C_t value from the target PCR C_t value. From this ΔC_t value, the relative expression level to *GAPDH* in arbitrary units (AU) for each target PCR can be calculated using the following equation: relative mRNA expression $= 2^{-\Delta Ct} \times 100$.

Statistics

Probability of disease-free survival (pDFS) was calculated from the date of diagnosis to the date of non-response, relapse or last contact. The failure to achieve complete remission (CR) at day 56 (non-response) was considered an event at day 56. pDFS curves

were calculated according the Kaplan-Meier method and compared by the Cox proportional-hazard regression model. Multivariate analysis was performed with the Cox proportional-hazard regression model. Statistical tests were performed at a two-tailed significance level of 0.05. Differences in the distribution of variables between groups of patients were analyzed using the Mann-Whitney U test. Bivariate correlations were calculated using the Spearman's rank correlation test.

RESULTS

The mRNA expression levels of *TEL, AML1, TEL-AML1* and *AML1-TEL* were measured in 45 t(12;21) positive pediatric ALL samples, that were validated by FISH analysis. A control group of 26 t(12;21) negative ALL samples was selected by matching for the following criteria: age 1-10 years, immunophenotype, no hyperdiploidy (> 50 chromosomes), no *MLL* rearrangements and no t(9;22). Furthermore, a non-leukemic control group containing both bone marrow and peripheral blood samples (n = 14) was selected in order to compare *TEL* and *AML1* expression in normal and leukemic cells.

TEL-AML1, AML1-TEL, AML1 and TEL mRNA expression and drug resistance

Expression of the fusion product *TEL-AML1* was present in all 45 t(12;21) positive ALL patients tested, whereas the *AML1-TEL* expression was present in only 76% of these cases. We compared the data on the presence of an extra der(21)t(12;21) to the mRNA expression of *TEL-AML1* and *AML1-TEL* (Figure 1). The expression of these fusion genes did not differ between patients with and without an extra der(21)t(12;21) ($p = 0.5$ and $p = 0.3$ respectively). No significant correlation was found between the expression of these fusion genes and sensitivity to L-Asparaginase, prednisolone or vincristine ($-0.233 > R_s < 0.102$, $p > 0.05$).

Expression of *AML1* did not significantly differ between 45 t(12;21) positive and 26 t(12;21) negative ALL ($p = 0.9$; Figure 2A). However, the mRNA expression of *AML1* in these 71 ALL samples (median 5.15 AU) is 2-fold higher compared to 14 normal control bone marrow or peripheral blood samples (median 2.30 AU; $p = 0.02$). Patients with an extra copy of *AML1* in t(12;21) positive ALL do not have a higher expression of *AML1* (median 1.3-fold difference, $p = 0.4$), as shown in Figure 2B. No correlations were found between *AML1* mRNA expression and sensitivity to L-Asparaginase, prednisolone and vincristine within neither t(12;21) positive ($0.154 > R_s < 0.256$, $p > 0.05$) or t(12;21) negative ALL samples ($-0.055 > R_s < 0.172$, $p > 0.05$).

A significant 3.5-fold lower expression of *TEL* mRNA was observed in 33 t(12;21) positive ALL (median 0.10 AU) compared to 23 t(12;21) negative ALL (median 0.33 AU; $p = 0.006$) and 13 normal bone marrow or peripheral blood samples (median 0.39 AU;

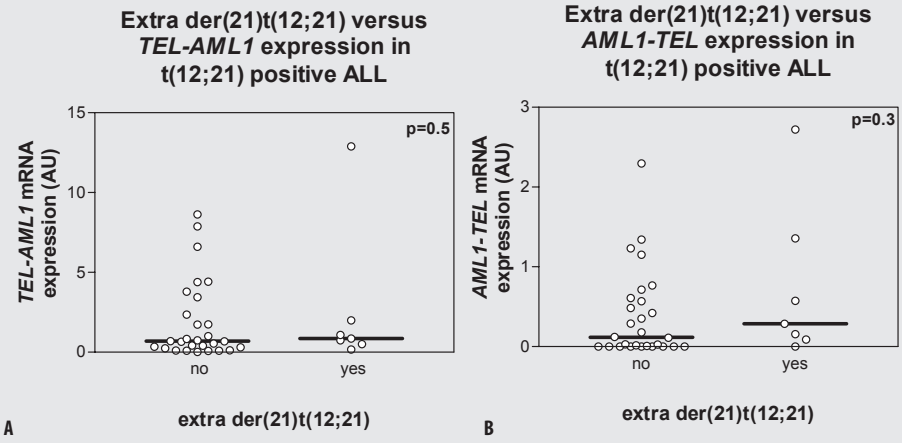

Figure 1. Relationship between an extra copy of der(21)t(12;21) and expression of fusion products of t(12;21) positive ALL.

A. *TEL-AML1*.

mRNA expression of *TEL-AML1* in t(12;21) positive ALL patients with (n=7; median 0.85) and without (n=28; median 0.69) an extra der(21)t(12;21), p=0.5. Arbitrary units (AU, see methods)

B. *AML1-TEL*.

mRNA expression of *AML1-TEL* relative to *GAPDH* in t(12;21) positive ALL patients with (n=7; median 0.29) and without (n=28; median 0.116) an extra der(21)t(12;21), p=0.3. Arbitrary units (AU)

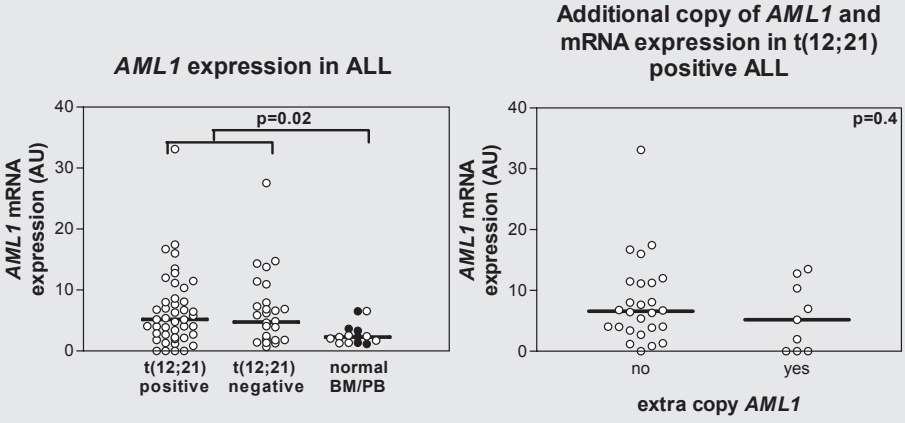

Figure 2.

A. *AML1* expression in ALL.

mRNA expression of *AML1* in 45 t(12;21) positive and 26 negative ALL patients as well as 14 normal controls. Lines indicate the median value; open circles represent bone marrow (BM) and closed circles represent peripheral blood (PB). t(12;21) positive versus negative patients (median 5.19 and 4.75 respectively, p=0.9); all ALL patients versus normal controls (median 5.15 and 2.30 respectively, p=0.02). Arbitrary units (AU)

B. Extra copy of *AML1* versus *AML1* expression.

mRNA expression of *AML1* in t(12;21) positive ALL patients with (n=9; median 5.19) and without an extra copy of *AML1*(n=26; median 6.58, p=0.4). Arbitrary units (AU)

p = 0.004) (Figure 3A). No significant difference in *TEL* mRNA expression was observed between 23 t(12;21) negative ALL and 13 normal bone marrow or peripheral blood samples (p = 0.3). FISH analysis of t(12;21) positive ALL samples indicated that in 70% of the cases a deletion of the non-translocated *TEL* allele had occurred. Interestingly, patients with a deletion of the non-translocated *TEL* allele still demonstrated detectable *TEL* mRNA expression levels (Figure 3B). Further examination revealed that this mRNA is derived from contaminating normal cells in the t(12;21) positive ALL samples

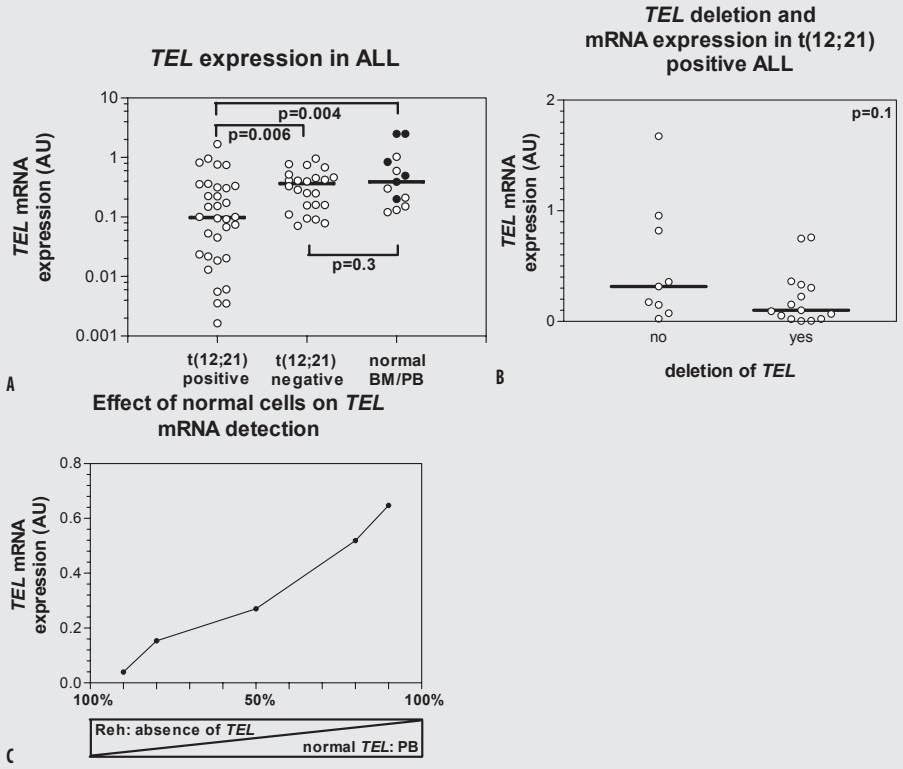

Figure 3.

A. *TEL* expression in ALL

mRNA expression of *TEL* in 33 t(12;21) positive and 23 negative ALL patients as well as in 13 normal controls. Lines indicate the median value; open circles represent bone marrow (BM) and closed circles represent peripheral blood (PB). t(12;21) positive versus negative patients (median 0.10 and 0.33 respectively, p=0.006); t(12;21) positive versus normal controls (median 0.10 and 0.39 respectively, p=0.004); t(12;21) negative patients versus normal controls (median 0.33 and 0.39 respectively, p=0.3). Arbitrary units (AU)

B. Deletion of *TEL* versus *TEL* expression

mRNA expression of *TEL* in t(12;21) positive ALL patients with (n=15; median 0.10) or without (n=9; median 0.32) a deletion of the non-translocated *TEL* allele, p=0.1. Arbitrary units (AU)

C. Effect of normal cells on *TEL* mRNA detection

A dilution series of 0% *TEL* expression (Reh cell line: 100% t(12;21) positive blasts with a deletion of the non-translocated *TEL* allele) to 100% *TEL* expression in normal peripheral blood (PB). Already low amounts (10%) of normal PB cells result into detectable *TEL* expression levels, comparable to the median expression observed in the t(12;21) positive ALL group with a deletion of *TEL* (Figure 3B). Arbitrary units (AU)

(Figure 3C). Despite the fact that our samples were purified towards >90% leukemic cells, the presence of <10% contaminating normal cells contributed to detectable *TEL* mRNA levels. Furthermore, FISH analysis demonstrated that a deletion of the second *TEL* allele is not present in 100% of the leukemic cells. Besides the dominant t(12;21) positive clone, smaller t(12;21) positive clones were observed that had retained the non-translocated *TEL* allele. Therefore expression of *TEL* in t(12;21) positive patients with a deletion of the non-translocated *TEL* allele in the dominant t(12;21) positive clone is probably the result of *TEL* expression in contaminating non-leukemic cells and small t(12;21) positive subclones which retained the *TEL* allele. No correlations were found between *TEL* expression and sensitivity to L-Asparaginase, prednisolone and vincristine within both t(12;21) positive ($-0.105 > R_s < 0.379$, $p > 0.05$) and t(12;21) negative ALL ($-0.041 > R_s < 0.362$, $p > 0.05$).

TEL-AML1, AML1-TEL and AML1 mRNA expression and clinical outcome

Patients with a high expression of *TEL-AML1* (mRNA expression above 75th percentile of total group) had a poorer outcome (3-yrs pDFS 30% ± 25%) than those with low expression levels of *TEL-AML1* (mRNA expression below 75th percentile of total group; 3-yrs pDFS 93% ± 5%; $p = 0.004$) (Figure 4A). Also, high expression of *AML1-TEL* (3-yrs pDFS 51% ± 25%; $p = 0.008$) (Figure 4B) and *AML1* (3-yrs pDFS 25% ± 22%; $p = 0.004$) (Figure 4C) were related to a poor outcome. Cox regression analysis using the mRNA expression levels of *TEL-AML1, AML1-TEL* and *AML1* as continuous variables also indicated that an increase in expression is associated with an increase in relapse risk in t(12;21) positive ALL, whereas an increase in *AML1* expression in t(12;21) negative ALL does not relate with an increased relapse risk (Table 1). In addition, a multivariate analysis including also the known prognostic factors age and WBC at diagnosis was performed (Table 2). In this analysis, only increased expression of *AML1-TEL* was associated with a poor prognosis in t(12;21) positive ALL (hazard ratio 7.02, 95% CI: 2.01-24.52, $p = 0.002$). High expression of *TEL-AML1* mRNA was correlated with high expression of *AML1* ($R_s = 0.524$; $p < 0.001$). This can explain the fact that both *TEL-*

Table 1. Predictive value of fusion-gene and *AML1* expression in t(12;21) positive and negative ALL.

Variable	N	Hazard ratio*	95% CI	P-value
t(12;21) positive ALL patients:				
TEL-AML1 expression	45	1.32	1.10-1.57	0.003
AML1-TEL expression	45	4.97	1.99-12.40	0.001
AML1 expression	45	1.12	1.03-1.22	0.006
t(12;21) negative ALL patients:				
AML1 expression	26	0.87	0.74-1.03	0.1

* Univariate cox proportional-hazard analysis using mRNA expression levels as continuous variable.

Table 2. Multivariate analysis of risk factors in t(12;21) positive ALL.

Variable	Unfavourable feature	Hazard ratio*	95% CI	P-value
Age at diagnosis (yrs)	>10	9.05	0.47-173.31	0.14
WBC at diagnosis (*10^9cells/L)	>25	4.70	0.53-41.90	0.17
TEL-AML1 expression		1.23	0.79-1.93	0.36
AML1-TEL expression		7.02	2.01-24.52	0.002
AML1 expression		1.10	0.84-1.43	0.49

* Cox proportional-hazard analysis using mRNA expression levels as continuous variable.

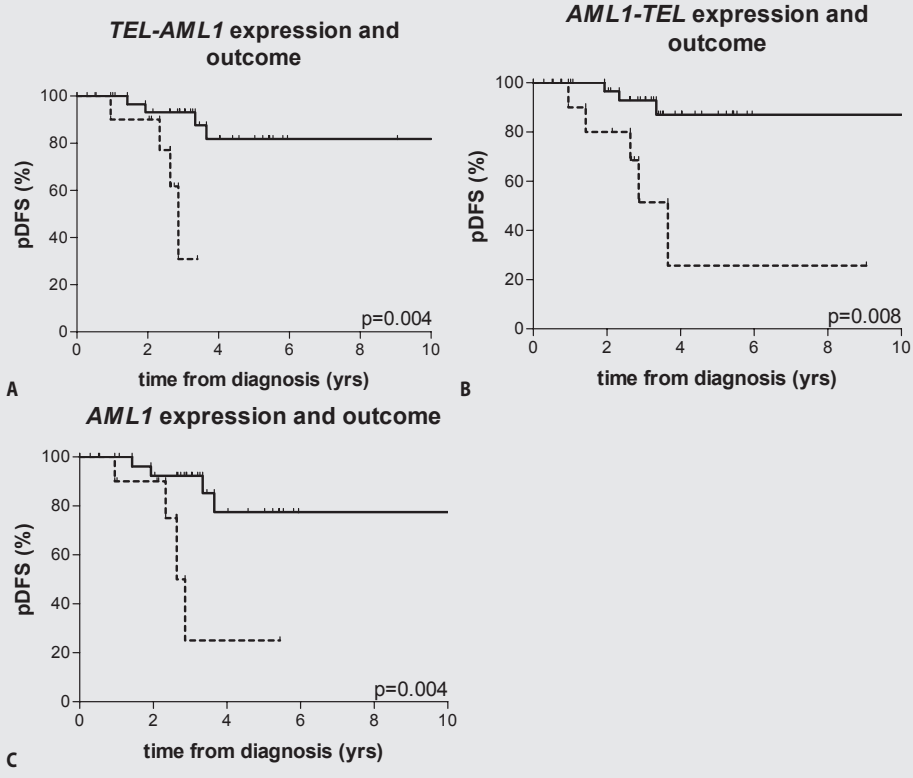

Figure 4. Disease free survival analysis in t(12;21) positive ALL.
Patients were divided into two groups by the 75th percentile for the expression of *TEL-AML1* (**A**), *AML1-TEL* (**B**) and *AML1* (**C**). High expression (P>75) dashed line; low expression (P < 75) solid line. Differences in pDFS were analyzed by the Cox proportional-hazard regression model (p_{cox}).
A. High expression is associated with an unfavourable prognosis (3-yrs pDFS 30% ± 25%) compared to low expression (3-yrs pDFS 93% ± 5%; p_{cox}=0.004; $p_{cox\ continous\ variable}$=0.003, see Table 2). **B**. High expression is associated with an unfavourable prognosis (3-yrs pDFS 51% ± 25%) compared to low expression (3-yrs pDFS 93% ± 6%; p_{cox}=0.008; $p_{cox\ continous\ variable}$=0.001, see Table 2). *C*. High expression is associated with an unfavourable prognosis (3-yrs pDFS 25% ± 22%) compared to low expression (3-yrs pDFS 93% ± 5%; p_{cox}=0.004; $p_{cox\ continous\ variable}$=0.006, see Table 2).

AML1 and *AML1* are no independent prognostic factors in the multivariate analysis. The expression of *AML1-TEL* was not related to either *TEL-AML1* or *AML1* expression ($R_s = 0.126$; $p = 0.4$ and $R_s = 0.097$; $p = 0.5$ respectively).

DISCUSSION

In the present study we examined the relation between *TEL, AML1, TEL-AML1* and *AML1-TEL* mRNA expression, the additional genetic changes in *TEL* and *AML1* genes, the in vitro sensitivity to L-Asparaginase, prednisolone and vincristine and clinical outcome in children with t(12;21) positive ALL at initial diagnosis.

A significantly lower expression of *TEL* was found in t(12;21) positive ALL patients compared to t(12;21) negative ALL patients and normal controls. This can be explained by the fact that the non-translocated *TEL* allele is frequently (± 70%) deleted in t(12;21) positive ALL[114-16]. So far, only one earlier study analyzed the mRNA expression of wild-type *TEL* in childhood acute leukemia[17]. In contrast to our results, Patel et al. found no difference in *TEL* expression between t(12;21) positive ALL and a control group, but these authors did not specify the percentage of blasts in their t(12;21) positive ALL samples. In the present study we observed that low expression levels of *TEL* may originate from contaminating non-leukemic cells present in t(12;21) positive ALL samples with a deletion of the second *TEL* allele, and from small t(12;21) positive subclones retaining the *TEL* allele.

No difference in *AML1* expression was found between t(12;21) positive ALL patients (with or without an additional copy of *AML1*) and t(12;21) negative ALL patients, although only in t(12;21) positive ALL patients high *AML1* expression is related to a poor outcome. Our data show that *AML1* expression is significantly elevated in both ALL subgroups compared to the normal control group. This might be due to the fact that expression of *AML1* is required for proliferation since *AML1* regulates G_1 to S cell cycle transition[18,19]. Although the AML1 expression in t(12;21) positive and negative ALL is comparable, differences in cell cycle are present in these two groups of ALL. The percentage of cells in S phase is lower in t(12;21) positive ALL compared to t(12;21) negative ALL[20]. This might be explained by the fact that in t(12;21) positive patients TEL-AML1 competes with wild-type AML1 to bind the DNA and acts as a transcriptional repressor of AML1 target genes[21].

Conflicting data on the prognostic relevance of t(12;21) positive ALL have been reported ranging from 60%-100% survival (reviewed by Loh et al.)[5,22-25]. Initially, studies reported favourable outcome of t(12;21) positive ALL patients compared to t(12;21) negative ALL patients. Later on, several studies could not confirm this prognostic relevance, among which DCOG-treated t(12;21) positive ALL[6,7]. The intensity of treatment

given to t(12;21) positive ALL patients seems to contribute to a favourable outcome[26-28]. Our data show that a high expression of *TEL-AML1*, *AML1-TEL* and *AML1* are related to a poor prognosis in pediatric t(12;21) positive ALL. However, only the expression of *AML1-TEL* is an independent prognostic factor in t(12;21) positive pediatric ALL. Current research focuses on determining the function of the TEL-AML1 fusion protein in leukemogenesis, since the *TEL-AML1* fusion product is expressed in all t(12;21) positive ALL cases whereas the reciprocal fusion product *AML1-TEL* is not. In mouse models, TEL-AML1 alone is insufficient for leukemogenesis, but may result in leukemia when additional mutations are present[29,30]. The presence of AML1-TEL expression did not make a difference in inducing hematologic disease in transgenic mice[29]. The *TEL-AML1* fusion product was detected in neonatal blood spots and cord blood samples at a hundred times higher frequency than expected from the corresponding leukemia incidence[31,32]. This finding together with the mouse model studies suggests that secondary additional genetic changes are required for leukemogenesis. As the second *TEL* allele is most frequently deleted in t(12;21) positive ALL, it is speculated that wild-type *TEL* acts as a tumor suppressor gene and its deletion in t(12;21) positive ALL is the second hit required for leukemogenesis. However, 30% of the t(12;21) positive ALL patients do not show a deletion of *TEL* indicating that another genetic abnormality is necessary for leukemogenesis. In a previous study, we showed the absence of additional genetic changes in *TEL* and *AML1* genes as well as an extra der(21)t(12;21) are associated with an unfavourable prognosis in pediatric t(12;21) positive ALL[7]. As shown in the present study, expression levels of *TEL-AML1* and *AML1* were not increased in patients with an extra der(21)t(12;21) or an additional copy of *AML1* respectively. However, this might be due to the fact that the discrimination level of the RTQ-PCR is minimal 2-fold.

As shown in the present study, *AML1-TEL* expression levels are associated with outcome. Resistance to prednisolone, vincristine or L-Asparaginase cannot explain this predictive value. Therefore, *AML1-TEL* may be involved in cell regrowth rather than in toxic response pathways. The *AML1-TEL* fusion product contains exon 1 or exon 1 and 2 of *AML1*, in which no functional domain is present and the last three exons of *TEL*, which contains the ETS domain. In the *TEL* gene, a repression domain was identified which encompasses the ETS domain and the 50 amino-acids immediately upstream of the ETS domain[1]. It is hypothesized that ETS proteins with transcriptional repression activity (like TEL) are primarily involved in ensuring the balance between cellular proliferation and differentiation in different cell types and developmental stages, in response to extracellular signals[33]. The isolated ETS domain of TEL binds conventional ETS binding sites (EBS) in vitro and regulates EBS driven transcription[34-36]. It can be hypothesized that AML1-TEL acts comparable to an isolated ETS domain and competes for binding with the endogenous TEL or acts like TEL in the absence of wild-type TEL. Therefore, it seems unlikely that AML1-TEL will not have a function in t(12;21)

positive ALL. This is the first study showing that *AML1-TEL* expression is associated with prognosis in t(12;21) positive ALL. Further characterization and validation of *AML1-TEL* expression is required to determine the therapeutic implications of the *AML1-TEL* expression levels in t(12;21) positive ALL.

ACKNOWLEDGEMENTS

We wish to express our gratitude to the members of the DCOG and the German COALL study group for their support to this study by providing leukemic samples.

REFERENCES

1. Lopez RG, Carron C, Oury C, Gardellin P, Bernard O, Ghysdael J. TEL is a sequence-specific transcriptional repressor. J Biol Chem. 1999;274:30132-30138.
2. Michaud J, Scott HS, Escher R. AML1 interconnected pathways of leukemogenesis. Cancer Invest. 2003;21:105-136.
3. Hart SM, Foroni L. Core binding factor genes and human leukemia. Haematologica. 2002;87:1307-1323.
4. Golub TR. TEL gene rearrangements in myeloid malignancy. Hematol Oncol Clin North Am. 1997;11:1207-1220.
5. Loh ML, Rubnitz JE. TEL/AML1-positive pediatric leukemia: prognostic significance and therapeutic approaches. Curr Opin Hematol. 2002;9:345-352.
6. Hubeek I, Ramakers-van Woerden NL, Pieters R, et al. TEL/AML1 fusion is not a prognostic factor in Dutch childhood acute lymphoblastic leukaemia. Br J Haematol. 2001;113: 254-255.
7. Stams WA, Beverloo HB, den Boer ML, et al. Incidence of additional genetic changes in the TEL and AML1 genes in t(12;21) positive pediatric ALL and their relation with drug sensitivity and clinical outcome. submitted.
8. Kaspers GJ, Veerman AJ, Pieters R, et al. Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay. Br J Cancer. 1994;70:1047-1052.
9. Hagemeijer A, Buijs A, Smit E, et al. Translocation of BCR to chromosome 9: a new cytogenetic variant detected by FISH in two Ph-negative, BCR-positive patients with chronic myeloid leukemia. Genes Chromosomes Cancer. 1993;8:237-245.
10. Sacchi N, Nisson PE, Watkins PC, Faustinella F, Wijsman J, Hagemeijer A. AML1 fusion transcripts in t(3;21) positive leukemia: evidence of molecular heterogeneity and usage of splicing sites frequently involved in the generation of normal AML1 transcripts. Genes Chromosomes Cancer. 1994;11:226-236.
11. Wlodarska I, Baens M, Peeters P, et al. Biallelic alterations of both ETV6 and CDKN1B genes in a t(12;21) childhood acute lymphoblastic leukemia case. Cancer Res. 1996;56: 2655-2661.
12. Den Boer ML, Harms DO, Pieters R, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol. 2003;21:3262-3268.
13. Stams WA, den Boer ML, Beverloo HB, et al. Sensitivity to L-asparaginase is not associated with expression levels of asparagine synthetase in t(12;21)+ pediatric ALL. Blood. 2003;101:2743-2747.
14. Raynaud S, Cave H, Baens M, et al. The 12;21 translocation involving TEL and deletion of the other TEL allele: two frequently associated alterations found in childhood acute lymphoblastic leukemia. Blood. 1996;87:2891-2899.
15. Romana SP, Le Coniat M, Poirel H, Marynen P, Bernard O, Berger R. Deletion of the short arm of chromosome 12 is a secondary event in acute lymphoblastic leukemia with t(12;21). Leukemia. 1996;10:167-170.
16. Cave H, Cacheux V, Raynaud S, et al. ETV6 is the target of chromosome 12p deletions in t(12;21) childhood acute lymphocytic leukemia. Leukemia. 1997;11:1459-1464.

17. Patel N, Goff LK, Clark T, et al. Expression profile of wild-type ETV6 in childhood acute leukaemia. Br J Haematol. 2003;122:94-98.
18. Strom DK, Nip J, Westendorf JJ, et al. Expression of the AML-1 oncogene shortens the G(1) phase of the cell cycle. J Biol Chem. 2000;275:3438-3445.
19. Bernardin-Fried F, Kummalue T, Leijen S, Collector MI, Ravid K, Friedman AD. AML1/RUNX1 increases during G1 to S cell cycle progression independent of cytokine-dependent phosphorylation and induces cyclin D3 gene expression. J Biol Chem. 2004;279:15678-15687.
20. Stams WA, den Boer ML, Beverloo HB, van Wering ER, Pieters R. Upregulation of AS and cell cycle arrest in t(12;21) positive ALL. Leukemia. 2005;19:318-9.
21. Hiebert SW, Sun W, Davis JN, et al. The t(12;21) translocation converts AML-1B from an activator to a repressor of transcription. Mol Cell Biol. 1996;16:1349-1355.
22. Veiga LB, Coser VM, Cavalli LR, et al. High frequency of t(12;21)(p13;q22) in children with acute lymphoblastic leukemia and known clinical outcome in southern Brazil. Leuk Res. 2004;28:1033-1038.
23. Zen PR, Lima MC, Coser VM, et al. Prevalence of TEL/AML1 fusion gene in Brazilian pediatric patients with acute lymphoblastic leukemia. Cancer Genet Cytogenet. 2004;151:68-72.
24. Sazawal S, Bhatia K, Gutierrez MI, Saxena R, Arya LS, Bhargava M. Paucity of TEL-AML 1 translocation, by multiplex RT-PCR, in B-lineage acute lymphoblastic leukemia (ALL) in Indian patients. Am J Hematol. 2004;76:80-82.
25. Kanerva J, Saarinen-Pihkala UM, Niini T, et al. Favorable outcome in 20-year follow-up of children with very-low-risk ALL and minimal standard therapy, with special reference to TEL-AML1 fusion. Pediatr Blood Cancer. 2004;42:30-35.
26. Takahashi Y, Horibe K, Kiyoi H, et al. Prognostic significance of TEL/AML1 fusion transcript in childhood B-precursor acute lymphoblastic leukemia. J Pediatr Hematol Oncol. 1998;20:190-195.
27. Maloney K, McGavran L, Murphy J, et al. TEL-AML1 fusion identifies a subset of children with standard risk acute lymphoblastic leukemia who have an excellent prognosis when treated with therapy that includes a single delayed intensification. Leukemia. 1999;13:1708-1712.
28. Avigad S, Kuperstein G, Zilberstein J, et al. TEL-AML1 fusion transcript designates a favorable outcome with an intensified protocol in childhood acute lymphoblastic leukemia. Leukemia. 1999;13:481-483.
29. Andreasson P, Schwaller J, Anastasiadou E, Aster J, Gilliland DG. The expression of ETV6/CBFA2 (TEL/AML1) is not sufficient for the transformation of hematopoietic cell lines in vitro or the induction of hematologic disease in vivo. Cancer Genet Cytogenet. 2001;130:93-104.
30. Bernardin F, Yang Y, Cleaves R, et al. TEL-AML1, expressed from t(12;21) in human acute lymphocytic leukemia, induces acute leukemia in mice. Cancer Res. 2002;62:3904-3908.
31. Wiemels JL, Cazzaniga G, Daniotti M, et al. Prenatal origin of acute lymphoblastic leukaemia in children. Lancet. 1999;354:1499-1503.
32. Mori H, Colman SM, Xiao Z, et al. Chromosome translocations and covert leukemic clones are generated during normal fetal development. Proc Natl Acad Sci U S A. 2002;99:8242-8247.

33. Mavrothalassitis G, Ghysdael J. Proteins of the ETS family with transcriptional repressor activity. Oncogene. 2000;19:6524-6532.
34. Poirel H, Oury C, Carron C, et al. The TEL gene products: nuclear phosphoproteins with DNA binding properties. Oncogene. 1997;14:349-357.
35. Poirel H, Lopez RG, Lacronique V, et al. Characterization of a novel ETS gene, TELB, encoding a protein structurally and functionally related to TEL. Oncogene. 2000;19:4802-4806.
36. Potter MD, Buijs A, Kreider B, van Rompaey L, Grosveld GC. Identification and characterization of a new human ETS-family transcription factor, TEL2, that is expressed in hematopoietic tissues and can associate with TEL1/ETV6. Blood. 2000;95:3341-3348.

Part B

Causes of sensitivity and resistance to L-Asparaginase in t(12;21) positive ALL

Chapter 4

Sensitivity to L-Asparaginase is not associated with expression levels of asparagine synthetase in t(12;21) positive pediatric ALL

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Blood 2003;101(7):2743-7

ABSTRACT

The (12;21) translocation resulting in *TEL-AML1* gene fusion is present in ~25% of childhood precursor B-lineage acute lymphoblastic leukemia (ALL) and is associated with a good prognosis and a high cellular sensitivity to L-Asparaginase. ALL cells are thought to be sensitive to L-Asparaginase due to lower asparagine synthetase (AS) levels. Resistance to L-Asparaginase may be caused by an elevated cellular level of AS and/or by the ability of resistant cells to rapidly induce the expression of the *AS* gene upon L-Asparaginase exposure. *AS* may be a target regulated by t(12;21). We studied the relationship between t(12;21) and the mRNA level of *AS* in order to investigate a possible mechanism underlying L-Asparaginase sensitivity. Real-time quantitative RT-PCR analysis surprisingly revealed that 30 t(12;21) positive patients expressed 5-fold more *AS* mRNA compared to 17 t(12;21) negative patients ($p = 0.008$) and 11 samples of healthy controls ($p = 0.016$). The mRNA levels of *AS* between t(12;21) negative ALL and healthy controls did not differ. No difference was found between t(12;21) positive and negative ALL patients in the capacity to upregulate *AS* after *in vitro* L-Asparaginase exposure, excluding a defective capacity for t(12;21) cells in upregulating *AS* upon L-Asparaginase exposure. Moreover, no correlation was observed between *AS* mRNA expression and sensitivity to L-Asparaginase. We conclude that the sensitivity of t(12;21) positive childhood ALL to L-Asparaginase is not associated with the expression level of the *AS* gene. Furthermore, we contradict the general thought that leukemic cells specifically lack AS compared to normal bone marrow and blood cells.

INTRODUCTION

The t(12;21) occurs in ~25% of childhood acute lymphoblastic leukemia (ALL), and is restricted to precursor B-cell lineage leukemia. The t(12;21) involves fusion of the *TEL(ETV6)* gene at 12p13 with the *AML1(RUNX1)* gene at 21q22. The *TEL* gene is a member of the Ets family of transcription factors and functions as a sequence-specific DNA-binding transcription regulator[1]. *AML1* encodes a transcription factor that binds the enhancer core sequence, TGTGGT[2]. The DNA-binding affinity of AML1 is increased through heterodimerization with the Core Binding Factor (CBF) β protein, forming the CBF. This complex regulates the transcription of numerous genes involved in hematopoiesis.

t(12;21) positive ALL has a relatively favourable outcome[3-9] which might be related to the finding that this type of ALL is significantly more sensitive *in vitro* to L-Asparaginase[10]. L-Asparaginase is an enzyme-derived drug widely used in chemotherapeutic protocols for treatment of children with ALL. *In vitro* resistance to L-Asparaginase is correlated with a relative poor prognosis *in vivo*[11,12]. The proposed mechanism of action of L-Asparaginase is the depletion of asparagine and glutamine in the blood leading to cellular efflux and depletion of these amino acids within cells[13]. ALL cells are thought to be particularly sensitive to L-Asparaginase treatment because of a relative low capacity to synthesize sufficient asparagine due to intrinsic lower asparagine synthetase (AS) levels[14,15]. Resistance to L-Asparaginase is suggested to be caused by an elevated cellular level of AS and/or by the ability of resistant cells to rapidly induce the expression of the *AS* gene upon L-Asparaginase exposure[16].

The enhancer core sequence of AML1 is required for the transcription of several hematopoietic-specific genes, including the T-cell receptor beta (TCRβ) enhancer. Although TEL-AML1 can bind to the enhancer core motif, and interacts with the AML1-binding protein, CBFβ, it fails to activate transcription but rather inhibits the basal activity of this enhancer[17]. Since the *AS* gene contains an enhancer core sequence in the promotor region, this gene may become transcriptionally repressed by TEL-AML1 through a similar mechanism. The resulting inhibition of the basal activity of AS would explain the sensitivity to L-Asparaginase for t(12;21) positive ALL compared to t(12;21) negative ALL. In the present study we investigated whether this hypothesis is valid in pediatric ALL. We determined basal *AS* mRNA expression levels and possible upregulation of *AS* levels in cultured blood/bone marrow samples of t(12;21) positive and t(12;21) negative children with ALL and a healthy pediatric control group. In the ALL cases these *AS* expression levels were related to L-Asparaginase sensitivity.

MATERIALS AND METHODS

Patient samples

Bone marrow and/or peripheral blood samples from untreated children with common/pre B-ALL at initial diagnosis were collected from the Erasmus MC / Sophia children's hospital, the Dutch Childhood Leukemia Study Group (DCLSG) and the German COALL study group. After informed consent bone marrow and/or peripheral blood samples from healthy children were included as controls from 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 min 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; Life Technologies, Gaithersburg, USA), 20% fetal calf serum (Integro, Zaandam, the Netherlands), 2mM L-glutamine (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 pencillin, 100 μg/ml streptomycin, 0.125 μg/ml fungizone (Gibco BRL, Life Technologies) and 0.2 mg/ml gentamycin (Life Technologies). Contaminating non-leukemic cells in the ALL samples were removed by immunomagnetic beads as described earlier[18]. All resulting samples contained ≥ 90% leukemic cells, as determined morphologically on May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins. For RNA extraction, a minimum of 5×10^6 leukemic cells were lysed in Trizol reagent (Life Technologies) and stored at -80°C. 0.025×10^6 leukemic cells were used for cytospin preparations for Fluorescence in situ Hybridization (FISH) analysis and stored at -20°C.

FISH analysis

The presence of the t(12;21) was determined with dual colored FISH using a digoxigenin labeled cosmid from intron 1 to exon 2 of *TEL* (50F4), together with a biotinylated cosmid for the first 5 exons of AML1 (CO664). Probe 50F4 was detected with Texas Red and probe CO664 with avidin-FITC. In t(12;21) positive patients a yellow fusion spot will be seen denoting the der(21), one green signal for the normal *AML1* on chromosome 21 and one red signal for the normal *TEL* on chromosome 12. The FISH protocol was based on that described previously[19]. In all instances two independent observers examined 100-300 interphase nuclei.

In vitro L-Asparaginase cytotoxicity assay

In vitro L-Asparaginase cytotoxicity was determined using the MTT assay as described previously[20]. Briefly, 100 μl aliquots of cell suspension (1.6×10^5 cells) were cultured in round-bottomed 96 well microtitre plates in the presence of six different concentra-

tions of L-Asparaginase (Paronal, Christiaens B.V., Breda, The Netherlands) ranging from 0.0032 – 10 IU/ml in duplicate. Control cells were cultured without L-Asparaginase. 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-diphenyltetrazoliumbromide (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[21], was measured spectrophotometrically at 562 nm. After subtraction of blank values, the leukemic cell survival (LCS) was calculated by the equation:

$$LCS = (OD_{Day4} \text{ treated well / mean } OD_{Day4} \text{ control wells}) \times 100\%$$

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 OD was ≥ 0.050[20].

RNA extraction and cDNA synthesis

Total cellular RNA was extracted from a minimum of 5×10^6 (≥ 90% leukemic) cells using Trizol reagent (Life Technologies) according to the manufacturer's protocol, with 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 (10mM Tris-HCl, 1 mM EDTA, pH = 8.0). The concentration of RNA was quantitated spectrophotometrically. Following a denaturation step of 5 min at 70°C, 1 µg of RNA was reversely transcribed into single stranded cDNA. The RT reaction was performed in a total volume of 25 µl containing 0.2 mM of random hexamers and 0.2 mM oligo dT primers (Amersham Pharmacia Biotech, Piscataway NJ, USA), 200 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison Wisconsin, USA) and 25 U RNAsin (Promega) and was incubated at 37°C for 30 min, 42°C for 15 min and 94°C for 5 min. The obtained cDNA was diluted to a final concentration of 8 ng/µl and stored at -80°C.

Real-time quantitative PCR

The mRNA expression levels of *AS* and an endogenous housekeeping gene encoding for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference, were quanti-

Table 1. Primer and probe combinations used for the real-time quantitative PCR

AS	Forward	5'-GCC CAT GGT CTT GAA CT-3'
	Reverse	5'-TTT GGT CGC CAG AGA AT-3'
	Probe	5'-(FAM)-CTT GTC TCT GCC ACC AGA AAT GA-(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'

fied using real-time PCR analysis (Taqman chemistry) on a ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster City, USA). Amplification of specific PCR products was detected using dual-fluorescent non-extendable probes (hybridizing in between primer pairs) labeled with 6-carboxyfluorescein (FAM) at the 5'-end and 6-carboxytetramethylrhodamine (TAMRA) at the 3'-end. The primers and probe combinations (table 1) were designed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, CO, USA) and purchased from Eurogentec (Belgium). All primers had a melting temperature (T_m; nearest neighbor method) of 65 ± 1°C. Both internal probes had a T_m of 75 ± 1°C. All PCRs performed with comparable efficiencies of ≥ 95%. The real-time quantitative PCR was performed in a total reaction volume of 50 µl containing 1 × Taqman buffer A (Applied Biosystems), 4 mM $MgCl_2$, 200 µM of each dNTP, 300 nM forward and reverse primer, 50 nM dual-labeled fluorogenic internal probe, 1.25 U Ampli*Taq* Gold DNA polymerase and 40 ng of cDNA template, in a MicroAmp optical 96-well plate covered with optical adhesive covers (Applied Biosystems). Samples were heated for 10 min at 95°C and amplified for 40 cycles of 15 sec at 95°C and 60 sec 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) in dH_2O was amplified in parallel to verify the amplification efficiency within each experiment. Since all PCRs were performed with equal efficiencies, relative mRNA expression levels of *AS* for each patient can directly be normalized for input RNA using *GAPDH* expression of the patient. The relative mRNA expression level of the target gene in each patient was calculated using comparative cycle time (C_t) method[22]. Briefly, the target PCR C_t values, i.e. the cycle number at which emitted fluorescence exceeds the 10 × standard deviation of base-line emissions as measured from cycles 3 to 12, is normalized 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 expression level to *GAPDH* for each target PCR can be calculated using the following equation:

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

Upregulation of AS expression levels after in vitro L-Asparaginase exposure

Leukemic samples with a purity of at least 90% leukemic cells were exposed to 0 IU/ml (control), 0.4 IU/ml and 10 IU/ml L-Asparaginase (Paronal, Christiaens B.V.) for 0, 18 and 42 hours. A total of 10×10^6 cells suspended in a concentration of 2.0×10^6 cells/ml in culture medium for each concentration and time point were placed into culture flasks. After 18 and 42 hours incubation, the samples still contained ≥ 90% leukemic cells. For RNA extraction, cells were lysed in Trizol reagent (Life Technologies) and stored at -80°C.

Statistics

Differences in mRNA expression between two groups were analyzed using the Mann-Whitney U test. The correlation between mRNA expression of *AS* and L-Asparaginase sensitivity were calculated using the Spearman's rank correlation test. Statistical tests were performed at a two-tailed significance level of 0.05.

RESULTS

Leukemic cells from a group of 82 children with the t(12;21) were compared to leukemic samples of 40 t(12;21) negative pediatric common or pre B-ALL for L-Asparaginase sensitivity. In this group we were able to confirm that t(12;21) positive patients are significantly more sensitive to L-Asparaginase than t(12;21) negative ALL, as described earlier[6]. Using real-time quantitative PCR the mRNA expression level of *AS* was measured in 30 t(12;21) positive pediatric ALL samples. For this t(12;21) positive group a control group of 17 t(12;21) negative ALL samples was selected by matching for the following criteria: age 1-10 yrs, immunophenotype, no hyperdiploidy (> 50), no *MLL* rearrangements, no t(9;22). A significant 5-fold higher expression of AS mRNA was observed in t(12;21) positive ALL compared to t(12;21) negative ALL ($p = 0.008$) (Figure 1).

Expression of *AS* mRNA in the t(12;21) positive ALL was also significant greater than in 11 normal healthy controls ($p = 0.019$) (Figure 1). No difference in mRNA expression of *AS* between t(12;21) negative ALL and healthy pediatric controls was found (Figure 1). Bone marrow and peripheral blood cells in the leukemic samples as well as in the normal controls did not differ in *AS* mRNA expression and therefor pooled together with the bone marrow samples.

The t(12;21) positive ALL group could be divided in 3 subgroups based on sensitivity to L-Asparaginase using previously reported cut-off points[11,23]. From 14 sensitive, 10 intermediate sensitive and 6 resistant patients the mRNA expression of *AS* did not differ (Figure 2). Neither the total ALL group, including both t(12;21) positive and nega-

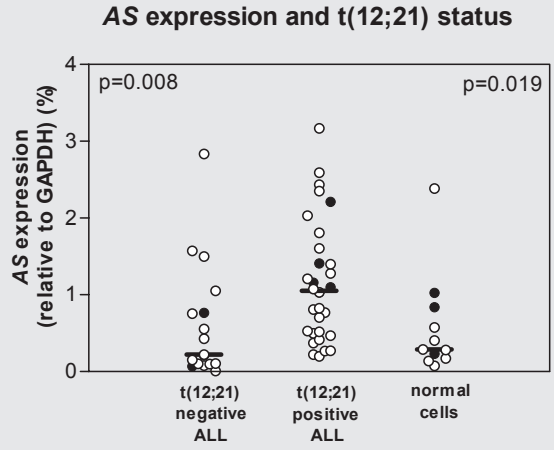

Figure 1. *AS* expression and t(12;21) status.
mRNA expression of *AS* relative to *GAPDH* in t(12;21) negative and positive ALL and in normal controls. Lines indicate the median value, open circles represent bone marrow of individual patients and closed circles represent peripheral blood of individual patients. P=0.008 relates to the comparison between the t(12;21) negative and positive patient groups; P=0.019 relates to the comparison between the t(12;21) positive patient group and normal controls.

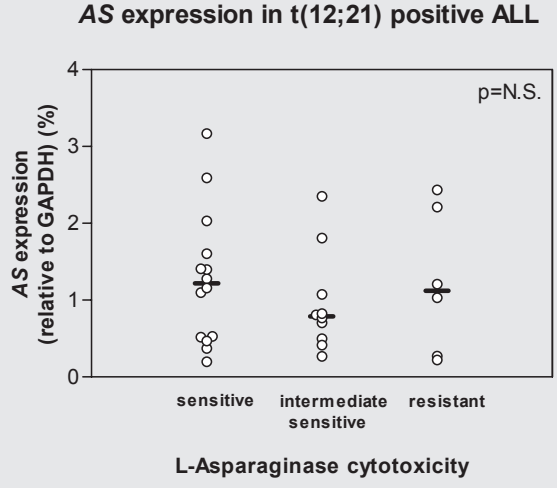

Figure 2. *AS* expression in t(12;21) ALL.
mRNA expression of *AS* relative to *GAPDH* in t(12;21) positive ALL patients who are *in vitro* sensitive, intermediate sensitive and resistant to L-Asparaginase. Lines indicate the median values, circles represent individual patients. The P-value indicates the difference between patient groups (N.S. = not significant).

tive samples, nor both groups separately, showed a correlation between L-Asparaginase sensitivity and the *AS* mRNA expression (Figure 3).

Hypothetically, t(12;21) positive ALL cells may be sensitive to L-Asparaginase due to a defective capacity to upregulate *AS* after L-Asparaginase exposure. Therefore, samples

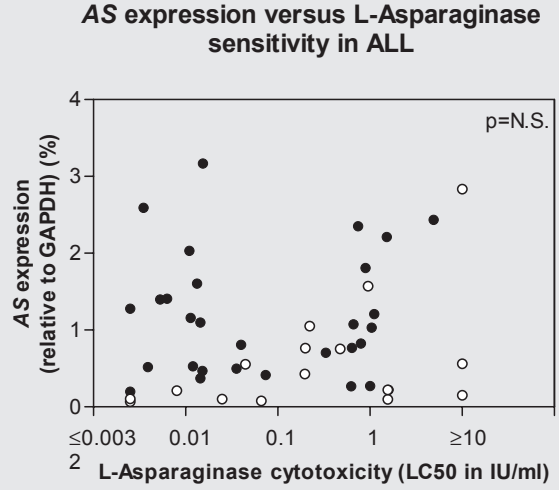

Figure 3. *AS* expression versus L-Asparaginase sensitivity in ALL.
Correlation between the mRNA expression of *AS* and the L-Asparaginase cytotoxicity. Open circles indicate individual t(12;21) negative patients, closed circles indicate individual t(12;21) positive patients.

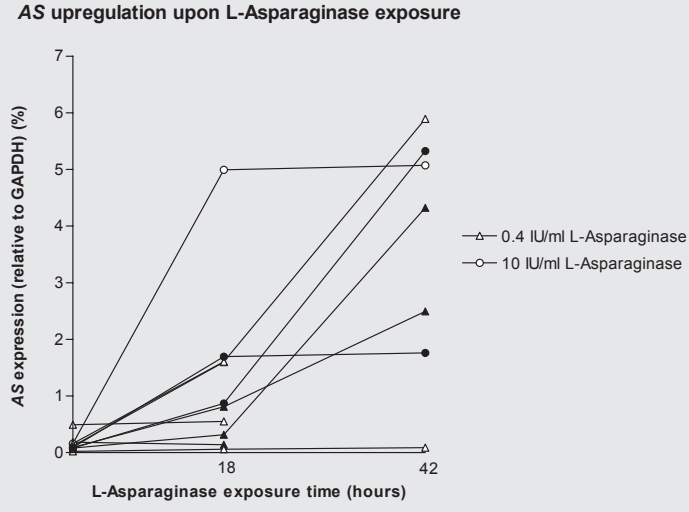

Figure 4. *AS* upregulation after L-Asparaginase exposure.
mRNA expression of *AS* relative to *GAPDH* in t(12;21) positive versus t(12;21) negative ALL after L-Asparaginase exposure. The open triangles or circles indicate t(12;21) negative patients, the closed triangles or circles indicate t(12;21) positive patients. Each connecting line represents a patient exposed to 0.4 IU/ml or 10 IU/ml (as indicated).

from 3 t(12;21) positive and 4 t(12;21) negative ALL patients were exposed *in vitro* to 0.4 IU/ml and 10 IU/ml L-Asparaginase. Within each group one patient did not show increased *AS* levels upon L-Asparaginase exposure. All other samples showed upregulation of *AS* which was independent from t(12;21) status and/or cellular sensitivity to

L-Asparaginase (Figure 4). Consequently, during *in vitro* exposure to L-Asparaginase no relation was observed between the mRNA expression of *AS* and the presence of t(12;21) or L-Asparaginase sensitivity.

DISCUSSION

Based largely on *in vitro* observations in (non-) human leukemia cell lines, it has been hypothesized that elevated AS activity is a cause of resistance to L-Asparaginase in human leukemia cells[16,24-28]. In the present study we analyzed a potential mechanism of L-Asparaginase sensitivity in t(12;21) positive childhood ALL, speculating that *TEL-AML1* represses the transcription of the *AS* gene. So far, only one study directly correlated AS expression and L-Asparaginase resistance in primary human leukemia cells. In 1969, Haskell and Canellos reported higher AS enzymatic activity in five L-Asparaginase resistant leukemia patients compared with four drug-sensitive patients during or after treatment[29]. However, besides the highly limited number of patients, the criteria used to determine whether the patient was resistant or sensitive to L-Asparaginase were not described in the paper. In addition this study was performed in a heterogeneous group including adult patients with either acute or chronic leukemia. In 2000 Dübbers et al.[30] reported a lower AS activity in pediatric B-lineage ALL and acute myelogenous leukemia (AML)-M5 compared to T-lineage ALL and other AML subgroups. However, the B-lineage ALL group showed a large heterogeneity in enzyme activity.

In the study presented here, the t(12;21) positive ALL group was matched with a t(12;21) negative, age 1-10 yrs, non-hyperdiploid (> 50), t(9;22) negative, non MLL rearranged, common / pre B-ALL group. We found that t(12;21) positive ALL cells express 5-fold more *AS* mRNA compared to the matched t(12;21) negative ALL. This stands in contrast to our hypothesis that TEL-AML1 might repress the *AS* gene and it also refutes the hypothesis that an elevated *AS* level is the most important determinant of L-Asparaginase resistance[17], since the present study and an earlier study[10] show that t(12;21) positive patients are significantly more sensitive to L-Asparaginase *in vitro*. Moreover, we found no correlation between *in vitro* sensitivity to L-Asparaginase and the mRNA expression of *AS* suggesting that the basal mRNA level of *AS* at initial diagnosis is not associated with L-Asparaginase sensitivity in pediatric ALL.

It could be argued that the mRNA expression level of *AS* does not relate to the protein level and enzyme activity. However, Hutson et al.[28] showed on human leukemia cell lines that complete amino acid deprivation resulted in a concerted increase in *AS* mRNA, protein, and enzymatic activity, suggesting that mRNA levels correspond to AS protein levels.

L-Asparaginase is an effective drug for newly diagnosed ALL. The effectiveness of this drug results from a rapid and complete depletion of cellular asparagine[13]. It was postulated years ago that leukemic cells depend on the external availability of the amino acid asparagine because of absence of endogenous AS[14,15]. Asparagine deficiency impairs protein synthesis and leads to a cessation of RNA and/or DNA synthesis, resulting in cell death. In our study however, we found no difference in *AS* mRNA expression between ALL and normal bone marrow or peripheral blood cells. This contradicts the general thought that leukemic cells specifically lack AS compared to normal bone marrow and peripheral blood cells.

In a small sample of patients we showed that leukemic cells from patients with or without the t(12;21) and resistant or sensitive to L-Asparaginase do not differ in their capacity to upregulate AS on i*n vitro* exposure to L-Asparaginase suggesting that resistance to L-Asparaginase is not caused by rapid induction of AS expression upon L-Asparaginase exposure[17]. However these findings need to be confirmed in a larger series of patients. The only difference we did find is a higher basal expression of *AS* in t(12;21) positive ALL compared with t(12;21) negative ALL and normal cells. We speculated that TEL-AML1 functions as a repressor for the transcription of *AS* comparable to the *TCRβ* enhancer. However, our data suggest the opposite. Therefore, based on these data, it might be hypothesized that *AS* is normally repressed by AML1 and that TEL-AML1 cancels the repression of *AS*.

The clinical role of L-Asparaginase in t(12;21) positive ALL is subject of discussion. Although most studies associate t(12;21) with a good prognosis, conflicting results are described[3,4,8,9,31-33]. These conflicting data might be due to differences in use of L-Asparaginase in the treatment protocols as t(12;21) positive ALL is highly sensitive to L-Asparaginase[10] and L-Asparaginase sensitive patients have a more favourable outcome[34]. The Dana-Farber Cancer Institute (DFCI) group showed a highly favourable outcome of t(12;21) positive ALL[3]. In the DFCI protocol a high-dose L-Asparaginase is used compared to other treatment protocols. However, it is possible that a general intensification of therapy, not only by L-Asparaginase but also by other drugs, might contribute to the fact that in some recent protocols t(12;21) has a favourable outcome. This has for instance been shown by a Japanese study, which reported no prognostic value for the presence of t(12;21) in an early study, however with intensified therapy in a newer protocol the t(12;21) positive patients did exceedingly well[33].

Summarizing, t(12;21) positive ALL, which *in vitro* is significantly more sensitive to L-Asparaginase, has a significantly higher *AS* mRNA expression level compared to t(12;21) negative ALL and normal lymphoid cells. So, the *AS* mRNA expression level does not explain the high sensitivity to L-Asparaginase of t(12;21) positive ALL. The mechanism that makes t(12;21) positive ALL patients more sensitive to L-Asparaginase remains unclear. An alternative explanation might be that t(12;21) positive ALL cells

are not able to provide sufficient amounts of the AS substrates, aspartate and glutamine. In 2001 Aslanian and Kilberg[35] illustrated that several adaptive processes occur to provide aspartate and glutamine to support the activity of AS. These substrates could come from an intracellular pool or may be acquired from the extracellular milieu by active transport across the plasma membrane via several amino acid transporters like Systems X_C, $X_{A,G}$, A, ASC and L. Another study in human leukemia cell lines showed that glutamine deprivation-dependent cell shrinkage induced activation of the CD95-mediated pathway[36]. This was also observed when L-Asparaginase was added to the medium. In 2001 Krishna Narla observed a higher expression level of the pro-apoptotic protein CD95 and lower levels of the anti-apoptotic protein Bcl-2 in t(12;21) positive ALL cells compared to t(12;21) negative ALL cells in children[37]. This suggests that L-Asparaginase sensitivity in t(12;21) positive ALL cells might be related to CD95 and/or Bcl-2 expression levels.

In conclusion, the mechanism of L-Asparaginase sensitivity in t(12;21) positive ALL is not related to AS expression and remains still unclear. Moreover, the present data clearly contradict an almost 35 year old theory that the therapeutic benefit of L-Asparaginase in leukemia is based upon the fact that leukemic cells lack sufficient AS compared to normal cells.

ACKNOWLEDGEMENTS

We wish to express our gratitude to the members of the DCLSG and the German COALL study group for their support to this study by providing leukemic samples.

REFERENCES

1. Poirel H, Oury C, Carron C, et al. The TEL gene products; nuclear phosphoproteins with DNA binding properties. Oncogene. 1997;14:349-357.
2. Meyers S, Downing JR, Hiebert SW. Identification of AML-1 and the (8;21) translocation protein (AML-1/ETO) as sequence specific DNA-binding proteins: the runt homology domain is required for DNA binding and protein-protein interactions. Mol Cell Biol. 1993;13:6336-6345.
3. Mc Lean TW, Ringold S, Neuberg D, et al. TEL/AML-1 dimerizes and is associated with a favorable outcome in childhood acute lymphoblastic leukemia. Blood. 1996;88(11):4252-4258.
4. Borkhardt A, Cazzaniga G, Viehmann S, et al. Incidence and clinical relevance of TEL/AML1 fusion genes in children with acute lymphoblastic leukemia enrolled in the German and Italian multicenter therapy trials. Associazione Italiana Ematologia Oncologia Pediatrica and the Berlin-Frankfurt-Munster Study Group. Blood. 1997;90(2):571-577.
5. Harbott J, Viehmann S, Borkhardt A, Henze G, Lampert F. Incidence of TEL/AML1 fusion gene analyzed consecutively in children with acute lymphoblastic leukemia in relapse. Blood. 1997;90:4933-4937.
6. Loh ML, Silverman LB, Young ML, et al. Incidence of TEL/AML1 fusion in children with relapsed acute lymphoblastic leukemia. Blood. 1998;92:4792-4797.
7. Seeger K, Adams HP, Buchwald D, et al. TEL-AML1 fusion transcript in relapsed childhood acute lymphoblastic leukemia: the Berlin-Frankfurt-Munster Study Group. Blood. 1998;91:1716-1722.
8. Rubnitz JE, Behm FG, Wichlan D, et al. Low frequency of TEL-AML1 in relapsed acute lymphoblastic leukemia supports a favorable prognosis for this genetic subgroup. Leukemia. 1999;13(1):19-21.
9. Jamil A, Theil KS, Kahwash S, Ruymann FB, Klopfenstein KJ. TEL-AML-1 fusion gene. Its frequency and prognostic significance in childhood acute lymphoblastic leukemia. Cancer Genet Cytogenet. 2000;122(2):73-78.
10. Ramakers-van Woerden NL, Pieters R, Loonen AH, et al. TEL/AML1 gene fusion is related to in vitro drug sensitivity for L-asparaginase in childhood acute lymphoblastic leukemia. Blood. 2000;96:1094-1099.
11. Kaspers GJL, Veerman AJP, Pieters R, et al. *In vitro* cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. Blood. 1997;90:2723-2729.
12. Asselin BL, Kreissman S, Coppola DJ, et al. Prognostic significance of early response to a single dose of asparaginase in childhood acute lymphoblastic leukemia. J Pediatr Hematol Oncol 1999;21:6-12.
13. Chakrabarti R, Schuster SM. L-Asparaginase: Perspectives on the mechanisms of action and resistance. Int J Pediatric Hematol Oncol. 1997;4(6):597-611.
14. Ohnuma T, Holland JF, Freeman A. Biochemical and pharmacological studies with asparaginase in man. Cancer Res. 1970;30:2297-2305.
15. Miller HK, Salser JS, Balis ME. Amino acid levels following L-asparaginase amidohydrolase (EC.3.5.1.1.) therapy. Cancer Res. 1969;29:183-187.
16. Aslanian AM, Fletcher BS, Kilberg MS. Asparagine synthetase expression alone is sufficient to induce L-asparaginase resistance in MOLT-4 human leukemia cells. Biochem J. 2001;357:321-328.

17. Hiebert SW, Sun WH, Davis JN, et al. The t(12;21) translocation converts AML-1B from an activator to a repressor of transcription. Mol Cell Biol. 1996;16:1349-1355.
18. Kaspers GJ, Veerman AJ, Pieters R, et al. Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay. Br J Cancer. 1994;70:1047-1052.
19. Hagemeijer A, Buijs A, Smit E, et al. Translocation of BCR to chromosome 9: a new cytogenetic variant detected by FISH in two Ph-negative, BCR-positive patients with chronic myeloid leukemia. Genes Chrom Canc. 1993;8:237-245.
20. Pieters R, Loonen AH, Huismans DR, et al. In vitro drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. Blood. 1990;76:2327-2336.
21. Pieters R, Huismans DR, Leyva A, Veerman AJ. Adaptation of the rapid automated tetrazolium dye based (MTT) assay for chemosensitivity testing in childhood leukemia. Cancer Lett. 1988;41:323-332.
22. Meijerink J, Mandigers C, van de Locht L, Tonissen E, Goodsaid F, Raemakers J. A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. J Mol Diagn. 2001;3:55-61.
23. Pieters R, Huismans DR, Loonen AH, et al. Relation of cellular drug resistance to long-term clinical outcome in childhood acute lymphoblastic leukaemia. Lancet. 1991;338:399-403.
24. Prager MD, Bachynsky N. Asparagine synthetase in asparaginase resistant and susceptible mouse lymphomas. Biochem Biophys Res Commun. 1968;31:43-47.
25. Kiriyama Y, Kubota M, Takimoto T, et al. Biochemical characterization of U937 cells resistant to L-asparaginase: the role of asparagine synthetase. Leukemia. 1989;3:294-297.
26. Worton KS, Kerbel RS, Andrulis IL. Hypomethylation and reactivation of the asparagine synthetase gene induced by L-asparaginase and ethyl methanesulfonate. Cancer Res. 1991;51:985-989.
27. Martin JK, Sun W, Maraga D, Schuster SM, Wylie DE. An investigation into the mechanism of L-asparaginase resistance in L5178Y murine leukemia cells. Amino Acids. 1993;5:51-69.
28. Hutson RG, Kitoh T, Amador DAM, Cosic S, Schuster SM, Kilberg MS. Amino acid control of asparagine synthetase: relation to asparaginase resistance in human leukemia cells. Am J Physiol. 1997;272:C1691-C1699.
29. Haskell CM, Canellos GP. L-asparaginase resistance in human leukemia-asparagine synthetase. Biochem Pharmacol. 1969;18:2578-2580.
30. Dübbers A, Würthwein G, Müller HJ, et al. Asparagine synthetase activity in paediatric acute leukemias: AML-M5 subtype shows lowest activity. Br J Hemat. 2000;109:427-429.
31. Tsang KS, Li CK, Chik KW, et al. TEL/AML1 rearrangement and the prognostic significance in childhood acute lymphoblastic leukemia in Hong Kong. Am J Hematol. 2001;68(2):91-98.
32. Hubeek I, Ramakers-van Woerden NL, Pieters R, et al. TEL-AML1 fusion is not a prognostic factor in Dutch childhood acute lymphoblastic leukemia. Br J Haematol. 2001;113:254-255.
33. Takahashi Y, Horibe K, Kiyoi H, et al. Prognostic significance of TEL/AML1 fusion transcript in childhood B-precursor Acute Lymphoblastic Leukemia. J Pediatr Hematol Oncol. 1998:20(3):190-195.
34. Kaspers GJL, Veerman AJP, Pieters R, et al. In vitro cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. Blood. 1997;90(7):2723-2729.

35. Aslanian AM, Kilberg MS. Multiple adaptive mechanisms affect asparagine synthetase substrate availability in asparaginase-resistant MOLT-4 human leukaemia cells. Biochem J. 2001;358:59-67.
36. Fumarola C, Zerbini A, Guidotti GG. Glutamine deprivation-mediated cell shrinkage induces ligand-independent CD95 receptor signaling and apoptosis. Cell Death Differ. 2001;8:1004-1013.
37. Krishna Narla R, Navara C, Sarquis M, Uckun FM. Chemosensitivity of TEL-AML1 fusion transcript positive acute lymphoblastic leukemia cells. Leuk lymphoma. 2001;41:615-623.

Chapter 5

Upregulation of asparagine synthetase and cell cycle arrest in t(12;21) positive ALL

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Leukemia 2005;19(2):318-9

The (12;21)(p13;q22) translocation resulting in *TEL-AML1* gene fusion is generally associated with a good prognosis and a high cellular sensitivity to L-Asparaginase[1,2]. L-Asparaginase hydrolyses asparagine and glutamine resulting into cellular depletion of these amino acids.

Krejci and coworkers studied changes in cell proliferation of t(12;21) positive and t(12;21) negative ALL cells after exposure to L-Asparaginase[3]. In correspondence with our data[2], their data indicate that leukemic cells are able to upregulate asparagine synthetase (*AS)* gene expression upon nutrient stress induced by L-Asparaginase. In addition, our work and Krejci's work show that *AS* was unexpectedly higher expressed in t(12;21) positive ALL cells compared to t(12;21) negative patients. These important findings contradict the general hypothesis that resistance to L-Asparaginase is caused by an elevated basic cellular level of AS and/or by the selective ability of only resistant cells to rapidly induce the expression of the *AS* gene upon L-Asparaginase exposure[4], since t(12;21) positive ALL cells are more sensitive to L-Asparaginase compared to t(12;21) negative ALL patients[1,2]. Besides induction of expression of *AS*, Krejci and coworkers observed that L-Asparaginase selectively prevents S-phase entry of a t(12;21) positive cell line, but not a t(12;21) negative cell line. Since AML1 is involved in the regulation of the G_1 to S cell cycle transition[5,6] and TEL-AML1 converts AML1 from functioning as a transcriptional activator to a transcriptional repressor[7], we investigated whether t(12;21) positive patients already show this cell cycle arrest at initial diagnosis independent from L-Asparaginase treatment.

To this aim, the cell cycle of 75 t(12;21) positive and 116 t(12;21) negative ALL patients at initial diagnosis obtained from the DCOG was analyzed by propium-iodide

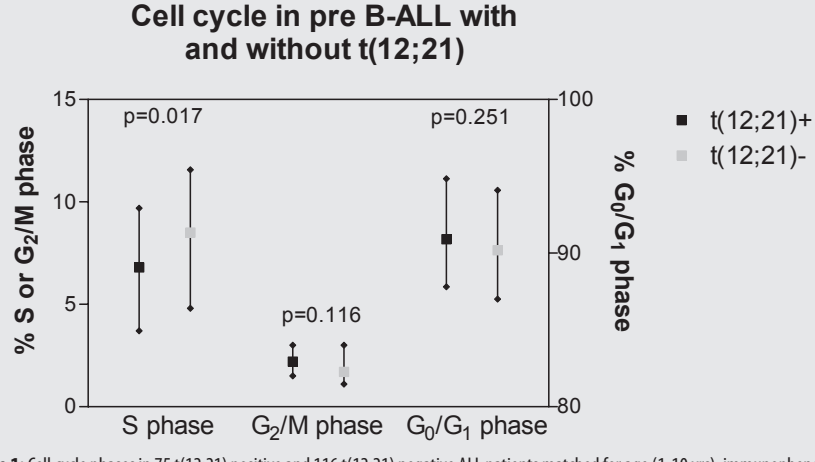

Figure 1: Cell cycle phases in 75 t(12;21) positive and 116 t(12;21) negative ALL patients matched for age (1-10 yrs), immunophenotype, absence of hyperdiploidy (>50 chromosomes), absence of *MLL* rearrangements and absence of t(9;22). Squares indicate medians, diamonds indicate 25th and 75th percentile. Cell cycle phase was measured in propidium-iodide stained nuclei using flow cytometry.

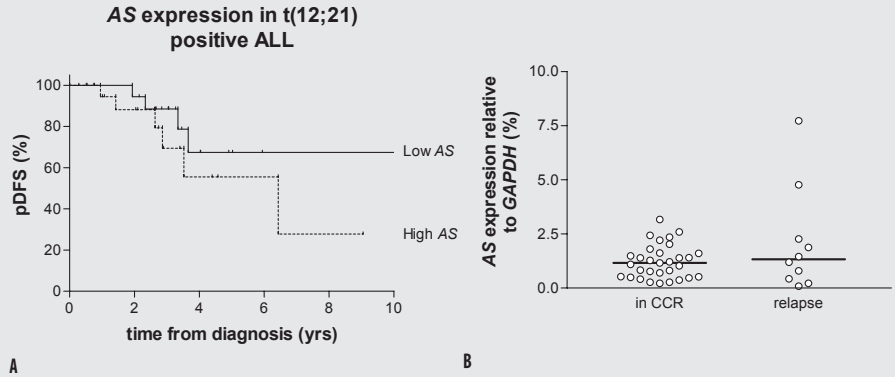

Figure 2: Probability of disease-free survival analysis (pDFS) in t(12;21) positive ALL with low (n=21, 5-yrs pDFS 67% ± 22%) and high (n=20, 5-yrs pDFS 56% ± 21%) expression of *AS*. The median value (1.2) was used as a cut off for the separation into high and low expression of *AS* (**A**). *AS* expression in t(12;21) positive ALL patients at diagnosis with (n=10) subsequent relapse compared to t(12;21) positive ALL patients in continuous complete remission (CCR) (n=31) (MWU: p>0.05) (**B**). *AS* mRNA was determined by real-time quantitative PCR using the same conditions as described previously[2].

staining of nuclei, as measured by flow cytometry (Figure 1). The percentage of cells in S phase is lower in t(12;21) positive ALL (median: 6.8%) compared to t(12;21) negative ALL (median: 8.5%; p = 0.017). This finding might also contribute to our and Krejci's observations that *AS* expression is higher in t(12;21) positive ALL compared to t(12;21) negative ALL at diagnosis, as *AS* was described to be compensationally increased when cells could not proceed through the cell cycle[8].

Krejci et al showed that high *AS* expression is associated with a favourable outcome within t(12;21) positive ALL. In our cohort of 41 t(12;21) positive ALL, which is comparable to Krejci's cohort, however, we could not confirm this: high *AS* expression did not correlate with a higher disease-free survival (Figure 2A) and *AS* expression did not differ between t(12;21) positive ALL patients with and without subsequent relapse (Figure 2B).

We hypothesize that t(12;21) positive ALL cells already at diagnosis have impaired potential to proceed from G_1 into S phase due to the fact that TEL-AML1 represses the target (i.e. cell cycle-associated) genes of AML1. This matches perfectly with the higher *AS* expression in these cells at diagnosis compared to t(12;21) negative ALL cells, since *AS* transcription is increased in cells that are hampered to proceed through the cell cycle. Nutrition stress caused by L-Asparaginase even enhances this increase in *AS* expression[2,3]. We could not confirm the findings of Krejci in relation to low *AS* expression in t(12;21) positive ALL and an unfavourable prognosis.

REFERENCES

1. Ramakers-van Woerden NL, Pieters R, Loonen AH, et al. TEL/AML1 gene fusion is related to in vitro drug sensitivity for L-asparaginase in childhood acute lymphoblastic leukemia. Blood. 2000;96:1094-1099.
2. Stams WA, den Boer ML, Beverloo HB, et al. Sensitivity to L-asparaginase is not associated with expression levels of asparagine synthetase in t(12;21)+ pediatric ALL. Blood. 2003;101:2743-2747.
3. Krejci O, Starkova J, Otova B, et al. Upregulation of asparagine synthetase fails to avert cell cycle arrest induced by L-asparaginase in TEL/AML1-positive leukaemic cells. Leukemia. 2004;18:434-441.
4. Aslanian AM, Fletcher BS, Kilberg MS. Asparagine synthetase expression alone is sufficient to induce l-asparaginase resistance in MOLT-4 human leukaemia cells. Biochem J. 2001;357:321-328.
5. Strom DK, Nip J, Westendorf JJ, et al. Expression of the AML-1 oncogene shortens the G(1) phase of the cell cycle. J Biol Chem. 2000;275:3438-3445.
6. Bernardin-Fried F, Kummalue T, Leijen S, Collector MI, Ravid K, Friedman AD. AML1/RUNX1 increases during G1 to S cell cycle progression independent of cytokine-dependent phosphorylation and induces cyclin D3 gene expression. J Biol Chem. 2004;279:15678-15687.
7. Hiebert SW, Sun W, Davis JN, et al. The t(12;21) translocation converts AML-1B from an activator to a repressor of transcription. Mol Cell Biol. 1996;16:1349-1355.
8. Colletta G, Cirafici AM. TSH is able to induce cell cycle-related gene expression in rat thyroid cell. Biochem Biophys Res Commun. 1992;183:265-272.

Chapter 6

Asparagine synthetase expression is linked with L-Asparaginase resistance in TEL-AML1 negative, but not in TEL-AML1 positive pediatric acute lymphoblastic leukemia

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Blood 2005;105(11):4223-5

ABSTRACT

Resistance to L-Asparaginase in leukemic cells may be caused by an elevated cellular expression of asparagine synthetase (AS). Previously, we reported that high *AS* expression did not correlate to L-Asparaginase resistance in TEL-AML1 positive B-lineage ALL. In the present study, we confirmed this finding in TEL-AML1 positive patients (n = 28) using microarrays. In contrast, 35 L-Asparaginase resistant TEL-AML1 negative B-lineage ALL patients had a significant 3.5-fold higher *AS* expression than 43 sensitive patients (p = 0.0004). Using RTQ-PCR, this finding was confirmed in an independent group of 39 TEL-AML1 negative B-lineage ALL patients (p = 0.03). High expression of *AS* was associated with poor prognosis (4-yrs pDFS 58% ± 11%) compared to low expression (4-yrs pDFS 83% ± 7%; p = 0.009). We conclude that resistance to L-Asparaginase and relapse-risk are associated with high expression of *AS* in TEL-AML1 negative but not in TEL-AML1 positive B-lineage ALL.

INTRODUCTION

L-Asparaginase is an enzyme widely used in chemotherapeutic protocols for treatment of children with acute lymphoblastic leukemia (ALL). *In vitro* and *in vivo* resistance to L-Asparaginase correlates with a relative poor prognosis in ALL[1,2]. L-Asparaginase depletes asparagine and glutamine in blood cells[3]. Impaired capacity to synthesize sufficient amounts of asparagine due to reduced asparagine synthetase (AS) levels is thought to explain the L-Asparaginase sensitivity of ALL cells to L-Asparaginase treatment[4,5]. L-Asparaginase resistance is suggested to be caused by an elevated cellular level of AS[6].

TEL-AML1 positive ALL patients are more sensitive to L-Asparaginase compared to TEL-AML1 negative patients[7,8]. However, we found a 5-fold higher expression of *AS* in the TEL-AML1 positive ALL patients compared to TEL-AML1 negative ALL patients[7], recently confirmed by Krejci et al.[9]. Therefore, within TEL-AML1 positive ALL resistance to L-Asparaginase is not associated with *AS* expression levels. Due to limited sample size in our previous study, it was unclear if this conclusion could be generalized for all other B-lineage ALL patients. Therefore, we investigated the relationship between *AS* expression and L-Asparaginase resistance in two independent and larger groups of TEL-AML1 negative ALL patients.

MATERIALS AND METHODS

Patient samples

Bone marrow and/or peripheral blood samples from 117 untreated children with TEL-AML1 negative and 28 untreated children with TEL-AML1 positive B-lineage ALL at initial diagnosis were collected at the Erasmus MC - Sophia Children's hospital, the Dutch Childhood Oncology Group and the German COALL study group. In addition, samples from 17 TEL-AML1 negative ALL patients used in our previous study were included[7]. Within 24 hours after sampling, mononuclear cells were isolated and total cellular RNA was extracted from a minimum of 5×10^6 ($\geq 90\%$ leukemic cells) using Trizol reagent, as described before[7].

Microarray analysis

RNA processing and hybridization to the U133A GeneChip® oligonucleotide microarray (Affymetrix) was performed according to manufacturer's protocol. Data analysis was performed as described before[10]. For this study, we limited the analysis to *AS* data in *in vitro* L-Asparaginase sensitive and resistant TEL-AML1 positive (23 and 5 patients respectively) and TEL-AML1 negative patients (43 and 35 patients respectively).

Real-time quantitative PCR (RTQ-PCR)

The mRNA expression level of *AS* and an endogenous housekeeping gene encoding for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference, were quantified using real-time PCR analysis (Taqman chemistry) with efficiencies ≥ 95, as described before[7]. The relative expression level of *AS* to *GAPDH* is calculated using the comparative cycle time method[7].

In vitro L-Asparaginase cytotoxicity assay

In vitro L-Asparaginase (Paronal, Christiaens B.V., Breda, The Netherlands) cytotoxicity was determined using the MTT assay as described previously[7]. Cut-off criteria for sensitive (LC_{50} < 0.033 IU/ml), intermediate sensitive and resistant (LC_{50} > 0.912 IU/ml) towards L-Asparaginase were as described previously[11]. These 3 groups were associated with prognosis of ALL patients[11].

Statistics

Differences in mRNA expression between groups of patients were analyzed using the Mann-Whitney U test. The correlation between *AS* expression and L-Asparaginase resistance was calculated using the Spearman's rank correlation test. Probability of disease-free survival (pDFS) was calculated from the date of diagnosis to the date of non-response, relapse or last contact using the Kaplan-Meier method and compared by log-rank test. Multivariate analysis was performed with the Cox proportional-hazard regression model.

RESULTS AND DISCUSSION

AS expression was analyzed with micro-array technology in 78 TEL-AML1 negative and 28 TEL-AML1 positive ALL patients. No difference in *AS* expression between L-Asparaginase resistant (n = 5, median = 828 AU, P25-P75: 164-1871) and L-Asparaginase sensitive (n = 23, median = 516 AU, P25-P75: 141-689; p = 0.3) patients was found within TEL-AML1 positive ALL patients, which is in concordance with earlier findings from our lab and others[7,9]. However, in TEL-AML1 negative ALL patients a significant 3.5 fold difference in *AS* expression was shown between L-Asparaginase resistant (n = 35; median = 946 AU, P25-P75: 293 – 1617) and sensitive (n = 43; median = 244 AU, P25-P75: 92 – 787) patients (p = 0.0004) (Figure 1).

The original TEL-AML1 negative ALL patient group (n = 17) from our previous study[7] was enlarged to a total of 39 patients (different patients than used for micro-array analysis) and *AS* expression was analyzed by RTQ-PCR. A significant positive correlation was found between *AS* expression and L-Asparaginase resistance (R_s = 0.39;

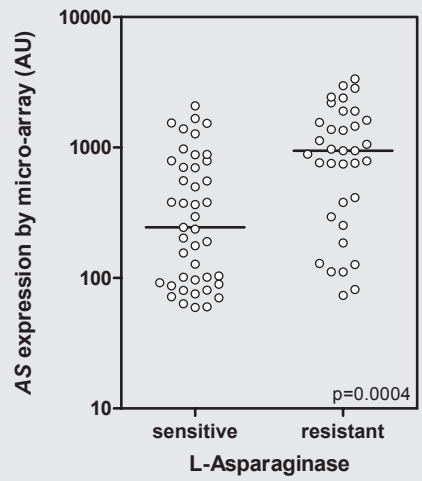

Figure 1. *AS* expression in TEL-AML1 negative ALL determined by micro-array
AS expression in TEL-AML1 negative ALL patients sensitive (n=43) and resistant to L-Asparaginase (n=35; p=0.0004) as determined by micro-array analysis. Circles represent individual patients; bars represent the median values. AU=Arbitrary units defined as scaled fluorescence measured on micro-array.

p = 0.017). Patients were divided in 3 subgroups based on *in vitro* cellular cytotoxicity to L-Asparaginase, i.e.: 10 sensitive, 15 intermediate sensitive and 14 resistant patients to L-Asparaginase. A significant 3.5-fold higher *AS* expression was shown in L-Asparaginase resistant compared to L-Asparaginase sensitive TEL-AML1 negative ALL patients (p = 0.03; Figure 2), which is in concordance with our micro-array data (Figure 1).

The mRNA expression level of *AS* does not necessarily correlate with protein level and enzyme activity of AS. However, Hutson et al.[12] showed in human leukemia cell lines that complete amino acid deprivation resulted in a simultaneous increase in *AS* mRNA, protein, and enzymatic activity, suggesting that mRNA corresponds to protein and activity levels.

Based largely on *in vitro* observations in (non-) human leukemia cell lines, it has been hypothesized that elevated AS activity is a cause of L-Asparaginase resistance in human leukemia cells lines[6,12-17]. Only a few studies have reported this relation between *AS* expression and L-Asparaginase sensitivity in patient materials[18,19]. We and others showed previously that *AS* expression is not related to L-Asparaginase sensitivity in TEL-AML1 positive ALL[7,9]. We now confirmed this with a second method (i.e. micro-array analysis). In contrast, we show in two independent groups of patients studied by two different techniques (micro-array and RTQ-PCR) that elevated *AS* expression is

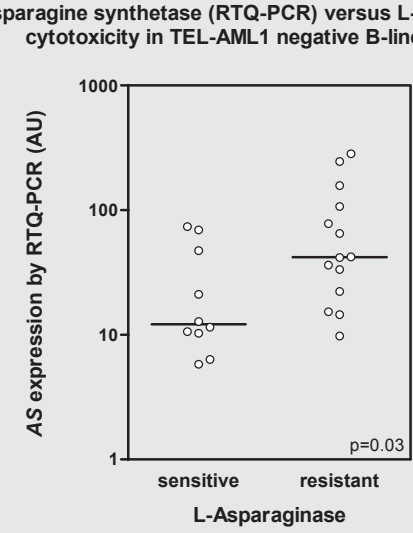

Figure 2. *AS* expression in TEL-AML1 negative ALL determined by RTQ-PCR
AS expression in TEL-AML1 negative ALL patients sensitive (n=10) and resistant to L-Asparaginase (n=14; p=0.03) as determined by RTQ-PCR. *AS* mRNA expression is indicated in arbitrary units (AU), and defined as the mRNA expression of *AS* relative to *GAPDH* * 100. Circles represent individual patients; bars represent the median values.

related to L-Asparaginase resistance in TEL-AML1 negative ALL patients. This is in line with the *in vitro* observations in (non-) human leukemia cell lines[6,12-17].

The 78 TEL-AML1 negative patients analyzed by micro-array had a median follow-up of patients at risk of 4.4 (range: 0.6-9.1) years. The clinical characteristics of L-Asparaginase resistant and sensitive patients were respectively: WBC of median 25.6 $*10^9$/L (P25-P75: 7.7-52.0) and 26.8$*10^9$/L (P25-P75: 8.8-73.2) p = 0.8, age of median 8.0 (P25-P75: 4.5-11.5) and 4.0 years (P25-P75: 2.0-7.3) p < 0.0001. High *AS* expression (median value) was associated with a poor prognosis (4-yrs pDFS 58%±11%) compared to low *AS* expression (4-yrs pDFS 83%±7%; p = 0.009). Furthermore, the *AS* expression in patients who relapsed (946 AU, P25-P75: 305-2153) was 2.5 fold higher compared to patients who did not relapse so far (379 AU, P25-P75: 101-932; p = 0.01). Multivariate analysis including WBC and age revealed that high *AS* expression is independently related to a poor prognosis (Hazard ratio: 3.0, 95% CI: 1.1-7.9, p = 0.03). In contrast, we recently showed that the expression of *AS* in TEL-AML1 positive ALL is not related to outcome[20]. Krejci et al.[9] even found the opposite: a relation between high *AS* expression and a good prognosis in TEL-AML1 positive ALL patients. These data suggest that the role of AS for L-Asparaginase resistance and therapy is different between both genetic subtypes. Since the TEL-AML1 genotype is associated with an increased L-Asparaginase sensitivity[7,8], the mechanism of action (and hence cause of resistance) may be different. A possibility might be the higher expression levels of the pro-apoptotic

protein CD95 in TEL-AML1 positive ALL cells compared to TEL-AML1 negative ALL cells[21], since the CD95-mediated pathway is activated by cell shrinkage which can be mediated by L-Asparaginase[22]. An alternative explanation might be that TEL-AML1 positive ALL cells are not able to provide sufficient amounts of the AS substrates, aspartate and glutamine, due to different amino acid metabolism and/or transmembrane amino acid transporters that contribute to the function of AS[23]. Further detailed studies are required to understand the observed difference between TEL-AML1 negative and positive subtypes.

ACKNOWLEDGEMENTS

We wish to express our gratitude to the members of the DCOG and the German COALL study group for their support to this study by providing leukemic samples.

REFERENCES

1. Kaspers GJ, Veerman AJ, Pieters R, et al. In vitro cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. Blood. 1997;90:2723-2729.
2. Asselin BL, Kreissman S, Coppola DJ, et al. Prognostic significance of early response to a single dose of asparaginase in childhood acute lymphoblastic leukemia. J Pediatr Hematol Oncol. 1999;21:6-12.
3. Chakrabarti R, Schuster SM. L-Asparaginase: Perspectives on the mechanisms of action and resistance. Int J Pediatric Hematol Oncol. 1997;4:597-611.
4. Ohnuma T, Holland JF, Freeman A, Sinks LF. Biochemical and pharmacological studies with asparaginase in man. Cancer Res. 1970;30:2297-2305.
5. Miller HK, Salser JS, Balis ME. Amino acid levels following L-asparagine amidohydrolase (EC.3.5.1.1) therapy. Cancer Res. 1969;29:183-187.
6. Aslanian AM, Fletcher BS, Kilberg MS. Asparagine synthetase expression alone is sufficient to induce l-asparaginase resistance in MOLT-4 human leukaemia cells. Biochem J. 2001;357:321-328.
7. Stams WA, den Boer ML, Beverloo HB, et al. Sensitivity to L-asparaginase is not associated with expression levels of asparagine synthetase in t(12;21)+ pediatric ALL. Blood. 2003;101:2743-2747.
8. Ramakers-van Woerden NL, Pieters R, Loonen AH, et al. TEL/AML1 gene fusion is related to in vitro drug sensitivity for L-asparaginase in childhood acute lymphoblastic leukemia. Blood. 2000;96:1094-1099.
9. Krejci O, Starkova J, Otova B, et al. Upregulation of asparagine synthetase fails to avert cell cycle arrest induced by L-asparaginase in TEL/AML1-positive leukaemic cells. Leukemia. 2004;18:434-441.
10. Holleman A, Cheok MH, den Boer ML, et al. Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. N Engl J Med. 2004;351:533-542.
11. Den Boer ML, Harms DO, Pieters R, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol. 2003;21:3262-3268.
12. Hutson RG, Kitoh T, Moraga Amador DA, Cosic S, Schuster SM, Kilberg MS. Amino acid control of asparagine synthetase: relation to asparaginase resistance in human leukemia cells. Am J Physiol. 1997;272:C1691-1699.
13. Prager MD, Bachynsky N. Asparagine synthetase in asparaginase resistant and susceptible mouse lymphomas. Biochem Biophys Res Commun. 1968;31:43-47.
14. Kiriyama Y, Kubota M, Takimoto T, et al. Biochemical characterization of U937 cells resistant to L-asparaginase: the role of asparagine synthetase. Leukemia. 1989;3:294-297.
15. Worton KS, Kerbel RS, Andrulis IL. Hypomethylation and reactivation of the asparagine synthetase gene induced by L-asparaginase and ethyl methanesulfonate. Cancer Res. 1991;51:985-989.
16. Martin JK, Sun W, Maraga D, Schuster SM, Wylie DE. An investigation into the mechanism of L-asparaginase resistance in L5178Y murine leukemia cells. Amino Acids. 1993;5:51-69.
17. Scherf U, Ross DT, Waltham M, et al. A gene expression database for the molecular pharmacology of cancer. Nat Genet. 2000;24:236-244.

18. Haskell CM, Canellos GP. l-asparaginase resistance in human leukemia--asparagine synthetase. Biochem Pharmacol. 1969;18:2578-2580.
19. Dubbers A, Wurthwein G, Muller HJ, et al. Asparagine synthetase activity in paediatric acute leukaemias: AML-M5 subtype shows lowest activity. Br J Haematol. 2000;109:427-429.
20. Stams WA, den Boer ML, Beverloo HB, van Wering ER, Pieters R. Upregulation of asparagine synthetase and cell cycle arrest in t(12;21)-positive ALL. Leukemia. 2005;19:318-319.
21. Fumarola C, Zerbini A, Guidotti GG. Glutamine deprivation-mediated cell shrinkage induces ligand-independent CD95 receptor signaling and apoptosis. Cell Death Differ. 2001;8:1004-1013.
22. Krishna Narla R, Navara C, Sarquis M, Uckun FM. Chemosensitivity of TEL-AML1 fusion transcript positive acute lymphoblastic leukemia cells. Leuk Lymphoma. 2001;41:615-623.
23. Aslanian AM, Kilberg MS. Multiple adaptive mechanisms affect asparagine synthetase substrate availability in asparaginase-resistant MOLT-4 human leukaemia cells. Biochem J. 2001;358:59-67.

Chapter 7

Identification of L-Asparaginase resistance and prognosis associated genes in TEL-AML1 positive ALL by gene expression profiling

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Submitted

ABSTRACT

TEL-AML1 positive acute lymphoblastic leukemia (ALL) is in general characterized by sensitivity towards L-Asparaginase. However, in individual patients resistance to this drug is observed which is associated with an un favourable prognosis. This study aimed at gaining more insight into genes related to L-Asparaginase resistance and the risk of relapse within TEL-AML1 positive ALL. Gene-expression profiling revealed 70 probe sets representing 52 unique genes (and 3 expressed sequence taqs) that discriminated between L-Asparaginase sensitive and resistant TEL-AML1 positive patients ($p < 0.001$) with a false-discovery rate of less than 10%. Ten-fold cross validation revealed prediction accuracy of 100% ($p = 0.004$) of resistant patients by means of a support vector machine. Resistance to L-Asparaginase in TEL-AML1 positive ALL was linked to an overrepresentation of genes involved in cell death and lipid metabolism. None of these genes has been previously linked to L-Asparaginase. Moreover, none of these genes has been linked to L-Asparaginase resistance in a previous study in TEL-AML1 negative ALL, indicating that the mechanism of L-Asparaginase differs between ALL genotypes. Genes predictive of relapse ($p < 0.001$) in TEL-AML1 positive ALL were only found with a high false discovery rate of 63%, but numbers of patients were low. This study reveals novel putative causes of L-Asparaginase resistance in TEL-AML1 positive ALL that may point to new ways to improve treatment results in this major subtype of childhood ALL.

INTRODUCTION

The most common chromosomal translocation observed in pediatric ALL is the t(12;21)(p13;q22) translocation restricted to precursor B-cell leukemia. This genetic subtype of ALL is found in approximately 25% of precursor B-lineage ALL[1,2]. It involves fusion of the *TEL* (also named *ETV6*) gene at chromosome 12p13 with the *AML1* (also named *CBFA2* or *RUNX1*) gene at chromosome 21q22. The *TEL* gene is a member of the ETS-domain transcription family[3-5]. ETS proteins control the expression of genes that are critical for several biological processes, including cellular proliferation, differentiation, and apoptosis[6]. The *AML1* gene is a transcription factor that forms one of the two subunits of the core binding factor[7-9]. AML1 is normally expressed in all hematopoietic lineages and regulates the transcription of myeloid and lymphoid lineage-specific genes[10]. The core binding factor is essential for definitive hematopoiesis of all lineages[11-13].

Conflicting data on the clinical outcome of t(12;21) positive ALL have been reported ranging from 60% to 100% 5-year event-free survival[14-18]. The incidence of TEL-AML1 rearrangements found at relapse varies from 6% to 24%[14]. Additional genetic changes in the TEL and/or AML1 genes occur in approximately 80% of all TEL-AML1 positive ALL patients[19]. Together, these data suggest that TEL-AML1 positive leukemia is a heterogeneous disease and the outcome may depend on the intensity and schedule of the treatment protocol. The t(12;21) positive ALL patients as a group are *in vitro* more sensitive to L-Asparaginase compared to t(12;21) negative ALL patients[20,21], although large interindividual differences in L-Asparaginase cytotoxicity are described. So, a number of TEL-AML1 positive ALL patients do suffer from a relapse and/or are resistant to L-Asparaginase.

The proposed mechanism of action of L-Asparaginase is the depletion of asparagine and glutamine in the blood leading to the intracellular depletion of these amino acids[22]. Lack or decreased expression of endogenous asparagine synthetase will result into an impaired capacity to re-synthesize sufficient amounts of asparagine upon L-Asparaginase exposure[23,24]. Resistance to L-Asparaginase may be caused by an elevated endogenous expression of asparagine synthetase[25]. Recently, we reported that increased expression of asparagine synthetase is linked with L-Asparaginase resistance in TEL-AML1 negative ALL[26]. However, this relationship was absent in children with TEL-AML1 positive ALL[21,26]. Gene expression profiling in childhood precursor B-lineage ALL in general revealed that resistance to L-Asparaginase is associated with increased expression of genes encoding ribosomal proteins and translation factors[27]. It is yet unknown whether similar genes are also involved in L-Asparaginase resistance of TEL-AML1 positive cases. The present study used an extensive genome-wide approach to get insight into putative causes of L-Asparaginase resistance and therapy failure in TEL-AML1 positive cases.

MATERIALS AND METHODS

Patient samples

Bone marrow and/or peripheral blood samples from 69 untreated children with B-lineage ALL (50 TEL-AML1 positive (33 eligible for L-Asparaginase resistance study and 27 included for relapse-prediction study, n = 10 in both studies) and 19 TEL-AML1 negative cases) at initial diagnosis were collected after informed consent at the Erasmus MC - Sophia Children's Hospital (n = 8), the Dutch Childhood Oncology Group (DCOG) (n = 32), the German COALL study group (n = 26) and the Pediatric Oncology Institute of Cancer Research Sutton, Surrey, United Kingdom (n = 3). Dutch patients were treated according to the DCOG ALL-7/8 and 9 protocol and German patients according to the COALL-97 protocol. TEL-AML1 presence was determined by FISH analysis as described before[21]. Within 24 hours after sampling, mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (density 1.077 g/ml; Nycomed Pharma, Oslo, Norway) and kept in culture medium consisting of RPMI 1640 medium (Dutch modification without L-glutamine; Life Technologies, Gaithersburg, USA), 20% fetal calf serum (Integro, Zaandam, The Netherlands), 2mM L-glutamine (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 pencillin, 100 μg/ml streptomycin, 0.125 μg/ml fungizone (Gibco BRL, Life Technologies) and 0.2 mg/ml gentamycin (Life Technologies). If necessary, ALL samples were enriched to achieve more than 90 percent blasts by removing non-malignant cells via immunomagnetic beads (DynaBeads), as described before[28].

In vitro L-Asparaginase cytotoxicity assay

In vitro L-Asparaginasecytotoxicity was determined using the MTT assay as described previously[21]. Briefly, cell suspensions were cultured in the presence of six concentrations of L-Asparaginase (Paronal, Christiaens B.V., Breda, The Netherlands) ranging from 0.0032 – 10 IU/ml. Control cells were cultured without L-Asparaginase. After four days of incubation, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT, 5 mg/ml; Sigma Aldrich, Zwijndrecht, The Netherlands) was added. During a 6-hour incubation, the yellow MTT tetrazolium salt is reduced to purple-blue formazan crystals by viable cells only. Criteria for sensitive ($LC_{50} < 0.033$ IU/ml) and resistant ($LC_{50} > 0.912$ IU/ml) towards L-Asparaginase were as described previously. These criteria were of predictive value in ALL patients[29].

RNA purification, labeling and hybridization to Affymetrix GeneChips

Total cellular RNA was extracted from a minimum of 5×10^6 leukemia cells using Trizol reagent (GIBCO BRL). RNA was additionally purified with phenol-chloroform-

isoamylalcohol (25:24:1), and RNA integrity was assessed using RNA 6000 Nano Assay LabChips (Agilent Technologies) as previously described[30]. RNA processing and hybridization to the U133A GeneChip oligonucleotide microarray (Affymetrix, Santa Clara, CA, USA) were performed as described earlier[30].

Analysis of gene expression data

Gene-expression values were calculated using Affymetrix Microarray Suite version 5.0. Arrays were excluded if the ratio of 3' to 5' probe sets for β-actin or glyceraldehyde-3-phosphate dehydrogenase was greater than 3. Unscaled expression signals were normalized using the vsn package in the statistical program R version 2.0.1[31]. All 22,283 probe sets were used for subsequent analysis.

Genes discriminative for resistance to L-Asparaginase or occurrence of a relapse were identified using the Wilcoxon rank-sums test. P-values were corrected for multiple testing using the false discovery rate (FDR) step-up procedure suggested by Benjamini & Hochberg[32]. This method compares the obtained list of p-values for all genes with what would be expected if no genes were differentially expressed between sensitive and resistant patients. This correction was run using the "multtest" package for R 2.0.1 (see www.r-project.org).

The identification of genes discriminative for L-Asparaginase resistance independently of TEL-AML1 status was performed by a gene specific regression model: gene expression = $\alpha + \beta \times I(\text{TEL-AML1}) + \gamma \times I(\text{L-Asparaginase resistance})$, where I(TEL-AML1) and I(L-Asparaginase resistance) represent variables indicating whether or not the patient is TEL-AML1 positive and L-Asparaginase resistant, respectively; and γ is the parameter representing the effect of L-Asparaginase resistance after controlling for TEL-AML1 status. Using this model, we also investigated the interaction between TEL-AML1 positivity and L-Asparaginase resistance[33,34]. The expression of genes selected by this model in L-Asparaginase resistant and sensitive ALL patients was compared within each ALL genotype using the Mann-Whitney U test.

The predictive accuracy using all probe sets and the top 70 discriminating probe sets for L-Asparaginase sensitivity compared to L-Asparaginase resistance (Wilcox $p < 0.001$) was assessed by randomly dividing the patients into two groups of L-Asparaginase sensitive and resistant, as if resistance status was unknown. Then 9/10 of the patients were used to build the model and 1/10 to assess the accuracy of the model (10-fold cross-validation) using a support vector machine. This classification tool is similar to logistic regression but is designed to exploit the nonlinear relationship among data points[27,35]. The overall significance of the estimated predictive accuracy was computed as the probability of observing equal or lower predictive accuracies on the basis of 1000 random permutations of the patients between the sensitive and resistant groups.

The degree of overrepresentation or underrepresentation of discriminating genes for L-Asparaginase resistance in TEL-AML1 positive ALL compared with the genes on the U133A GeneChip was determined using the Gene Ontology Mining Tool (https://www.affymetrix.com). The p-values were calculated using the chi-square test.

Probability of disease-free survival (pDFS) was calculated from the date of diagnosis to the occurrence of an event (non-response or relapse) or date of last contact (censored). The pDFS curves were calculated according the Kaplan-Meier method and analyzed by the log-rank test.

RESULTS

Prediction of L-Asparaginase resistance in TEL-AML1 positive patients

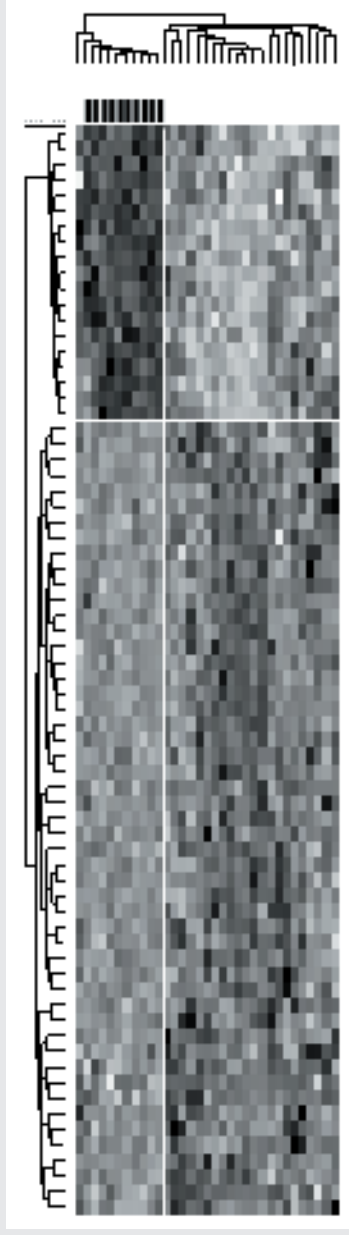
The Wilcoxon rank-sums test was used as a supervised method to identify genes that differed in expression between L-Asparaginase sensitive (n = 23) and resistant (n = 10) TEL-AML1 positive ALL patients. With a false discovery rate ≤ 10%, 70 probe sets were identified that were differentially expressed between L-Asparaginase sensitive and resistant patients ($P_{wilcoxon} < 0.001$). These 70 probe sets correspond to 52 unique genes and 3 expressed sequence taqs and are listed in Figure 1. 19 probe sets were underexpressed in L-Asparaginase resistant cases whereas 51 probe sets were relatively overexpressed in resistant TEL-AML1 positive ALL cases.

Supervised clustering and principal component analysis

These 70 probe sets discriminative for L-Asparaginase resistance in TEL-AML1 positive ALL patients were used for a hierarchical clustering. Hierarchical clustering assigned all resistant patients into one cluster and 22 of 23 sensitive patients in the second cluster (Figure 1). Similarly, principal component analysis, a technique for visualizing high-dimensional data sets, correctly separated all sensitive from resistant patients. Using 10-fold cross-validation, the accuracy to discriminate resistant from sensitive cases (using the top 70 of probe sets) was 100% (p = 0.004).

Gene ontology functional classification of discriminating genes

From the discriminative 70 probe sets for L-Asparaginase resistance in TEL-AML1 positive ALL, 42 probe sets were annotated in the Gene Ontology (GO) database. Genes involved in lipid metabolism (16%; *FDPS, HSD17B4, LDLR, UGCG, DPAGT1, PPAP2B, SPHK2*) and cell death (17%; *HDAC1, TIA1, HTATIP2, BCL2L13, SPHK2*) were overrepresented in these 70 probe sets in comparison with the probe sets that are present on the U133A GeneChip (each 5%; p < 0.001) (Figure 2). Since *BCL2L13* was recently described to be related to both L-Asparaginaseresistance and outcome in



Probe Set ID	Gene Symbol	R/S
220694_at	---	0.89
201206_s_at	RRBP1	0.76
222252_x_at	---	0.89
212226_s_at	PPAP2B	0.80
202068_s_at	LDLR	0.76
210369_at	SWAP70	0.74
217965_s_at	HCNGP	0.89
213404_s_at	RHEB	0.88
214766_s_at	ELYS	0.85
216563_at	---	0.81
212644_s_at	C14orf32	0.88
212808_at	FLJ14639	0.90
204881_s_at	UGCG	0.89
209136_s_at	USP10	0.89
211503_s_at	RAB14	0.91
216361_s_at	MYST3	0.71
211000_s_at	IL6ST	0.79
213638_at	PHACTR1	0.94
215623_x_at	SMC4L1	0.85
219570_at	C20orf23	1.25
219405_at	RNF137	1.08
204125_at	NDUFAF1	1.15
208304_at	CCR3	1.55
203227_s_at	SAS	1.21
219383_at	FLJ14213	1.20
218685_s_at	SMUG1	1.37
217647_at	DHODH	1.78
218361_at	GPP34R	1.38
218832_x_at	ARRB1	1.52
219907_at	FRS3	1.09
40273_at	SPHK2	1.15
207180_s_at	HTATIP2	1.13
45297_at	EHD2	1.28
204718_at	EPHB6	1.18
218893_at	FLJ23469	1.08
210347_s_at	BCL11A	1.15
205012_s_at	HAGH	1.18
219806_s_at	FN5	1.22
212429_s_at	GTF3C2	1.08
209448_at	HTATIP2	1.22
209586_s_at	HTCD37	1.12
201931_at	ETFA	1.14
211595_s_at	MRPS11	1.20
208670_s_at	CRI1	1.15
221744_at	HAN11	1.16
209268_at	VPS45A	1.12
217955_at	BCL2L13	1.20
201310_s_at	C5orf13	1.11
202297_s_at	RER1	1.10
218491_s_at	THY28	1.18
204683_at	ICAM2	1.16
212175_s_at	AK2	1.14
218572_at	C14orf123	1.16
209509_s_at	DPAGT1	1.15
220068_at	VPREB3	1.12
204839_at	POP5	1.11
202857_at	TMEM4	1.13
203259_s_at	C6orf74	1.18
211609_x_at	PSMD4	1.12
218270_at	MRPL24	1.24
201209_at	HDAC1	1.11
201448_at	TIA1	1.25
218452_at	SMARCAL1	1.78
201275_at	FDPS	1.09
204288_s_at	ARGBP2	1.81
219497_s_at	BCL11A	1.18
201450_s_at	TIA1	1.17
209781_s_at	KHDRBS3	1.10
218983_at	C1RL	1.26
201413_at	HSD17B4	1.18

Figure 1. Supervised hierarchical clustering using probe sets discriminative for L-Asparaginase resistance in TEL-AML1 positive ALL. The 70 probe sets (representing 52 unique genes and 3 expressed sequence taqs) differentially expressed between L-Asparaginase resistant and sensitive TEL-AML1 positive ALL cases used to perform a hierarchical clustering (Pearson-based). Each column represents a TEL-AML1 positive ALL sample, labelled black if resistant to L-Asparaginase. Each row represents a probe set. Probe sets that are relatively overexpressed compared to the mean expression are depicted in white or light grey and those that are relatively underexpressed are depicted in black or dark grey. In the right panel, the probe set ID, gene symbol and the ratio between the median expression of the gene probe in L-Asparaginase resistant compared to L-Asparaginase sensitive TEL-AML1 positive patients is given (R/S ratio). All probe sets have a Wilcoxon $p<0.001$ and $FDR<0.01$.

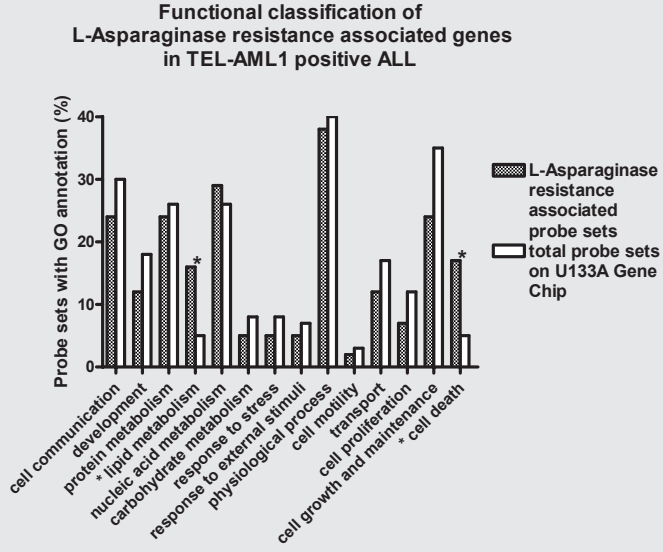


Figure 2. Functional classification of genes discriminative between L-Asparaginase resistant and sensitive TEL-AML1 positive ALL cases. Classification of probe sets into functional categories was performed using gene ontology database (GO). The functional GO classification of probe sets discriminative between L-Asparaginase resistant and sensitive TEL-AML1 positive ALL was compared with the probe sets present on the U133A GeneChip (22,283 probe sets on U133A GeneChip of which 14,430 with GO annotation). From the 70 probe sets associated with L-Asparaginase resistance, 42 probe sets were annotated in the GO database. Functional categories that are proportionally overrepresented in the probe sets discriminative for L-Asparaginase, compared to the entire U133A GeneChip, are indicated by an asterisk ($P<0.001$)

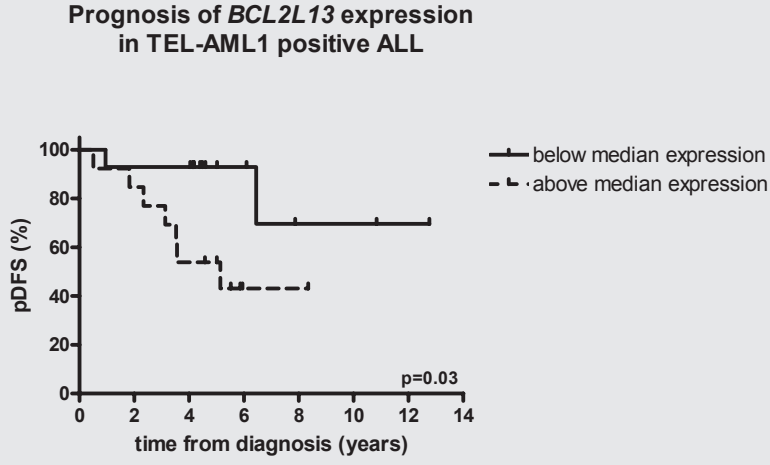


Figure 3. Disease-free survival analysis of BCL2L13 expression in TEL-AML1 positive ALL
Probability of disease-free survival (pDFS) in TEL-AML1 positive ALL according to high (above median expression value, $P>50$) and low (below median expression value, $P<50$) expression of *BCL2L13*. Black solid line, $P<50$, 5-yrs pDFS 93% ± 9%; n=14, 1 event). Dotted line, $P>50$, 5-yrs pDFS 54% ± 14%; n=13, 7 events; $p=0.03$).

pediatric B-lineage ALL[36], we performed a survival analysis for this particular gene. A high expression (above median value) of *BCL2L13* was significantly related to a poor outcome (5-yrs pDFS 54% ± 14%) compared to low expression of *BCL2L13* (5-yrs pDFS 93% ± 9%; p = 0.03) (Figure 3) in this independent group of TEL-AML1 positive ALL patients.

Interaction between TEL-AML1 status and L-Asparaginase resistance

In a separate analysis including both TEL-AML1 positive and negative samples, we used a regression model to address which genes were discriminative for L-Asparaginase resistance in these patients and if these genes were dependent of TEL-AML1 status. We found 16 genes with differential expression between L-Asparaginase sensitive and resistant patients (FDR < 10%), in different directions depending on TEL-AML1 status. Of these, 2 genes were discriminative for L-Asparaginase resistance in TEL-AML1 negative but not in TEL-AML1 positive ALL patients, and the other 14 genes were discriminative for L-Asparaginase resistance within both groups, but with opposite expression levels (Table 1).

Relapse prediction in TEL-AML1 positive ALL

The Wilcoxon rank-sums test was used to identify genes that are linked to the occurrence of a relapse. This analysis was performed on gene expression profiles generated at initial diagnosis of only 27 TEL-AML1 positive ALL patients with a minimum follow-up of 4 years (median follow-up of patients at risk of 5 years, range 4.0-12.8). Nine of these 27 patients suffered a relapse (median time to relapse 3.1 years, range 0.5-6.4). With a FDR of 63%, only 4 discriminative probe sets were identified ($P_{wilcoxon} < 0.001$; Table 2). These 4 probe sets showed no overlap with the 70 probe sets discriminative for L-Asparaginase resistance. Disease-free survival analysis for each probe set individually showed that relative overexpression (above median expression) of *KIAA1026* (probe set 213403_at) and *MGC11332* (probe set 213478_at) is associated with an unfavourable outcome (5 yrs DFS of 46% ± 14% and 50% ± 13%, respectively) compared to patients with a relative underexpression (5 yrs DFS of 100%, p < 0.001 for both probe sets). Relative underexpression (below median expression) of *KLK5* (probe set 222242_s_at) and *MGC3196* (probe set 203326_x_at) is associated with an unfavourable outcome (5 yrs DFS of 50% ± 13% for both probe sets) compared to patients with a relative overexpression (5 yrs DFS of 100%, p < 0.001 for both probe sets). Multivariate analysis including each individual probe set and other prognostic factors (age, WBC) indicated that none of these probe sets was of independent prognostic value in this group of TEL-AML1 positive ALL patients.

Table 1. The 16 genes differentially expressed between L-Asparaginase sensitive and resistant patients independent of TEL-AML1 status

		TEL-AML1 positive ALL				TEL-AML1 negative ALL			
probeset ID	**gene symbol**	**L-Asp sensitive***	**L-Asp resistant***	**ratio****	**p-value*****	**L-Asp sensitive***	**L-Asp resistant***	**ratio****	**p-value*****
201819_at	SCARB1	4.46 (4.15-4.77)	5.01 (4.81-5.38)	0.89	0.001	4.23 (3.88-4.94)	3.60 (3.44-33.93)	1.18	0.020
202493_x_at	CSH1	1.16 (1.02-1.50)	1.66 (1.36-2.82)	0.70	0.007	2.02 (1.56-3.15)	1.23 (1.00-1.51)	1.64	0.009
202861_at	PER1	4.98 (4.81-5.30)	4.47 (4.06-4.99)	1.11	0.020	2.71 (2.00-3.94)	4.83 (4.17-5.25)	0.56	0.020
205463_s_at	PDGFA	3.46 (2.88-3.81)	2.74 (2.32-3.12)	1.26	0.022	2.44 (2.12-3.91)	4.50 (4.03-5.15)	0.54	0.020
205524_s_at	HAPLN1	1.27 (1.21-1.42)	1.31 (1.05-1.50)	0.97	0.862	1.93 (1.77-2.41)	1.35 (1.23-1.54)	1.43	0.002
205978_at	KL	1.92 (1.72-2.07)	0.83 (0.55-1.24)	2.31	<0.001	1.11 (0.78-1.31)	1.85 (1.44-2.07)	0.60	0.027
206983_at	CCR6	2.13 (1.95-2.47)	1.56 (1.04-2.12)	1.37	0.014	1.18 (0.64-1.66)	1.89 (1.72-2.49)	0.62	0.006
207945_s_at	CSNK1D	5.34 (4.88-5.64)	4.35 (4.18-4.86)	1.23	0.004	3.82 (3.47-4.40)	5.39 (4.24-5.90)	0.71	0.020
208499_s_at	DNAJC3	3.42 (3.14-3.72)	2.79 (1.67-3.06)	1.23	0.001	2.67 (1.98-3.21)	3.52 (3.16-3.81)	0.76	0.037
208622_s_at	VIL2	6.48 (5.91-6.95)	5.77 (5.20-6.10)	1.12	0.022	5.23 (5.09-6.14)	6.97 (6.31-7.31)	0.75	0.006
208869_s_at	GABARAPL1	3.73 (3.35-4.19)	3.38 (2.99-3.67)	1.10	0.028	3.03 (2.61-3.65)	4.18 (3.92-4.27)	0.72	0.004
213548_s_at	H41	3.99 (3.68-4.18)	3.59 (3.10-3.85)	1.11	0.022	1.90 (1.49-2.36)	3.87 (3.00-4.17)	0.49	0.002
217523_at	CD44	4.34 (3.43-4.58)	2.97 (2.62-3.80)	1.46	0.018	3.38 (2.74-4.21)	5.29 (4.87-5.99)	0.64	0.006
220012_at	ERO1LB	3.78 (3.52-3.95)	3.50 (2.97-3.95)	1.08	0.207	2.78 (1.50-3.18)	3.70 (3.39-4.33)	0.75	0.002
221708_s_at	SMAP-1	4.15 (3.72-4.21)	4.25 (4.17-4.28)	0.98	0.008	4.36 (4.23-4.36)	3.74 (3.51-3.96)	1.17	0.001
221969_at	PAX5	5.78 (5.49-5.86)	6.09 (5.82-6.22)	0.95	0.003	5.75 (5.65-6.06)	4.92 (4.34-5.56)	1.17	0.009

*median normalized expression (P25-P75) in arbitrary units.

**ratio between L-Asparaginase (L-Asp) sensitive and L-Asp resistant in TEL-AML1 positive and negative ALL

***MWU analysis between L-Asp sensitive and resistant patients in both TEL-AML1 positive and negative ALL

Table 2. Identification of probe sets of which expression level at initial diagnosis is predictive for clinical outcome in TEL-AML1 positive ALL.

probeset ID	gene symbol	Pts in CCR*	Pts who suffer from relapse*	Ratio	p-value**
213403_at	KIAA1026	2.17 (1.74-2.39)	2.84 (2.74-3.10)	0.76	p<0.001
222242_s_at	KLK5	3.53 (3.46-3.70)	2.90 (2.72-3.30)	1.22	p<0.001
203326_x_at	MGC3196	2.22 (1.97-2.53)	1.79 (1.71-1.90)	1.24	p<0.001
213478_at	MGC11332	2.53 (1.99-2.90)	3.37 (2.96-3.60)	0.75	p<0.001

*median normalized expression (P25-P75) in arbitrary units; CCR: complete continuous remission
**Wilcoxon p-value

DISCUSSION

The present study used an extensive genome-wide approach to get insight into putative causes of L-Asparaginase resistance and therapy failure in TEL-AML1 positive cases. We have identified genes that are differentially expressed between L-Asparaginase sensitive and resistant cases with TEL-AML1 positive ALL. The mechanism(s) of L-Asparaginase resistance in ALL cells is unclear. L-Asparaginase causes depletion of asparagine and glutamine and high expression of asparagine synthetase may therefore rescue leukemic cells from asparagine deficiency, a phenomenon seen in several cell[25,26,37-42] lines. A gene expression profiling study by Fine et al. showed that genes predictive for L-Asparaginase resistance in leukemic cell lines were not predictive for L-Asparaginase resistance in a mixed group of ALL patients[43]. Asparagine synthetase was the best predictor of L-Asparaginase resistance and asparagine synthetase was more highly expressed in L-Asparaginase resistant cell lines, but not in ALL patients[43]. These data may be explained by the fact that the causes of L-Asparaginase resistance may differ between genetic subtypes. We recently showed that expression of asparagine synthetase is linked to L-Asparaginase resistance in leukemic cells of TEL-AML1 negative ALL patients, but not in TEL-AML1 positive ALL patients[21,26]. This indicates that the mechanism of L-Asparaginase resistance differs between ALL genotypes.

Recently, we found that especially genes involved in protein synthesis were overrepresented in L-Asparaginase resistant mainly TEL-AML1 negative precursor B-lineage ALL patients[27]. Also a discordant resistance between vincristine and L-Asparaginase was described, which indicated that ALL cells that are L-Asparaginase sensitive and vincristine resistant are likely to be TEL-AML1 positive[44]. The genes discriminating for L-Asparaginase resistance in pediatric B-lineage ALL encoded merely cytoplasmic ribosomal proteins. In contrast, in the TEL-AML1 positive patients studied here the number of protein synthesis-associated probe sets were limited (n = 10) and linked to mitochondrial instead of cytoplasmatic ribosomes. In TEL-AML1 positive ALL, functional analysis by gene ontology indicated that resistance to L-Asparaginase is presumably more linked to defects in the apoptosis machinery (*HDAC1, TIA1, HTATIP2, BCL2L13, SPHK2*) and lipid metabolism (*FDPS, HSD17B4, LDLR, UGCG, DPAGT1,*

PPAP2B, SPHK2). Taken together our and previous studies, the absence of an overlap in L-Asparaginase resistance associated genes suggests different causes of resistance to this drug between genotypes. In correspondence with this, we found L-Asparaginase resistance associated genes whose expression levels were oppositely associated with L-Asparaginase resistance in TEL-AML1 positive and negative ALL.

In the present study, we addressed which genes may be important for L-Asparaginase resistance in TEL-AML1 positive ALL subtype. We identified 5 genes involved in chromatin remodelling by histone (de)acetylation (i.e. *HDAC1*, *SMC4L1*, *HTATIP2*, *MYST3* and *SMARCAL1*) that were associated with L-Asparaginase resistance in TEL-AML1 positive ALL (Figure 1). Chromatin remodelling by histone acetylation and deacetylation is crucial in the regulation of gene expression. The TEL-AML1 fusion protein represses transcription of AML1 target genes through recruitment of histone deacetylases (HDACs) which induces closure of the chromatin[1,45-49]. HDAC inhibitors, therefore, might enhance the efficacy of cytotoxic agents like L-Asparaginase. Recently, we observed that both TEL-AML1 positive and negative cases became more sensitive to L-Asparaginase upon exposure to the HDAC inhibitor FK228 (depsipeptide)[50]. This sensitizing effect was not due to synergism between FK228 and L-Asparaginase, but was caused by the additive toxicity of FK288 itself. This suggests that FK228 may not be the first choice to reverse L-Asparaginase resistance in pediatric ALL. More insight into the biological function of the yet identified genes may elucidate whether chromatin remodelling plays a role in L-Asparaginase resistance in TEL-AML1 positive ALL.

Genes involved in lipid metabolism (*FDPS, HSD17B4, LDLR, UGCG, DPAGT1, PPAP2B, SPHK2*) were related to L-Asparaginase resistance in TEL-AML1 positive ALL patients. *FDPS* and *LDLR* are involved in cholesterol synthesis. Cholesterol synthesis has been related to sensitivity of several drugs in AML cells, but not in ALL cells[51]. Furthermore, genes involved in the ceramide metabolism pathway (*UGCG* and *SPHK2*) seem important, since 2 out of 7 genes on the total GeneChip involved in the ceramide pathway were discriminative for L-Asparaginase cytotoxicity. Ceramide expression on its turn can induce apoptosis[52].

Differential expression of apoptosis genes in childhood ALL is associated with lineage, genetic subtype, in vitro drug resistance and clinical outcome[36]. L-Asparaginase resistance is associated with decreased induction of apoptosis in childhood ALL, but with unknown causes[53]. We found *HDAC1, TIA1, HTATIP2, BCL2L13* and *SPHK2* to be linked to L-Asparaginase resistance in TEL-AML1 positive ALL. BCL2L13 (Bcl-rambo) is a recently discovered member of the pro-apoptotic Bcl-2 family[54,55], and is associated with in vitro L-Asparaginase resistance and an un favourable long-term outcome in children with B-lineage ALL[36]. In the present and independent study, we also found that high expression of *BCL2L13* was associated with L-Asparaginase resistance in TEL-AML1 positive ALL. Furthermore, high expression of *BCL2L13* was related to a

poor prognosis in TEL-AML1 patients (Figure 3). Therefore, *BCL2L13* may represent a new risk factor in childhood ALL independent of genetic subtype.

In the present study we found 4 genes to be linked with the occurrence of a relapse in TEL-AML1 positive ALL, although with a very high false discovery rate of 63%. This indicates that either no genes exist that can predict relapse or that the differences in expression of these genes is small and more patients should be analyzed to gain statistical power. Another possibility is that the clonal evolution of an underlying pre-leukemic subclone gives rise to a novel leukemia, which is falsely regarded as a relapse of the original major clone[56]. Despite the high false discovery rate, one of these 4 genes, Kallikrein 5 (*KLK5*), a serine protease, has been shown before to be associated with an unfavourable prognosis in several forms of cancer[57-59]. Remarkably, however, in the present study a low expression is associated with a poor prognosis in TEL-AML1 positive ALL. This suggests a different functional role for the serine protease KLK5 in primary leukemic cells of children compared to carcinoma cells.

In conclusion, we found 70 probe sets representing 52 unique genes and 3 expressed sequence taqs, which discriminated between L-Asparaginase sensitive and resistant TEL-AML1 positive ALL patients. These genes are different from the genes found in TEL-AML1 negative ALL, strengthening the hypothesis that the mechanism of L-Asparaginase action and/or resistance differs between both genetic subgroups. Studying the biological function of these genes may reveal that new insights in putative targets can be used to improve the treatment of TEL-AML1 positive leukemia.

ACKNOWLEDGEMENTS

We wish to express our gratitude to the members of the DCOG and the German COALL study group for their support to this study by providing leukemic samples.

REFERENCES

1. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. N Engl J Med. 2004;350:1535-1548.
2. Sawinska M, Ladon D. Mechanism, detection and clinical significance of the reciprocal translocation t(12;21)(p12;q22) in the children suffering from acute lymphoblastic leukaemia. Leuk Res. 2004;28:35-42.
3. Sharrocks AD, Brown AL, Ling Y, Yates PR. The ETS-domain transcription factor family. Int J Biochem Cell Biol. 1997;29:1371-1387.
4. Graves BJ, Petersen JM. Specificity within the ets family of transcription factors. Adv Cancer Res. 1998;75:1-55.
5. Sharrocks AD. The ETS-domain transcription factor family. Nat Rev Mol Cell Biol. 2001;2:827-837.
6. Sementchenko VI, Watson DK. Ets target genes: past, present and future. Oncogene. 2000;19:6533-6548.
7. Miyoshi H, Shimizu K, Kozu T, Maseki N, Kaneko Y, Ohki M. t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. Proc Natl Acad Sci U S A. 1991;88:10431-10434.
8. Levanon D, Negreanu V, Bernstein Y, Bar-Am I, Avivi L, Groner Y. AML1, AML2, and AML3, the human members of the runt domain gene-family: cDNA structure, expression, and chromosomal localization. Genomics. 1994;23:425-432.
9. Speck NA, Stacy T, Wang Q, et al. Core-binding factor: a central player in hematopoiesis and leukemia. Cancer Res. 1999;59:1789s-1793s.
10. Lutterbach B, Hiebert SW. Role of the transcription factor AML-1 in acute leukemia and hematopoietic differentiation. Gene. 2000;245:223-235.
11. Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH, Speck NA. Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc Natl Acad Sci U S A. 1996;93:3444-3449.
12. Sasaki K, Yagi H, Bronson RT, et al. Absence of fetal liver hematopoiesis in mice deficient in transcriptional coactivator core binding factor beta. Proc Natl Acad Sci U S A. 1996;93:12359-12363.
13. Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell. 1996;84:321-330.
14. Loh ML, Rubnitz JE. TEL/AML1-positive pediatric leukemia: prognostic significance and therapeutic approaches. Curr Opin Hematol. 2002;9:345-352.
15. Veiga LB, Coser VM, Cavalli LR, et al. High frequency of t(12;21)(p13;q22) in children with acute lymphoblastic leukemia and known clinical outcome in southern Brazil. Leuk Res. 2004;28:1033-1038.
16. Zen PR, Lima MC, Coser VM, et al. Prevalence of TEL/AML1 fusion gene in Brazilian pediatric patients with acute lymphoblastic leukemia. Cancer Genet Cytogenet. 2004;151:68-72.
17. Sazawal S, Bhatia K, Gutierrez MI, Saxena R, Arya LS, Bhargava M. Paucity of TEL-AML 1 translocation, by multiplex RT-PCR, in B-lineage acute lymphoblastic leukemia (ALL) in Indian patients. Am J Hematol. 2004;76:80-82.

18. Kanerva J, Saarinen-Pihkala UM, Niini T, et al. Favorable outcome in 20-year follow-up of children with very-low-risk ALL and minimal standard therapy, with special reference to TEL-AML1 fusion. Pediatr Blood Cancer. 2004;42:30-35.
19. Stams WA, Beverloo HB, den Boer ML, et al. Incidence of additional genetic changes in the TEL and AML1 genes in t(12;21) positive pediatric ALL and their relation with drug sensitivity and clinical outcome. submitted.
20. Ramakers-van Woerden NL, Pieters R, Loonen AH, et al. TEL/AML1 gene fusion is related to in vitro drug sensitivity for L-asparaginase in childhood acute lymphoblastic leukemia. Blood. 2000;96:1094-1099.
21. Stams WA, den Boer ML, Beverloo HB, et al. Sensitivity to L-asparaginase is not associated with expression levels of asparagine synthetase in t(12;21)+ pediatric ALL. Blood. 2003;101:2743-2747.
22. Chakrabarti R, Schuster SM. L-Asparaginase: Perspectives on the mechanisms of action and resistance. Int J Pediatric Hematol Oncol. 1997;4:597-611.
23. Ohnuma T, Holland JF, Freeman A, Sinks LF. Biochemical and pharmacological studies with asparaginase in man. Cancer Res. 1970;30:2297-2305.
24. Miller HK, Salser JS, Balis ME. Amino acid levels following L-asparagine amidohydrolase (EC.3.5.1.1) therapy. Cancer Res. 1969;29:183-187.
25. Aslanian AM, Fletcher BS, Kilberg MS. Asparagine synthetase expression alone is sufficient to induce l-asparaginase resistance in MOLT-4 human leukaemia cells. Biochem J. 2001;357:321-328.
26. Stams WA, den Boer ML, Holleman A, et al. Asparagine synthetase expression is linked with L-asparaginase resistance in TEL-AML1-negative but not TEL-AML1-positive pediatric acute lymphoblastic leukemia. Blood. 2005;105:4223-4225.
27. Holleman A, Cheok MH, den Boer ML, et al. Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. N Engl J Med. 2004;351:533-542.
28. Kaspers GJ, Veerman AJ, Pieters R, et al. Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay. Br J Cancer. 1994;70:1047-1052.
29. Den Boer ML, Harms DO, Pieters R, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol. 2003;21:3262-3268.
30. Armstrong SA, Staunton JE, Silverman LB, et al. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. Nat Genet. 2002;30:41-47.
31. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics. 2002;18 Suppl 1:S96-104.
32. Klipper-Aurbach Y, Wasserman M, Braunspiegel-Weintrob N, et al. Mathematical formulae for the prediction of the residual beta cell function during the first two years of disease in children and adolescents with insulin-dependent diabetes mellitus. Med Hypotheses. 1995;45:486-490.
33. Cardoso J, Molenaar L, de Menezes RX, et al. Genomic profiling by DNA amplification of laser capture microdissected tissues and array CGH. Nucleic Acids Res. 2004;32:e146.
34. t Hoen PA, Turk R, Boer JM, et al. Intensity-based analysis of two-colour microarrays enables efficient and flexible hybridization designs. Nucleic Acids Res. 2004;32:e41.

35. Yeoh EJ, Ross ME, Shurtleff SA, et al. Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. Cancer Cell. 2002;1:133-143.
36. Holleman A, den Boer ML, Menezes RX, et al. The expression of 70 apoptosis genes in relation to lineage, genetic subtype, cellular drug resistance, and outcome in childhood acute lymphoblastic leukemia. submitted.
37. Prager MD, Bachynsky N. Asparagine synthetase in asparaginase resistant and susceptible mouse lymphomas. Biochem Biophys Res Commun. 1968;31:43-47.
38. Kiriyama Y, Kubota M, Takimoto T, et al. Biochemical characterization of U937 cells resistant to L-asparaginase: the role of asparagine synthetase. Leukemia. 1989;3:294-297.
39. Worton KS, Kerbel RS, Andrulis IL. Hypomethylation and reactivation of the asparagine synthetase gene induced by L-asparaginase and ethyl methanesulfonate. Cancer Res. 1991;51:985-989.
40. Martin JK, Sun W, Maraga D, Schuster SM, Wylie DE. An investigation into the machanism of L-asparaginase resistance in L5178Y murine leukemia cells. Amino Acids. 1993;5:51-69.
41. Hutson RG, Kitoh T, Moraga Amador DA, Cosic S, Schuster SM, Kilberg MS. Amino acid control of asparagine synthetase: relation to asparaginase resistance in human leukemia cells. Am J Physiol. 1997;272:C1691-1699.
42. Scherf U, Ross DT, Waltham M, et al. A gene expression database for the molecular pharmacology of cancer. Nat Genet. 2000;24:236-244.
43. Fine BM, Kaspers GJ, Ho M, Loonen AH, Boxer LM. A genome-wide view of the in vitro response to l-asparaginase in acute lymphoblastic leukemia. Cancer Res. 2005;65:291-299.
44. Lugthart S, Cheok MH, den Boer ML, et al. Identification of genes conferring cross-resistance in childhood acute lymphoblastic leukemia. Cancer Cell. 2005;7:375-86.
45. Chakrabarti SR, Sood R, Nandi S, Nucifora G. Posttranslational modification of TEL and TEL/AML1 by SUMO-1 and cell-cycle-dependent assembly into nuclear bodies. Proc Natl Acad Sci U S A. 2000;97:13281-13285.
46. Hiebert SW, Sun W, Davis JN, et al. The t(12;21) translocation converts AML-1B from an activator to a repressor of transcription. Mol Cell Biol. 1996;16:1349-1355.
47. Zelent A, Greaves M, Enver T. Role of the TEL-AML1 fusion gene in the molecular pathogenesis of childhood acute lymphoblastic leukaemia. Oncogene. 2004;23:4275-4283.
48. Fenrick R, Amann JM, Lutterbach B, et al. Both TEL and AML-1 contribute repression domains to the t(12;21) fusion protein. Mol Cell Biol. 1999;19:6566-6574.
49. Guidez F, Petrie K, Ford AM, et al. Recruitment of the nuclear receptor corepressor N-CoR by the TEL moiety of the childhood leukemia-associated TEL-AML1 oncoprotein. Blood. 2000;96:2557-2561.
50. Stams WA, den Boer ML, Beverloo HB, et al. Histone deacetylase inhibitor FK228 (FR901228, depsipeptide) induces B-cell differentiation in both TEL-AML1 positive and negative pediatric acute lymphoblastic leukemia. submitted.
51. Banker DE, Mayer SJ, Li HY, Willman CL, Appelbaum FR, Zager RA. Cholesterol synthesis and import contribute to protective cholesterol increments in acute myeloid leukemia cells. Blood. 2004;104:1816-1824.
52. Pettus BJ, Chalfant CE, Hannun YA. Ceramide in apoptosis: an overview and current perspectives. Biochim Biophys Acta. 2002;1585:114-125.

53. Holleman A, den Boer ML, Kazemier KM, Janka-Schaub GE, Pieters R. Resistance to different classes of drugs is associated with impaired apoptosis in childhood acute lymphoblastic leukemia. Blood. 2003;102:4541-4546.
54. Kataoka T, Holler N, Micheau O, et al. Bcl-rambo, a novel Bcl-2 homologue that induces apoptosis via its unique C-terminal extension. J Biol Chem. 2001;276:19548-19554.
55. Yi P, Zhang W, Zhai Z, Miao L, Wang Y, Wu M. Bcl-rambo beta, a special splicing variant with an insertion of an Alu-like cassette, promotes etoposide- and Taxol-induced cell death. FEBS Lett. 2003;534:61-68.
56. Ford AM, Fasching K, Panzer-Grumayer ER, Koenig M, Haas OA, Greaves MF. Origins of "late" relapse in childhood acute lymphoblastic leukemia with TEL-AML1 fusion genes. Blood. 2001;98:558-564.
57. Diamandis EP, Borgono CA, Scorilas A, et al. Immunofluorometric quantification of human kallikrein 5 expression in ovarian cancer cytosols and its association with unfavorable patient prognosis. Tumour Biol. 2003;24:299-309.
58. Planque C, de Monte M, Guyetant S, et al. KLK5 and KLK7, two members of the human tissue kallikrein family, are differentially expressed in lung cancer. Biochem Biophys Res Commun. 2005;329:1260-1266.
59. Michael IP, Sotiropoulou G, Pampalakis G, et al. Biochemical and enzymatic characterization of human kallikrein 5 (hK5), a novel serine protease potentially involved in cancer progression. J Biol Chem. 2005;280:14628-14635.

Part C

Specificity of targeted drugs for t(12;21) positive ALL

Chapter 8

Histone deacetylase inhibitor FK228 (FR901228, depsipeptide) induces B-cell differentiation in both TEL-AML1 positive and negative pediatric acute lymphoblastic leukaemia

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Accepted for publication in Haematologica

ABSTRACT

PURPOSE: The most common translocation in childhood pediatric acute lymphoblastic leukemia involves fusion of *TEL* and *AML1* genes. The fusion protein TEL-AML1 recruits co-repressors and histone deacetylases (HDACs), which transrepress AML1 target genes. FK228 (FR901228 or depsipeptide) is a promising HDAC inhibitor that inhibits cell proliferation and induces differentiation and apoptosis of cancer cells. We investigated the specificity of FK228 as putative new drug in the treatment of TEL-AML1 positive ALL.

EXPERIMENTAL DESIGN: The cytotoxicity of FK228 in TEL-AML1 positive and negative ALL patient cells and normal bone marrow cells was determined using MTT assays. The induction of B-cell differentiation by FK228 exposure in TEL-AML1 positive and negative patient cells was determined by analyzing the expression of nine B-cell markers by flow cytometry.

RESULTS: Normal bone marrow cells were in vitro median 2-fold more resistant to FK228 induced cell kill compared to primary TEL-AML1 positive and negative ALL patient cells ($p = 0.03$). FK228 induced dose-dependent B-cell differentiation in both TEL-AML1 positive and negative ALL cells. FK228 had an additive effect on the in vitro cytotoxicity of L-asparaginase in both genetic ALL subtypes.

CONCLUSION: FK228 induces differentiation in children with B-lineage ALL, but its effect is not selective for TEL-AML1 rearranged B-lineage ALL only.

INTRODUCTION

The t(12;21)(p13;q22) (*TEL-AML1*) is the most common (~25%) translocation in childhood acute lymphoblastic leukemia (ALL), and is restricted to precursor B-cell lineage leukemia. The prognosis of ALL patients with the *TEL-AML1* translocation is under discussion and seems to depend on the intensity of the treatment protocol[1]. In addition, TEL-AML1 positive ALL patients are more sensitive to L-Asparaginase compared to TEL-AML1 negative ALL patients[2,3].

TEL (ETV6) contains an N-terminal pointed (PNT) dimerization domain which mediates homodimerization[4,5]. The C-terminal DNA-binding domain (homologous domain in all Ets-family transcription factors) recognizes a purine-rich GGAA/T core motif within promoters and enhancers of various genes[6]. TEL contributes to angiogenesis and homing of hematopoietic progenitor cells to the bone marrow[7,8]. *AML1 (RUNX1)* encodes a transcription factor that binds the enhancer core sequence TGT/cGGT through its N-terminal Runt homology domain (RHD)[9]. The DNA-binding affinity of AML1 is increased by heterodimerization between the RHD and the core binding factor β protein, forming the core binding factor. Transcription of AML1-targeted genes is mediated by the C-terminal transactivation (TA) domain of AML1. The core binding factor is essential for definitive hematopoiesis of all lineages[10,11].

The TEL-AML1 fusion product contains the PNT domain of TEL and the RHD and TA domain of AML1. The PNT domain and the central region of TEL as well as the RHD and amino acids 216-290 within AML1, appear critical in inhibition of transcription via binding of co-repressors such as SMRT, mSin3A, N-CoR and HDAC-3[12-16]. Like AML1, the abnormal TEL-AML1 fusion protein can bind to core enhancer sequences, but instead of activating transcription through recruitment of co-activators and histone acetylases, it recruits co-repressors and histone deacetylases (HDACs). This induces closure of the chromatin resulting into inhibition of transcription[17-20]. These changes in the normal AML1-mediated transcriptional cascade alter both the self-renewal capacity and the differentiation capacity of hematopoietic cells[11,21]. TEL-AML1 positive ALL might benefit from treatment with HDAC inhibitors since the TEL-AML1-induced transcriptional repression was shown to be reversed by HDAC inhibitors[13,15]. Besides TEL-AML1 positive ALL cells, also in t(8;21)/[*AML1-ETO*], t(15;17)/[*PML-RARα*] and t(11;17)/[*PLZF-RARα*] positive acute myeloid leukemia differentiation was induced by HDAC inhibitors[22,23].

HDAC inhibitors are a new class of antineoplastic agents currently being evaluated in clinical trials. The class includes butyrates, which have traditionally been studied as differentiating agents, and a more recently developed bicyclic depsipeptide FK228 (FR901228)[24]. FK228 and butyrates have been shown to induce cell differentiation in myeloid leukemias[22,23,25,26], to inhibit cell proliferation[27] and to enhance apoptosis in

several types of cancer cells[27-33]. Recently, phase I clinical trials demonstrated that both FK228 and sodium butyrate are well tolerated, but FK228 is the only HDAC inhibitor so far that has shown clinical efficacy in specific tumor types[34-36]. Currently, a multi-institutional phase II trial of FK228 for patients with T-cell lymphomas is being coordinated by the American National Cancer Institute[24]. The present study was undertaken to gain more insight into the potential use of HDAC inhibitors in the treatment of TEL-AML1 positive ALL. To this aim, we studied the cytotoxic effect of HDAC inhibition alone and in combination with L-asparaginase in TEL-AML1 negative and positive leukemic cells of children with ALL at initial diagnosis. In addition, the effect of HDAC inhibition on B-cell differentiation of these leukemic cells was studied.

MATERIALS AND METHODS

Patient samples

Bone marrow and/or peripheral blood samples from 29 untreated children with common/pre B-ALL at initial diagnosis were collected at the Erasmus MC - Sophia Children's Hospital, the Dutch Childhood Oncology Group (DCOG) and the German COALL study group. Bone marrow samples from 4 healthy children were included as controls. Samples were collected after informed consent was obtained from patients, their parents or guardians, according to the Helsinki declaration. The informed consent has been approved by the Medical Ethics Committee Erasmus MC (i.e. Institutional Review Board of the Erasmus University Medical Center Rotterdam, The Netherlands). 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 min 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; Life Technologies, Gaithersburg, USA), 20% fetal calf serum (Integro, Zaandam, the Netherlands), 2 mM L-glutamine (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 pencillin, 100 µg/ml streptomycin, 0.125 µg/ml fungizone (Gibco BRL, Life Technologies) and 0.2 mg/ml gentamycin (Life Technologies). Contaminating non-leukemic cells in the ALL samples were removed by immunomagnetic beads as described earlier[37]. All resulting samples contained ≥ 90% leukemic cells, as determined morphologically on May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins.

In vitro cytotoxicity assay

In vitro cytotoxicity of the HDAC inhibitors FK228 and sodium butyrate as well as L-Asparaginase was determined using the MTT assay[38]. Briefly, 100 µl aliquots of cell suspension (1.6×10^5 cells) were cultured in round-bottomed 96 well microtitre plates in the presence of six concentrations of L-Asparaginase (Paronal, Christiaens B.V., Breda, The Netherlands) ranging from 0.00013 – 10 IU/ml, sodium butyrate (Sigma-Aldrich, St. Louis, MO, USA) ranging from 0.01 – 1000 µg/ml or FK228 (FR901228, Fujisawa Pharmaceutical Co., Osaka, Japan) ranging from 0.04 – 10 ng/ml in duplicate. To study the sensitizing effect of FK288, cells were pre-exposed to FK228 (0.1, 0.4 and 1.1 ng/ml) for 30 minutes before co-incubation with different concentrations of L-Asparaginase in a 96-wells plate. Control cells were cultured without drugs. After 4 days of (co)incubation at 37°C, 10 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT, 5 mg/ml; Sigma Aldrich, Zwijndrecht, The Netherlands) was added to each well. During a 6-hour incubation, the yellow MTT tetrazolium salt is reduced to purple-blue formazan crystals by viable cells. Samples with ≥ 70% leukemic cells in the control wells and an optical density higher than 0.050 arbitrary units (adjusted for blank values) were used to calculate the concentration of drug lethal to 50% of the cells (LC_{50}).

B-cell differentiation markers

After 4 days of exposure to FK228 or control medium, the expression of B-cell differentiation markers was measured by flow cytometry. Three selected quadruple immunostainings (1. TdT-FITC/CD19-PE/CD20-PerCP/CD10-APC; 2. CD34-FITC/CD19-PE/CD45-PerCP/CD22-APC; 3. SmIgM-PE/CyIgµ-FITC/CD19-PerCPCy5.5/CD34-APC; BD Biosciences, San Jose, CA, USA) were used to analyze FK228-induced changes in the differentiation stage of precursor B-cells[39]. As described before[40], 25 µl aliquots of FK228 exposed leukemic cells (20×10^6 cells/ml) were incubated for 10 minutes at room temperature with combinations of optimally titrated monoclonal antibodies (see reference 42 for details on the antibodies used). Analysis of marker expression was performed on gated living cells based on forward and sideward scatter characteristics. Quadruple labeling for membrane-bound antigens were directly analyzed on a FACSCalibur (BD Biosciences, San Jose, CA, USA) using Cell Quest Pro software. For staining of cytoplasmic µ chain (CyIgµ) and intranuclear staining of terminal deoxynucleotidyl transferase (TdT), we first analyzed the membrane-bound antigens followed by permeabilisation of the plasma and nuclear membranes using IntraPrep Permeabilisation Reagent (Immunotech, Marseille, France) and FACSBrand lysing solution (BD Biosciences; San Diego, CA, USA), respectively. The fluorescence index (FI) was calculated by dividing the mean fluorescence intensity of the specific antibody by the value of its isotypic control antibody. The fold-change value for each differentiation

marker represents the ratio between the FI of FK228 exposed cells compared to the FI of culture medium (control)-treated cells.

Statistics

The cytotoxicity of HDAC inhibitors between two groups was compared using the Mann-Whitney U test. The effect of FK228 exposure on L-Asparaginase cytotoxicity was analyzed by paired t-test of the area under the curve. Statistical tests were performed at a two-tailed significance level of 0.05.

RESULTS

FK228 and sodium butyrate cytotoxicity

Cytotoxicity of FK228 was measured in 14 TEL-AML1 positive ALL, 15 TEL-AML1 negative ALL and 4 normal bone marrow samples (Figure 1A). The cytotoxicity of FK228 did not differ between TEL-AML1 positive (median LC_{50} value 1.0 ng/ml, P25-P75: 0.8-1.3) and TEL-AML1 negative ALL cells (median LC_{50} value 0.8 ng/ml, P25-P75: 0.6-1.2; p = 0.3). Normal bone marrow samples were median 2-fold more resistant compared to ALL cases (median LC_{50} value 2.0 ng/ml, P25-P75: 1.1-2.6 and median LC_{50} value 1.0 ng/ml, P25-P75: 0.7-1.2 respectively; p = 0.03).

In correspondence with FK228, the cytotoxicity of sodium butyrate did not differ between TEL-AML1 positive and negative cases (median LC_{50} value 0.05 µg/ml, P25-

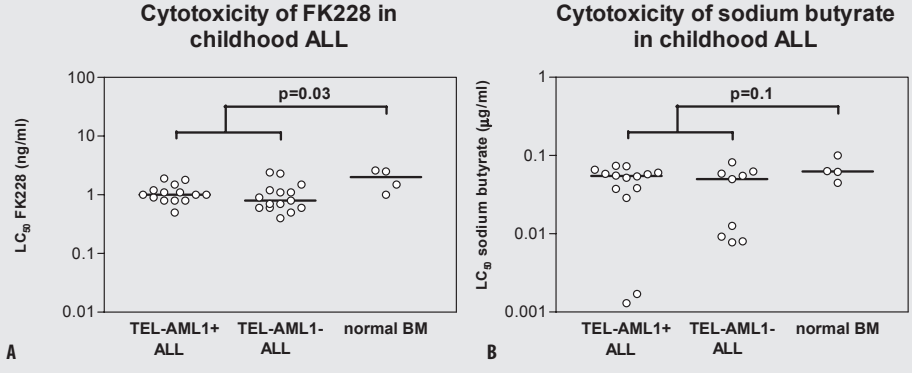

Figure 1. Cytotoxicity of HDAC inhibitors inchildhood ALL

A. FK228. Cytotoxicity of FK228 was measured in 14 TEL-AML1 positive (median LC_{50} value 1.0 ng/ml), 15 TEL-AML1 negative (median LC_{50} value 0.8 ng/ml) and 4 normal bone marrow (BM) samples (median LC_{50} value 2.0 ng/ml). TEL-AML1 positive versus negative ALL cells: p=0.3; TEL-AML1 positive and negative ALL cells versus normal BM samples: p=0.03.

B. Sodium butyrate. Cytotoxicity of sodium butyrate was measured in 14 TEL-AML1 positive (median LC_{50} value 0.05 µg/ml), 9 TEL-AML1 negative (median LC_{50} value 0.05 µg/ml) and 4 normal bone marrow (BM) samples (median LC_{50} value 0.06 µg/ml). TEL-AML1 positive versus negative ALL cells: p=0.6; TEL-AML1 positive and negative ALL cells versus normal BM samples: p=0.1.

P75 0.009-0.06 µg/ml and median LC_{50} value 0.05 µg/ml, P25-P75 0.04-0.06 µg/ml, respectively; Figure 1B). Normal bone marrow cells were ~1.2-fold more resistant to this drug compared to ALL cells (median LC_{50} value 0.06 µg/ml, P25-P75: 0.05-0.9 µg/ml and median LC_{50} value 0.05 µg/ml, P25-P75: 0.01-0.06 respectively; $p = 0.1$).

Effect of FK228 on L-Asparaginase cytotoxicity

The effect of FK228 exposure on L-Asparaginase sensitivity in leukemic cells was analyzed in 5 TEL-AML1 positive and 4 TEL-AML1 negative ALL samples. Co-incubation of 1.1 ng/ml FK228 and L-Asparaginase resulted into a strong decrease in cell survival compared to incubation with L-Asparaginase as single drug (Figure 2; $p = 0.03$). As shown in Figure 2, the effect of co-incubation did not differ between TEL-AML1 positive and negative cases. The effect of FK228 on L-Asparaginase sensitivity was additive only, since no difference in toxicity of L-Asparaginase was observed after correction for cell kill induced by FK288 itself in both genetic subgroups.

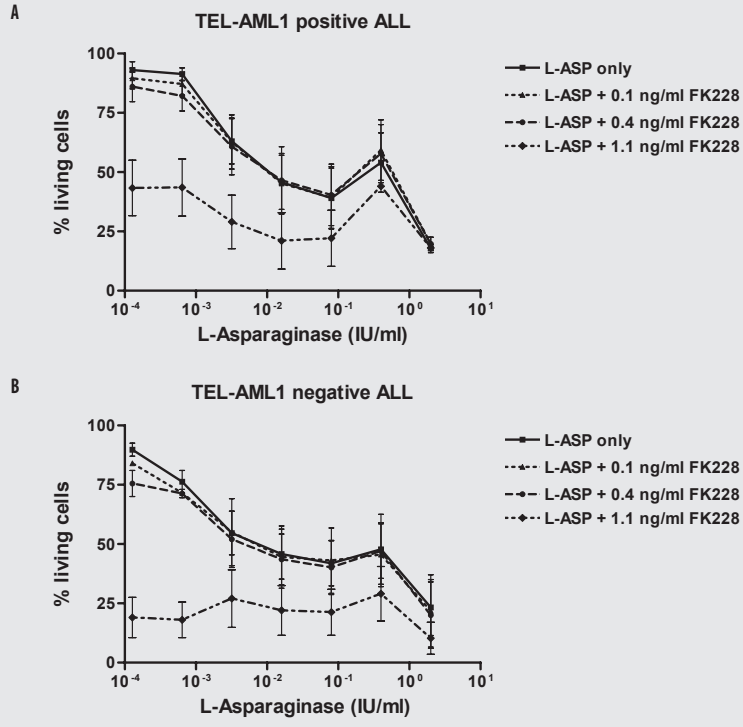

Figure 2. Sensitizing effect of FK228 on L-Asparaginase cytotoxicity in leukemic cells
The effect of 0.1, 0.4 and 1.1 ng/ml FK228 on L-Asparaginase cytotoxicity was studied in (**A**) 5 *TEL-AML1* positive and (**B**) 4 *TEL-AML1* negative ALL samples. Curves summarize the mean and standard error of the mean in percentage of surviving cells after incubation with L-asparaginase as single agent (solid line) and after co-incubation with FK288 (dashed lines).

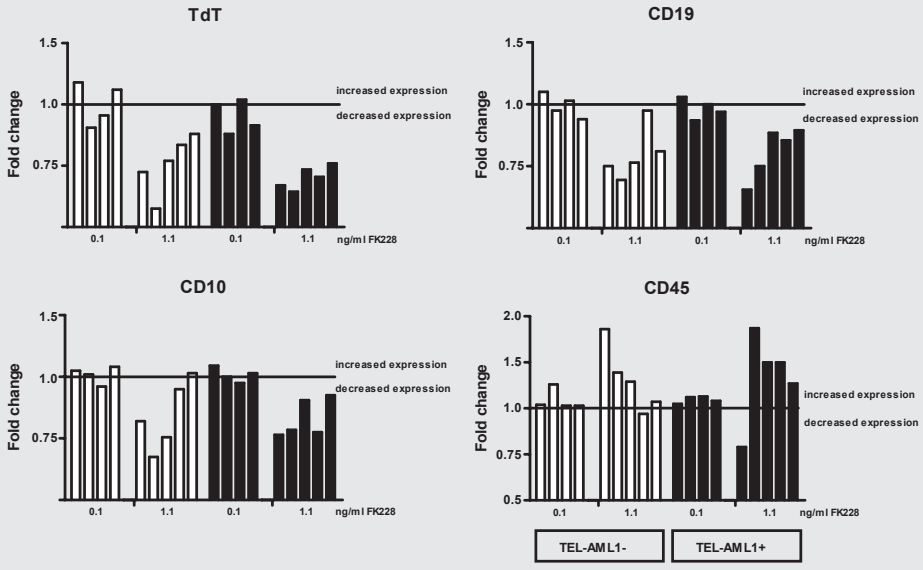

Figure 3. Effect of FK228 on B-cell differentiation expression in TEL-AML1 positive and negative ALL cells
Effect of exposure to 0.1 and 1.1 ng/ml FK228 in TEL-AML1 positive and negative ALL cells on the expression of B-cell differentiation markers TdT (2.6-fold decrease, $p < 0.001$), CD10 (1.4-fold decrease, $p=0.007$), CD19 (1.6-fold decrease, $p < 0.001$) and CD45 (1.1-fold increase, $p=0.09$) compared to controls cultured for the same time without FK228 exposure. There is no difference in effect of FK228 between TEL-AML1 positive and negative samples.

B-cell differentiation

Samples of five TEL-AML1 positive and five TEL-AML1 negative ALL patients (matched for age 1-10 yrs, common/preB immunophenotype, no hyperdiploidy (> 50 chromosomes), absence of *MLL* rearrangement and t(9;22)/[*BCR-ABL*]) were exposed to FK228 to study the effect on B-cell differentiation. The expression of none of the 9 tested differentiation markers changed after incubation with 0.1 and 0.4 ng/ml FK228 compared to control cells. However, exposure with 1.1 ng/ml FK228 resulted in a clear and significant effect on the expression of CD10, CD19, and TdT (Figure 3, Table 1). This effect of 1.1 ng/ml FK228 was comparable between TEL-AML1 positive and negative ALL cells (Figure 3). The expression after FK228 exposure was 1.4-fold decreased for CD10 ($p = 0.007$), 1.6-fold decreased for CD19 ($p < 0.001$) and 2.6-fold for TdT ($p < 0.001$;Table 1) compared to controls cultured for the same time without FK228 exposure. A slight increase of CD20 (1.8-fold), CD22 (1.1-fold) and CD45 (1.1-fold) was observed whereas the expression of CD34, CyIgμ and surface IgM (SmIgM) did not differ after exposure to FK228 (Table 1). The decrease in CD19, TdT, and CD10 as well as the slight increase in CD20, CD22 and CD45 expression corresponds to the induction of differentiation towards a more mature B-cell stadium[39].

Table 1. FK228-induced B-cell differentiation in childhood ALL

Marker	Fluorescence Index* unexposed median (P25-P75)		Fluorescence Index* exposed to 1.1 ng/ml FK228 median (P25-P75)		fold change	paired t-test p-value
CD10	751.1	(558.3-964.4)	550.3	(305.2-834.1)	-1.4	0.007
CD19	208.8	(136.7-358.1)	131.8	(76.1-242.2)	-1.6	<0.001
CD20	10.1	(3.8-24.2)	18.6	(5.4-24.2)	1.8	0.150
CD22	112.6	(100.3-316.1)	122.4	(83.3-273.2)	1.1	0.820
CD34	32.2	(8.6-85.1)	31.0	(13.3-70.5)	1.0	0.100
CD45	93.3	(42.7-111.2)	104.6	(64.9-130.9)	1.1	0.090
TdT	50.8	(26.1-67.4)	19.4	(12.2-31.2)	-2.6	<0.001
CyIgμ	24.8	(14.7-56.7)	26.3	(15.4-36.7)	1.1	0.150
SmIgM	10.6	(8.0-13.1)	9.0	(7.7-11.1)	1.2	0.160

* The fluorescence index (FI) is the mean fluorescence intensity of the specific antibody by its isotypic control antibody. Significant p-values are underlined.

DISCUSSION

The *AML1* gene is disrupted by the t(12;21) translocation in pediatric ALL. Normally, AML1 functions as the DNA-binding component of the core binding factor that activates the expression of genes required for proliferation and differentiation[41-43]. Numerous *AML1* translocations also occur in acute myeloid leukemia (AML)[44]. The common element of these fusion proteins is that they all fuse the enhancer core sequence binding RHD (runt homology domain) of AML1 with a second protein that inhibits AML1-dependent transcriptional activation[44]. Furthermore, most of these fusion products interact with the N-CoR complex, resulting into recruitment of Sin3 and HDACs to AML1-dependent promoters[12-16,23,45]. Since inhibitors of HDACs have been shown to abolish AML1-ETO-mediated transcriptional repression and to induce differentiation in t(8;21)/[*AML1-ETO*] positive AML[23], we studied if HDAC inhibitors also have an effect in ALL patients with a rearranged *AML1* gene, i.e. TEL-AML1 positive ALL.

Despite the presence of more binding sites for HDAC inhibitors on the *TEL-AML1* fusion gene compared to wild type *TEL* or *AML1* genes, we observed no difference between TEL-AML1 positive and negative cases in the cytotoxic effect of both HDAC inhibitors FK228 and sodium butyrate. Moreover, in both TEL-AML1 positive and negative cases, FK228 induced the differentiation into a more mature B-cell phenotype. This suggests that the effect of HDAC inhibitors is independent of the genetic subtype. Studies in acute myeloid leukemia also found that HDAC inhibition restores the differentiation process independent from genetic subtype[46]. It is conceivable to hypothesize that different genetic alterations may result in common patterns of deregulated gene expression, leading to blockage of differentiation and favoring leukemogenesis. For

both acute lymphoblastic and acute myeloid leukemia there are indications that the normal pattern of expression of *HOX* genes is disrupted (e.g. by rearrangement of *AML1, MLL, E2A or PBX1 genes*), causing a change in the self-renewal and growth of hematopoietic stem cells and committed progenitors[19]. It is of interest to study whether leukemogenesis is triggered when HDACs disrupt the HOX regulatory pathway of normal hematopoiesis.

TEL-AML1 positive ALL patients are known to be more sensitive to L-Asparaginase compared to TEL-AML1 negative ALL patients[2,3]. We recently showed that the expression level of asparagine synthetase (necessary for intracellular re-synthesis of asparagine) correlated to resistance for L-Asparaginase in TEL-AML1 negative cases but not in TEL-AML1 positive ALL patients[2,47]. This indicates that the mechanism of L-Asparaginase action (and hence cause of sensitivity or resistance) is different between both genetic subgroups of B-lineage ALL. Since both FK228 and L-Asparaginase induce apoptosis, it was of interest to determine the potentiating effect of FK228 on the cytotoxicity of L-Asparaginase. We observed that both TEL-AML1 positive and negative cases became more sensitive to L-Asparaginase upon FK228 exposure. However, this sensitizing effect was not caused by a synergism between FK228 and L-Asparaginase, but could be explained by an additive effect caused by the cytotoxicity of FK288 itself. Other HDAC inhibitors such as sodium butyrate have also been reported to induce an additive rather than a synergistic effect on drugs used in the treatment of ALL (e.g. vincristine, dexamethsone and daunorubicin) in several cancer types[48-50].

In the present study, we analyzed the cytotoxic and differentiating effect of the HDAC inhibitor FK228 in TEL-AML1 positive and negative ALL to gain more insights in the possibility to use this drug in the treatment of these patients. Clinical phase I/II trials suggest that FK228 is a promising HDAC inhibitor since it is well tolerated and already showed clinical efficacy in a few patients, such as a t(8;21) positive AML patient, one patient with renal cell carcinoma and several patients with a T-cell lymphoma[24]. Although our results showed that FK228 induced lymphoid differentiation, the effect of this HDAC inhibitor was not selective for TEL-AML positive ALL patients. However, the 2-fold difference in toxicity of FK228 between ALL and normal bone marrow samples may be indicative for a therapeutic advantage to use FK228 in the treatment of ALL. In addition, the non-TEL-AML1 restricted additive effect of FK228 on L-Asparaginase cytotoxicity as well as the induction of B-cell differentiation encourages further studies on the efficacy of HDAC inhibitors in the treatment of precursor B-ALL irrespective of TEL-AML1 status.

ACKNOWLEDGEMENTS

We wish to express our gratitude to the members of the DCOG and the German COALL study group for their support to this study by providing leukemic samples and to Fujisawa Pharmaceutical Co., Osaka, Japan for their support to this study by providing the HDAC inhibitor FK228.

REFERENCES

1. Loh ML, Rubnitz JE. TEL/AML1-positive pediatric leukemia: prognostic significance and therapeutic approaches. Curr Opin Hematol. 2002;9:345-352.
2. Stams WA, den Boer ML, Beverloo HB, et al. Sensitivity to L-asparaginase is not associated with expression levels of asparagine synthetase in t(12;21)+ pediatric ALL. Blood. 2003;101:2743-2747.
3. Ramakers-van Woerden NL, Pieters R, Loonen AH, et al. TEL/AML1 gene fusion is related to in vitro drug sensitivity for L-asparaginase in childhood acute lymphoblastic leukemia. Blood. 2000;96:1094-1099.
4. McLean TW, Ringold S, Neuberg D, et al. TEL/AML-1 dimerizes and is associated with a favorable outcome in childhood acute lymphoblastic leukemia. Blood. 1996;88:4252-4258.
5. Jousset C, Carron C, Boureux A, et al. A domain of TEL conserved in a subset of ETS proteins defines a specific oligomerization interface essential to the mitogenic properties of the TEL-PDGFR beta oncoprotein. Embo J. 1997;16:69-82.
6. Graves BJ, Petersen JM. Specificity within the ets family of transcription factors. Adv Cancer Res. 1998;75:1-55.
7. Sawinska M, Ladon D. Mechanism, detection and clinical significance of the reciprocal translocation t(12;21)(p12;q22) in the children suffering from acute lymphoblastic leukaemia. Leuk Res. 2004;28:35-42.
8. Wang LC, Swat W, Fujiwara Y, et al. The TEL/ETV6 gene is required specifically for hematopoiesis in the bone marrow. Genes Dev. 1998;12:2392-2402.
9. Meyers S, Downing JR, Hiebert SW. Identification of AML-1 and the (8;21) translocation protein (AML-1/ETO) as sequence-specific DNA-binding proteins: the runt homology domain is required for DNA binding and protein-protein interactions. Mol Cell Biol. 1993;13:6336-6345.
10. Hart SM, Foroni L. Core binding factor genes and human leukemia. Haematologica. 2002;87:1307-1323.
11. Speck NA, Gilliland DG. Core-binding factors in haematopoiesis and leukaemia. Nat Rev Cancer. 2002;2:502-513.
12. Chakrabarti SR, Nucifora G. The leukemia-associated gene TEL encodes a transcription repressor which associates with SMRT and mSin3A. Biochem Biophys Res Commun. 1999;264:871-877.
13. Guidez F, Petrie K, Ford AM, et al. Recruitment of the nuclear receptor corepressor N-CoR by the TEL moiety of the childhood leukemia-associated TEL-AML1 oncoprotein. Blood. 2000;96:2557-2561.
14. Wang L, Hiebert SW. TEL contacts multiple co-repressors and specifically associates with histone deacetylase-3. Oncogene. 2001;20:3716-3725.
15. Fenrick R, Amann JM, Lutterbach B, et al. Both TEL and AML-1 contribute repression domains to the t(12;21) fusion protein. Mol Cell Biol. 1999;19:6566-6574.
16. Uchida H, Downing JR, Miyazaki Y, Frank R, Zhang J, Nimer SD. Three distinct domains in TEL-AML1 are required for transcriptional repression of the IL-3 promoter. Oncogene. 1999;18:1015-1022.
17. Chakrabarti SR, Sood R, Nandi S, Nucifora G. Posttranslational modification of TEL and TEL/AML1 by SUMO-1 and cell-cycle-dependent assembly into nuclear bodies. Proc Natl Acad Sci U S A. 2000;97:13281-13285.

18. Hiebert SW, Sun W, Davis JN, et al. The t(12;21) translocation converts AML-1B from an activator to a repressor of transcription. Mol Cell Biol. 1996;16:1349-1355.
19. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. N Engl J Med. 2004;350:1535-1548.
20. Zelent A, Greaves M, Enver T. Role of the TEL-AML1 fusion gene in the molecular pathogenesis of childhood acute lymphoblastic leukaemia. Oncogene. 2004;23:4275-4283.
21. Downing JR. The core-binding factor leukemias: lessons learned from murine models. Curr Opin Genet Dev. 2003;13:48-54.
22. Kitamura K, Hoshi S, Koike M, Kiyoi H, Saito H, Naoe T. Histone deacetylase inhibitor but not arsenic trioxide differentiates acute promyelocytic leukaemia cells with t(11;17) in combination with all-trans retinoic acid. Br J Haematol. 2000;108:696-702.
23. Wang J, Saunthararajah Y, Redner RL, Liu JM. Inhibitors of histone deacetylase relieve ETO-mediated repression and induce differentiation of AML1-ETO leukemia cells. Cancer Res. 1999;59:2766-2769.
24. Piekarz R, Bates S. A review of depsipeptide and other histone deacetylase inhibitors in clinical trials. Curr Pharm Des. 2004;10:2289-2298.
25. Richon VM, Emiliani S, Verdin E, et al. A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. Proc Natl Acad Sci U S A. 1998;95:3003-3007.
26. Kosugi H, Towatari M, Hatano S, et al. Histone deacetylase inhibitors are the potent inducer/enhancer of differentiation in acute myeloid leukemia: a new approach to anti-leukemia therapy. Leukemia. 1999;13:1316-1324.
27. Tsurutani J, Soda H, Oka M, et al. Antiproliferative effects of the histone deacetylase inhibitor FR901228 on small-cell lung cancer lines and drug-resistant sublines. Int J Cancer. 2003;104:238-242.
28. Kosugi H, Ito M, Yamamoto Y, et al. In vivo effects of a histone deacetylase inhibitor, FK228, on human acute promyelocytic leukemia in NOD / Shi-scid/scid mice. Jpn J Cancer Res. 2001;92:529-536.
29. Byrd JC, Shinn C, Ravi R, et al. Depsipeptide (FR901228): a novel therapeutic agent with selective, in vitro activity against human B-cell chronic lymphocytic leukemia cells. Blood. 1999;94:1401-1408.
30. Ueda H, Manda T, Matsumoto S, et al. FR901228, a novel antitumor bicyclic depsipeptide produced by Chromobacterium violaceum No. 968. III. Antitumor activities on experimental tumors in mice. J Antibiot (Tokyo). 1994;47:315-323.
31. Adachi M, Zhang Y, Zhao X, et al. Synergistic effect of histone deacetylase inhibitors FK228 and m-carboxycinnamic acid bis-hydroxamide with proteasome inhibitors PSI and PS-341 against gastrointestinal adenocarcinoma cells. Clin Cancer Res. 2004;10:3853-3862.
32. Aron JL, Parthun MR, Marcucci G, et al. Depsipeptide (FR901228) induces histone acetylation and inhibition of histone deacetylase in chronic lymphocytic leukemia cells concurrent with activation of caspase 8-mediated apoptosis and down-regulation of c-FLIP protein. Blood. 2003;102:652-658.
33. Imai T, Adachi S, Nishijo K, et al. FR901228 induces tumor regression associated with induction of Fas ligand and activation of Fas signaling in human osteosarcoma cells. Oncogene. 2003;22:9231-9242.
34. Sandor V, Bakke S, Robey RW, et al. Phase I trial of the histone deacetylase inhibitor, depsipeptide (FR901228, NSC 630176), in patients with refractory neoplasms. Clin Cancer Res. 2002;8:718-728.

35. Marshall JL, Rizvi N, Kauh J, et al. A phase I trial of depsipeptide (FR901228) in patients with advanced cancer. J Exp Ther Oncol. 2002;2:325-332.
36. Piekarz RL, Robey R, Sandor V, et al. Inhibitor of histone deacetylation, depsipeptide (FR901228), in the treatment of peripheral and cutaneous T-cell lymphoma: a case report. Blood. 2001;98:2865-2868.
37. Kaspers GJ, Veerman AJ, Pieters R, et al. Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay. Br J Cancer. 1994;70:1047-1052.
38. Den Boer ML, Harms DO, Pieters R, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol. 2003;21:3262-3268.
39. van Lochem EG, van der Velden VH, Wind HK, te Marvelde JG, Westerdaal NA, van Dongen JJ. Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: reference patterns for age-related changes and disease-induced shifts. Cytometry. 2004;60B:1-13.
40. Noordzij JG, de Bruin-Versteeg S, Verkaik NS, et al. The immunophenotypic and immunogenotypic B-cell differentiation arrest in bone marrow of RAG-deficient SCID patients corresponds to residual recombination activities of mutated RAG proteins. Blood. 2002;100:2145-2152.
41. Strom DK, Nip J, Westendorf JJ, et al. Expression of the AML-1 oncogene shortens the G(1) phase of the cell cycle. J Biol Chem. 2000;275:3438-3445.
42. Bernardin-Fried F, Kummalue T, Leijen S, Collector MI, Ravid K, Friedman AD. AML1/RUNX1 increases during G1 to S cell cycle progression independent of cytokine-dependent phosphorylation and induces cyclin D3 gene expression. J Biol Chem. 2004;279:15678-15687.
43. Lenny N, Westendorf JJ, Hiebert SW. Transcriptional regulation during myelopoiesis. Mol Biol Rep. 1997;24:157-168.
44. Fenrick R, Hiebert SW. Role of histone deacetylases in acute leukemia. J Cell Biochem Suppl. 1998;30-31:194-202.
45. Wang J, Hoshino T, Redner RL, Kajigaya S, Liu JM. ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. Proc Natl Acad Sci U S A. 1998;95:10860-10865.
46. Ferrara FF, Fazi F, Bianchini A, et al. Histone deacetylase-targeted treatment restores retinoic acid signaling and differentiation in acute myeloid leukemia. Cancer Res. 2001;61:2-7.
47. Stams WA, den Boer ML, Holleman A, et al. Asparagine synthetase expression is linked with L-asparaginase resistance in TEL-AML1-negative but not TEL-AML1-positive pediatric acute lymphoblastic leukemia. Blood. 2005;105:4223-4225.
48. Conway RM, Madigan MC, Billson FA, Penfold PL. Vincristine- and cisplatin-induced apoptosis in human retinoblastoma. Potentiation by sodium butyrate. Eur J Cancer. 1998;34:1741-1748.
49. Niitsu N, Kasukabe T, Yokoyama A, et al. Anticancer derivative of butyric acid (Pivalyloxymethyl butyrate) specifically potentiates the cytotoxicity of doxorubicin and daunorubicin through the suppression of microsomal glycosidic activity. Mol Pharmacol. 2000;58:27-36.
50. Bernhard D, Loffler M, Hartmann BL, Yoshida M, Kofler R, Csordas A. Interaction between dexamethasone and butyrate in apoptosis induction: non-additive in thymocytes and synergistic in a T cell-derived leukemia cell line. Cell Death Differ. 1999;6:609-617.

Chapter 9

Summary

The t(12;21)(p13;q22) translocation resulting in a TEL-AML1 fusion is the most common translocation in pediatric ALL. In general, TEL-AML1 positive ALL is associated with a good prognosis. However, conflicting data on the percentage of patients entering relapse and the proportion of TEL-AML1 positive cases at relapse have been reported. Overall, the reported prognostic relevance of TEL-AML1 seems to depend on the intensity of the treatment protocol. A possible explanation for this finding can be ascribed to the fact that TEL-AML1 positive ALL cells are *in vitro* more sensitive to L-Asparaginase compared to TEL-AML1 negative ALL cells. However, within the t(12;21) positive ALL group, large interindividual differences in cellular *in vitro* sensitivity to L-Asparaginase were found. The clinical heterogeneity in response to therapy as well as the large heterogeneity in *in vitro* sensitivity to L-Asparaginase, suggests that additional genetic changes might be important for the differences in drug sensitivity and clinical outcome. Therefore in **chapter 2**, we studied the incidence of additional genetic changes in the *TEL* and *AML1* genes in t(12;21) positive ALL and their relation with drug sensitivity and clinical outcome. 83% of the patients showed one or more additional genetic changes in these two genes. The additional genetic changes could be divided into a complete deletion of the second *TEL* allele (62%), a partial deletion of the second *TEL* allele (8%), an extra *AML1* gene (23%) and an extra der(21)t(12;21) indicative for an extra copy of the TEL-AML1 fusion gene (10%). 20% of all t(12;21) positive ALL patients had more than one additional genetic abnormality in *TEL* and *AML1*. Patients with an extra der(21)t(12;21) were more resistant to prednisolone, but no difference was found for L-Asparaginase and multivariate analysis including other risk factors showed that only prednisolone resistance was an independent prognostic factor in TEL-AML1 positive ALL patients.

In **chapter 3** we showed that the level of mRNA expression of *TEL, AML1* and the fusion products *TEL-AML1* and *AML1-TEL* was not associated with drug sensitivity. High *AML1-TEL* expression level was predictive for an unfavourable outcome irrespective of the presence of other risk factors including cellular resistance to prednisolone, vincristine and L-Asparaginase.

We previously showed that TEL-AML1 positive ALL patients are in vitro more sensitive to L-Asparaginase compared to TEL-AML1 negative patients. L-Asparaginase is an enzyme used in chemotherapeutic protocols for children with ALL. L-Asparaginase depletes asparagine and glutamine in blood plasma and cells. Impaired capacity to synthesize asparagine due to reduced asparagine synthetase levels may explain the L-Asparaginase sensitivity of ALL cells. Cell line studies indicated that L-Asparaginase resistance may be caused by an increased expression of asparagine synthetase. Since the asparagine synthetase gene contains a binding site for *AML1* in its promoter region, this gene may become transcriptionally repressed by TEL-AML1. Therefore, a decreased expression of asparagine synthetase may explain the sensitivity to L-Aspara-

ginase in TEL-AML1 positive ALL compared to TEL-AML1 negative ALL. However, as shown in **chapter 4** we found that asparagine synthetase expression is not related to L-Asparaginase sensitivity in TEL-AML1 positive ALL. We even observed a 5-fold higher expression of asparagine synthetase in TEL-AML1 positive compared to TEL-AML1 negative patients. As described in **chapter 5** the high expression of asparagine synthetase is probably due to the impaired potential of TEL-AML1 positive ALL cells to proceed from G_1 into S phase, as TEL-AML1 represses the target genes of AML1 (i.e. cell cycle-associated). Interestingly, whereas increased expression of asparagine synthetase is not linked with L-Asparaginase resistance in TEL-AML1 positive ALL, we showed in **chapter 6** that increased expression of asparagine synthetase is related to L-Asparaginase resistance in TEL-AML1 negative pre-B ALL. These data suggest that the role of asparagine synthetase for L-Asparaginase resistance and consequently in treatment response is different between both genetic subtypes. The mechanism of increased L-Asparaginase sensitivity is still unclear for TEL-AML1 positive ALL. Allthough the group of TEL-AML1 positive ALL cases is sensitive to L-Asparaginase, there is considerable heterogeneity within this group with very sensitive and very resistant cases. To elucidate putative causes of L-Asparaginase resistance, a gene expression profiling study was conducted as described in **chapter 7.** Fifty-two unique genes were identified that discriminated between L-Asparaginase resistant and sensitive TEL-AML1 positive ALL patients. These genes were not comparable to genes previously described to be associated with L-Asparaginase resistance in mainly TEL-AML1 negative cases or in a diversity of ALL cell lines. Genes involved in lipid metabolism and cell death were over-represented in L-Asparaginase resistant cases compared to all genes represented on the U133A GeneChip. Furthermore, four genes were selected to be involved in prediction of relapse within TEL-AML1 positive ALL. However, the statistics on this analysis are not very strong indicating that the group has to be enlarged for convincing results.

TEL-AML1 acts as a transcriptional repressor of AML1 target genes by recruiting co-repressors and histone deacetylases (HDACs), which induce closure of the chromatin structure and, hence, inhibition of transcription. TEL-AML1 therefore, seems a promising target for treatment with HDAC inhibitors to reverse its transcriptional repression. In **chapter 8** the effect of HDAC inhibitors FK228 and sodium butyrate were studied. HDAC inhibitors were more toxic to both TEL-AML1 positive and negative ALL cells compared to normal bone marrow cells. FK228 induced B-cell differentiation and apoptosis in both TEL-AML1 positive and negative ALL cells. So in contrast to our hypothesis, there was no specific effect of HDAC inhibitors on TEL-AML1 positive ALL.

In conclusion, the work described in this thesis has improved our insights in the genetic and biological features of TEL-AML1 positive ALL cells. Still some issues (like the working mechanism of L-Asparaginase) remain unclear. Ongoing studies in TEL-

AML1 positive ALL address the functional role of L-Asparaginase resistance associated genes in more detail. This knowledge is required to develop more effective treatment regimes that improve outcome end reduce side-effects of therapy in TEL-AML1 positive ALL patients.

Chapter 10

General discussion

The success rate of treatment of ALL has improved considerably since the 1960s to a 5-years event-free-survival of nearly 80%. However, the last decade the prognosis has hardly been further improved. To cure the remaining 20% of patients, a more individual treatment is necessary based on patient characteristics. To do so, a better understanding in the pathogenesis and the cause of resistance to chemotherapy in ALL is needed. Pediatric ALL is a heterogeneous disease that differs markedly in the cellular and molecular characteristics of individual patients as well as their response to chemotherapy[1-3]. In this thesis we studied ALL patients with a t(12;21) resulting in a *TEL-AML1* fusion, which is the most common translocation in pediatric ALL. Conflicting data on the prognostic value of this translocation have been reported. This discrepancy may be explained by the fact that that an intensification of treatment induces a favourable outcome of TEL-AML1 positive ALL patients compared to TEL-AML1 negative ALL patients[4-6].

Since the discovery of TEL-AML1 positive ALL, a high frequency of additional genetic changes in the *TEL* and/or *AML1* genes have been described. We have found that these additional genetic changes are not related to the prognosis of TEL-AML1 positive ALL patients (chapter 2), which was recently also reported by Attarbaschi et al. in an independent study[7]. However, patients with an extra der(21)t(12;21), which results into an extra TEL-AML1 fusion product, were more resistant to prednisolone. Prednisolone resistance is related to an unfavourable outcome in TEL-AML1 positive (chapter 2) as well as TEL-AML1 negative ALL[8-11]. It can be hypothesized that TEL-AML1 interferes with genes or cellular processes involved in the mechanism of action by prednisolone e.g. by interaction with transcription factors. The exact molecular mechanisms of resistance to prednisolone in ALL cells is still unknown. Gene expression profile studies discriminated genes involved in Prednisolone resistance or sensitivity[12,13]. High expression of the glucocorticoid receptor is associated with sensitivity to prednisolone, but is probably not the main mechanism of action[14]. Future studies on the cellular function of these genes (i.e. by siRNA assays) may lead to an explanation for the relation with the TEL-AML1 fusion product.

TEL-AML1 mRNA is formed in all t(12;21) positive ALL cases, whereas the reciprocal fusion product *AML1-TEL* is formed in approximately 75% of all t(12;21) positive ALL cases. The TEL-AML1 fusion product contains the pointed (PNT) domain of TEL required for dimerization and transcriptional repression, the runt homology domain (RHD) of AML1 required for dimerization and DNA-binding and the transactivation (TA) domain of AML1. The AML1-TEL only contains the ETS domain of TEL required for dimerization and DNA-binding and exon 1 of AML1, which has an unknown function (Figure 3 of the introduction). As shown in chapter 3, high level expression of *AML1-TEL* mRNA is associated with a poor outcome. In this thesis, we postulate that

AML1-TEL may compete for binding with endogenous TEL or may act like TEL in the absence of wild-type TEL. ETS proteins are targets of the mitogen-activated protein kinase (MAPK) signal transduction pathway and are regulated by mitotic signals[15-17]. Microtubule-interfering agents (like vincristine) stimulate MAPKs and impair mitosis[18-20]. So, ETS proteins and the vincristine working mechanism might be related to each other via the MAPK signal transduction pathway.

Recently in a large cohort, a discordant resistance between vincristine and L-Asparaginase was associated with TEL-AML1 positive ALL [21]. A similar inversed correlation between L-Asparaginase and vincristine resistance was found in the TEL-AML1 positive ALL patients described in chapter 2. TEL-AML1 positive ALL patients are sensitive to L-Asparaginase but resistant to vincristine. Interestingly, we found also a trend for an inversed correlation between *AML1-TEL* expression and vincristine sensitivity in the limited number of patient samples described in chapter 3, which may contribute to the association of high *AML1-TEL* expression and a poor outcome. It would be interesting to study the relation between *AML1-TEL*, vincristine and L-Asparaginase cytotoxicity at initial diagnosis and long-term outcome in a large cohort of TEL-AML1 positive ALL patients. Insights in the importance of MAPKs in vincristine cytotoxicity in AML1-TEL expressing cells may point to ways to modulate vincristine resistance in these cells.

Drug resistance profiles that combine the in vitro cytotoxicity of prednisolone, vincristine, and L-Asparaginase (PVA profile) have prognostic value in childhood ALL[9,10,22]. The resistant PVA profile can discriminate patients at higher risk of early events (ie, non-response and relapse within 2.5 years after diagnosis) but is less suitable for the prediction of late relapses. Patients with a resistant PVA profile may benefit from an intensification of therapy. In the COALL-97 treatment protocol therapy of children with newly diagnosed ALL is based upon the individual drug resistance profile. Patients are first stratified using the conventional risk criteria of age, immunophenotype, and white blood cell count. A second stratification is based on the PVA profile. It is of interest to determine in future whether TEL-AML1 positive ALL patients without additional genetic changes in *TEL* and/or *AML1* or with an extra der(21)t(12;21) might benefit from an intensified treatment protocol like for patients with a resistant PVA profile, since these parameters were associated with the occurrence of early relapses (chapter 2).

TEL-AML1 positive ALL patients, as a group, are significantly more sensitive to L-Asparaginase compared to TEL-AML1 negative ALL patients. However, within the TEL-AML1 positive ALL patients large interindividual differences in *in vitro* sensitivity to L-Asparaginase were found (chapter 4)[23]. *In vitro* and *in vivo* resistance to L-Asparaginase is correlated with a relative poor prognosis[9,24]. L-Asparaginase induces asparagine and glutamine depletion in the blood leading to cellular efflux and deple-

tion of these amino acids within cells[25]. Since 1969, resistance to L-Asparaginase was thought to be caused by high asparagine synthetase levels in leukemic cells, since asparagine synthetase synthesizes asparagine from aspartic acid in cells[26-28]. In contrast to this general hypothesis, we surprisingly found a 5-fold higher expression of asparagine synthetase in TEL-AML1 positive ALL patients, which are *in vitro* more sensitive to L-Asparaginase, compared to TEL-AML1 negative ALL patients (chapter 4). Recently, Krejci et al. confirmed in an independent study that TEL-AML1 positive ALL patients express higher asparagine synthetase levels compared to other ALL patients[29]. Asparagine synthetase overexpression may be related to the fact that the cell cycle is repressed by TEL-AML1 (chapter 5). Asparagine synthetase transcription increases in cells that are hampered to proceed through the cell cycle.

In contrast to TEL-AML1 positive ALL, we found that high asparagine synthetase expression is related to L-Asparaginase resistance in TEL-AML1 negative ALL (chapter 6), which has also been found in leukemia cell lines[30-36] and patient samples[26,37]. Recently, Fine et al. reported a gene expression study on genes related to L-Asparaginase cytotoxicity in cell lines and patient samples[38]. Asparagine synthetase was found to be highly predictive for L-Asparaginase cytotoxicity in the cell lines. However, this could not be confirmed in the patient samples, which represented both TEL-AML1 positive and negative ALL patients. Furthermore, no gene profile was found that was predictive of L-Asparaginase cytotoxicity in the patient samples. In this study by Fine et al. the TEL-AML1 status of the ALL patients was not taken into account[38]. Our findings combined with the study of Fine et al.[38] indicate that the mechanism of L-Asparaginase resistance differs between TEL-AML1 positive and negative ALL subtypes.

To gain more insight in the possible mechanism of L-Asparaginase resistance in TEL-AML1 positive ALL, a genome-wide expression study was performed (chapter 7). We identified 70 probe sets associated with L-Asparaginase resistance with an overrepresentation of genes involved in cell death and lipid metabolism compared to genes represented on the total microarray chip. This is in contrast to a recent study by Holleman et al., who identified ribosomal protein genes to be related to L-Asparaginase resistance in a broader group of pediatric B-lineage ALL patients[12]. These differences in the identified genes again points to different mechanisms of L-Asparaginase resistance between TEL-AML1 positive and negative ALL patients, since the majority of patients in the study of Holleman et al. was TEL-AML1 negative. In our microarray study, we also identified genes, which may be predictive for relapse in TEL-AML1 positive ALL. One of the 4 genes selected, Kallikrein 5 (KLK5) a serine protease, seems a promising prognostic factor, since the expression of kallikrein 5 has been shown to be associated with an unfavourable prognosis in several forms of cancer[39-41]. Future independent studies have to confirm if KLK5 really is predictive for an unfavourable prognosis and

if KLK5 is a prognostic factor in only TEL-AML1 positive ALL or also in other ALL subtypes.

The overrepresented genes associated with L-Asparaginase resistance in TEL-AML1 positive ALL patients were involved in cell death and lipid metabolism. L-Asparaginase resistance is associated with decreased induction of apoptosis in childhood ALL, but the causes of this are unknown[42]. Apoptosis is a common mode of eukaryotic cell death, triggered by an inducible cascade of biochemical events leading to activation of endonucleases that disintegrate the DNA. TEL-AML1 positive ALL cells express high levels of the pro-apoptotic protein CD95 (member of the TNF receptor family)[43]. TNF and CD40, another TNF receptor family member, are also highly expressed in TEL-AML1 positive ALL patients compared to TEL-AML1 negative patients[44,45]. Therefore, it could be hypothesized that members of the TNF receptor family are more pronounced in TEL-AML1 positive cells making these cells more sensitive to apoptosis induced by L-Asparaginase.

Besides indications that TEL-AML1 positive ALL patients might benefit from intensification of drugs used in the induction treatment, there are also indications that TEL-AML1 positive ALL patients might also benefit from drugs like methotrexate (MTX) which is a major component in the maintenance treatment protocols for childhood ALL. The ability of cells to form and accumulate MTX polyglutamates is well recognized as a determinant of MTX cytotoxicity[46,47]. MTX polyglutamates accumulation has been shown to be lower in TEL-AML1 positive ALL compared to TEL-AML1 negative ALL[48]. Genes encoding for proteins involved in cellular folate / anti-folate homeostasis have distinct expression patterns across ALL subtypes[49]. *FPGS*, a gene involved MTX polyglutamylation, is lower expressed in TEL-AML1 positive ALL cells compared to other ALL subtypes[49]. Moreover, *ABCG2*, which encodes for ABC subfamily G member 2 (i.e. BCRP), was overexpressed in TEL-AML1 positive ALL cells[49]. ABCG2 is involved in MTX efflux from cells by exporting short-chain MTX polyglutamates[50]. In addition, recently TEL-AML1 positive ALL patients were found to have lower de novo purine synthesis[51], which is a target for antimetabolites like MTX. Purine nucleotides participate in a variety of cellular functions, including synthesis of DNA and RNA, regulation of enzymatic activity, protein synthesis and function, and mediation of energy transfer in cells. TEL-AML1 positive patients might benefit from an intensified treatment protocol including intensification of MTX, since it was shown before in T-ALL and AML patients that inefficient polyglutamylation of MTX can be overcome by continuous and high dose exposure[52,53].

An intensification of drugs used in the treatment of TEL-AML1 positive ALL leads to a favourable prognosis, but of course also leads to more side-effects of the drugs. Therefore, it would be useful if other drugs enhance the cytotoxicity (synergistically

or additive) of the currently used drugs specifically in TEL-AML1 positive ALL. A promising class of drugs seems HDAC inhibitors, which are currently being evaluated in clinical phase I/II trials. Inhibitors of HDACs have been shown to relieve the AML1-ETO-mediated repression of AML1 target genes in t(8;21) positive AML thereby inducing differentiation[54]. Therefore, ALL patients with a rearranged *AML1* gene like TEL-AML1 positive ALL patients may theoretically benefit from treatment with HDAC inhibitors. FK228 is a promising HDAC inhibitor since it is well tolerated in phase I clinical trials and already showed clinical efficacy in one t(8;21) positive AML patient, one patient with renal cell carcinoma and several patients with a T-cell lymphoma[55]. In this thesis, we found that FK228 induced lymphoid cell differentiation and was more toxic to both TEL-AML1 positive and negative leukemic cells compared to normal bone marrow cells (chapter 8). Moreover, an additive effect of FK228 was shown on L-Asparaginase cytotoxicity in both ALL subtypes. Therefore, HDAC inhibitors like FK228 may not specifically target TEL-AML1 positive ALL cells but also other ALL cells. The difference of both ALL types compared to normal bone marrow cells may point to a favourable therapeutic index for this drug. It needs to be explored whether this holds true for FK228 in clinical studies in both TEL-AML1 positive and negative ALL.

In conclusion, since the discovery of t(12;21) in 1995, most studies have addressed the relationship between this translocation and leukemogenesis, cellular drug resistance and prognosis. Studies showed that TEL-AML1 positive ALL patients benefit from an intensified treatment protocol, leading to a favourable outcome of these patients compared to TEL-AML1 negative ALL patients. We here report on the incidence of additional genetic changes in TEL-AML1 positive ALL and its fusion products in relation to prognosis and drug sensitivity. The general dogma that L-Asparaginase sensitivity is due to lack of asparagine synthetase expression was rejected in this thesis and shown to be different among TEL-AML1 positive and negative ALL patients. New genes were identified to be associated with L-Asparaginase resistance in TEL-AML1 positive ALL. Finally, HDAC inhibitors were shown to have a specific effect on TEL-AML1 positive ALL compared to normal cells but not compared to other ALL subtypes. In this thesis, we have identified new genetic and molecular features in TEL-AML1 positive ALL patients which point to new insight into prognosis and drug sensitivity of these patients. Ongoing functional studies need to address how these genetic and molecular features affect the efficacy and toxicity of antileukemic therapy, so new more effective or even individual treatment protocols can be developed that improve outcome and decrease side-effects of therapy in TEL-AML1 positive ALL patients.

REFERENCES

1. Yeoh EJ, Ross ME, Shurtleff SA, et al. Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. Cancer Cell. 2002;1:133-143.
2. Ross ME, Zhou X, Song G, et al. Classification of pediatric acute lymphoblastic leukemia by gene expression profiling. Blood. 2003;102:2951-2959.
3. Pui CH, Evans WE. Acute lymphoblastic leukemia. N Engl J Med. 1998;339:605-615.
4. Takahashi Y, Horibe K, Kiyoi H, et al. Prognostic significance of TEL/AML1 fusion transcript in childhood B-precursor acute lymphoblastic leukemia. J Pediatr Hematol Oncol. 1998;20:190-195.
5. Maloney K, McGavran L, Murphy J, et al. TEL-AML1 fusion identifies a subset of children with standard risk acute lymphoblastic leukemia who have an excellent prognosis when treated with therapy that includes a single delayed intensification. Leukemia. 1999;13:1708-1712.
6. Avigad S, Kuperstein G, Zilberstein J, et al. TEL-AML1 fusion transcript designates a favorable outcome with an intensified protocol in childhood acute lymphoblastic leukemia. Leukemia. 1999;13:481-483.
7. Attarbaschi A, Mann G, Konig M, et al. Incidence and relevance of secondary chromosome abnormalities in childhood TEL/AML1+ acute lymphoblastic leukemia: an interphase FISH analysis. Leukemia. 2004;18:1611-1616.
8. Pieters R, Huismans DR, Loonen AH, et al. Relation of cellular drug resistance to long-term clinical outcome in childhood acute lymphoblastic leukaemia. Lancet. 1991;338:399-403.
9. Kaspers GJ, Veerman AJ, Pieters R, et al. In vitro cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. Blood. 1997;90:2723-2729.
10. Hongo T, Yajima S, Sakurai M, Horikoshi Y, Hanada R. In vitro drug sensitivity testing can predict induction failure and early relapse of childhood acute lymphoblastic leukemia. Blood. 1997;89:2959-2965.
11. Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. Blood. 1998;92:259-266.
12. Holleman A, Cheok MH, den Boer ML, et al. Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. N Engl J Med. 2004;351:533-542.
13. Tissing WJ, Den Boer ML, Meijerink JP, et al. Genome wide identification of target genes regulated upon prednisolone exposure in primary acute lymphoblastic leukemia cells. Submitted.
14. Tissing WJ, Meijerink JP, den Boer ML, Pieters R. Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia. Leukemia. 2003;17:17-25.
15. Arai H, Maki K, Waga K, et al. Functional regulation of TEL by p38-induced phosphorylation. Biochem Biophys Res Commun. 2002;299:116-125.
16. Maki K, Arai H, Waga K, et al. Leukemia-related transcription factor TEL is negatively regulated through extracellular signal-regulated kinase-induced phosphorylation. Mol Cell Biol. 2004;24:3227-3237.

17. Foulds CE, Nelson ML, Blaszczak AG, Graves BJ. Ras/mitogen-activated protein kinase signaling activates Ets-1 and Ets-2 by CBP/p300 recruitment. Mol Cell Biol. 2004;24:10954-10964.
18. Wang TH, Wang HS, Ichijo H, et al. Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. J Biol Chem. 1998;273:4928-4936.
19. Subbaramaiah K, Hart JC, Norton L, Dannenberg AJ. Microtubule-interfering agents stimulate the transcription of cyclooxygenase-2. Evidence for involvement of ERK1/2 AND p38 mitogen-activated protein kinase pathways. J Biol Chem. 2000;275:14838-14845.
20. Jordan MA, Wilson L. Microtubules and actin filaments: dynamic targets for cancer chemotherapy. Curr Opin Cell Biol. 1998;10:123-130.
21. Lugthart S, Cheok MH, den Boer ML, et al. Identification of genes conferring cross-resistance in childhood acute lymphoblastic leukemia. submitted. 2005.
22. Den Boer ML, Harms DO, Pieters R, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol. 2003;21:3262-3268.
23. Ramakers-van Woerden NL, Pieters R, Loonen AH, et al. TEL/AML1 gene fusion is related to in vitro drug sensitivity for L-asparaginase in childhood acute lymphoblastic leukemia. Blood. 2000;96:1094-1099.
24. Asselin BL, Kreissman S, Coppola DJ, et al. Prognostic significance of early response to a single dose of asparaginase in childhood acute lymphoblastic leukemia. J Pediatr Hematol Oncol. 1999;21:6-12.
25. Chakrabarti R, Schuster SM. L-Asparaginase: Perspectives on the mechanisms of action and resistance. Int J Pediatric Hematol Oncol. 1997;4:597-611.
26. Haskell CM, Canellos GP. l-asparaginase resistance in human leukemia--asparagine synthetase. Biochem Pharmacol. 1969;18:2578-2580.
27. Ohnuma T, Holland JF, Freeman A, Sinks LF. Biochemical and pharmacological studies with asparaginase in man. Cancer Res. 1970;30:2297-2305.
28. Miller HK, Salser JS, Balis ME. Amino acid levels following L-asparagine amidohydrolase (EC.3.5.1.1) therapy. Cancer Res. 1969;29:183-187.
29. Krejci O, Starkova J, Otova B, et al. Upregulation of asparagine synthetase fails to avert cell cycle arrest induced by L-asparaginase in TEL/AML1-positive leukaemic cells. Leukemia. 2004;18:434-441.
30. Aslanian AM, Fletcher BS, Kilberg MS. Asparagine synthetase expression alone is sufficient to induce l-asparaginase resistance in MOLT-4 human leukaemia cells. Biochem J. 2001;357:321-328.
31. Prager MD, Bachynsky N. Asparagine synthetase in asparaginase resistant and susceptible mouse lymphomas. Biochem Biophys Res Commun. 1968;31:43-47.
32. Kiriyama Y, Kubota M, Takimoto T, et al. Biochemical characterization of U937 cells resistant to L-asparaginase: the role of asparagine synthetase. Leukemia. 1989;3:294-297.
33. Worton KS, Kerbel RS, Andrulis IL. Hypomethylation and reactivation of the asparagine synthetase gene induced by L-asparaginase and ethyl methanesulfonate. Cancer Res. 1991;51:985-989.
34. Martin JK, Sun W, Maraga D, Schuster SM, Wylie DE. An investigation into the mechanism of L-asparaginase resistance in L5178Y murine leukemia cells. Amino Acids. 1993;5:51-69.

35. Scherf U, Ross DT, Waltham M, et al. A gene expression database for the molecular pharmacology of cancer. Nat Genet. 2000;24:236-244.
36. Hutson RG, Kitoh T, Moraga Amador DA, Cosic S, Schuster SM, Kilberg MS. Amino acid control of asparagine synthetase: relation to asparaginase resistance in human leukemia cells. Am J Physiol. 1997;272:C1691-1699.
37. Dubbers A, Wurthwein G, Muller HJ, et al. Asparagine synthetase activity in paediatric acute leukaemias: AML-M5 subtype shows lowest activity. Br J Haematol. 2000;109:427-429.
38. Fine BM, Kaspers GJ, Ho M, Loonen AH, Boxer LM. A genome-wide view of the in vitro response to l-asparaginase in acute lymphoblastic leukemia. Cancer Res. 2005;65:291-299.
39. Diamandis EP, Borgono CA, Scorilas A, et al. Immunofluorometric quantification of human kallikrein 5 expression in ovarian cancer cytosols and its association with unfavorable patient prognosis. Tumour Biol. 2003;24:299-309.
40. Planque C, de Monte M, Guyetant S, et al. KLK5 and KLK7, two members of the human tissue kallikrein family, are differentially expressed in lung cancer. Biochem Biophys Res Commun. 2005;329:1260-1266.
41. Michael IP, Sotiropoulou G, Pampalakis G, et al. Biochemical and enzymatic characterization of human kallikrein 5 (hK5), a novel serine protease potentially involved in cancer progression. J Biol Chem. 2005;280:14628-14635.
42. Holleman A, den Boer ML, Kazemier KM, Janka-Schaub GE, Pieters R. Resistance to different classes of drugs is associated with impaired apoptosis in childhood acute lymphoblastic leukemia. Blood. 2003;102:4541-4546.
43. Krishna Narla R, Navara C, Sarquis M, Uckun FM. Chemosensitivity of TEL-AML1 fusion transcript positive acute lymphoblastic leukemia cells. Leuk Lymphoma. 2001;41:615-623.
44. Holleman A, Den Boer ML, Menezes RX, et al. The expression of 70 apoptosis genes in relation to lineage, genetic subtype, cellular drug resistance, and outcome in childhood acute lymphoblastic leukemia. submitted.
45. Alessandri AJ, Reid GS, Bader SA, Massing BG, Sorensen PH, Schultz KR. ETV6 (TEL)-AML1 pre-B acute lymphoblastic leukaemia cells are associated with a distinct antigen-presenting phenotype. Br J Haematol. 2002;116:266-272.
46. Masson E, Relling MV, Synold TW, et al. Accumulation of methotrexate polyglutamates in lymphoblasts is a determinant of antileukemic effects in vivo. A rationale for high-dose methotrexate. J Clin Invest. 1996;97:73-80.
47. Curt GA, Jolivet J, Carney DN, et al. Determinants of the sensitivity of human small-cell lung cancer cell lines to methotrexate. J Clin Invest. 1985;76:1323-1329.
48. Whitehead VM, Payment C, Cooley L, et al. The association of the TEL-AML1 chromosomal translocation with the accumulation of methotrexate polyglutamates in lymphoblasts and with ploidy in childhood B-progenitor cell acute lymphoblastic leukemia: a Pediatric Oncology Group study. Leukemia. 2001;15:1081-1088.
49. Kager L, Cheok M, Yang W, et al. Folate pathway gene expression differs in subtypes of acute lymphoblastic leukemia and influences methotrexate pharmacodynamics. J Clin Invest. 2005;115:110-117.
50. Volk EL, Schneider E. Wild-type breast cancer resistance protein (BCRP/ABCG2) is a methotrexate polyglutamate transporter. Cancer Res. 2003;63:5538-5543.

51. Zaza G, Yang W, Kager L, et al. Acute lymphoblastic leukemia with TEL-AML1 fusion has lower expression of genes involved in purine metabolism and lower de novo purine synthesis. Blood. 2004.
52. Rots MG, Pieters R, Peters GJ, et al. Circumvention of methotrexate resistance in childhood leukemia subtypes by rationally designed antifolates. Blood. 1999;94:3121-3128.
53. Rots MG, Pieters R, Jansen G, et al. A possible role for methotrexate in the treatment of childhood acute myeloid leukaemia, in particular for acute monocytic leukaemia. Eur J Cancer. 2001;37:492-498.
54. Wang J, Saunthararajah Y, Redner RL, Liu JM. Inhibitors of histone deacetylase relieve ETO-mediated repression and induce differentiation of AML1-ETO leukemia cells. Cancer Res. 1999;59:2766-2769.
55. Piekarz R, Bates S. A review of depsipeptide and other histone deacetylase inhibitors in clinical trials. Curr Pharm Des. 2004;10:2289-2298.

Chapter 11

Nederlandse samenvatting

Acute lymfatische leukemie (ALL) is een kwaadaardige aandoening waarbij witte bloedcellen in het beenmerg ontregeld groeien en niet goed uitrijpen tot normale bloedcellen. Door de woekering van deze onrijpe leukemie cellen wordt de aanmaak van normale bloedcellen verstoord en verdrongen. Dit leidt tot bloedarmoede, verhoogde bloedingsneiging en stoornissen in de afweer tegen infecties. De leukemiecellen verspreiden zich naar het bloed en worden ook in andere organen, zoals lymfeklieren, milt, lever, het zenuwstelsel en eierstokken of testikels gevonden. Zonder behandeling is dit een dodelijke aandoening.

ALL is de meest voorkomende vorm van kanker op de kinderleeftijd. Op volwassen leeftijd is het relatief zeldzaam. Leukemiecellen vertonen bijna altijd genetische afwijkingen, in het aantal chromosomen alsook in de chromosomale structuur. Als afwijking in de chromosomale structuur worden meestal translocaties gevonden. Bij een translocatie is er sprake van een uitwisseling van genetisch materiaal tussen 2 chromosomen. De meest voorkomende translocatie bij kinderen met ALL (bijna 25%) is een translocatie tussen chromosoom 12 en 21, ook wel aangeduid als t(12;21) positieve ALL. De term TEL-AML1 positieve ALL wordt ook gebruikt aangezien het breukpunt van de translocatie ligt in het *TEL* gen op chromosoom 12 en het *AML1* gen op chromosoom 21. Dit houdt in dat er een stuk van het *TEL* gen aan het *AML1* gen wordt gefuseerd, resulterend in het *TEL-AML1* fusiegen, en vice versa.

Sinds de ontdekking van de t(12;21) in 1995 is er veel gediscussieerd over de overlevingskans van kinderen met deze translocatie. Op dit moment lijkt de gunstige overlevingskans van kinderen met deze translocatie afhankelijk te zijn van de intensiteit van de behandeling. Oftewel hoe intensiever de behandeling, des te gunstiger de overlevingskans. Dit zou samen kunnen hangen met het feit dat TEL-AML1 positieve leukemiecellen gevoeliger zijn voor een aantal cytostatica (met name voor L-Asparaginase). Er zijn echter in de groep van patienten met TEL-AML1 positieve leukemie ook grote individuele verschillen in de gevoeligheid voor L-Asparaginase. Dit zou mogelijk een verband kunnen hebben met het feit dat er naast de translocatie ook vaak bijkomende genetische afwijkingen in het *TEL* en/of *AML1* gen gevonden worden in de leukemiecellen.

Het doel van de huidige studie was om genetische en biologische kenmerken te identificeren bij kinderen met TEL-AML1 positieve ALL die mogelijk een relatie kunnen hebben met de overlevingskans alsmede de gevoeligheid voor cytostatica.

In deel A werden de additionele genetische afwijkingen bestudeerd in relatie tot de overlevingskans en cytostatica gevoeligheid. In **hoofdstuk 2** werd in beenmerg materiaal van 143 TEL-AML1 positieve ALL patiënten geïnventariseerd welke additionele genetische afwijkingen in het *TEL* en/of *AML1* gen voorkomen en hoe frequent. Bovendien werd bestudeerd of er een relatie van deze additionele afwijkingen was met de

overlevingskans en cytostatica gevoeligheid van deze patiënten. In 83% van de patiënten bleek er sprake te zijn van een of meerdere additionele genetische afwijkingen. Deze additionele afwijkingen bestonden uit een deletie (verwijderd stuk genetische materiaal) van het niet getransloceerde *TEL* gen (62%), een partiele deletie van het *TEL* gen (8%), een extra *AML1* gen (23%) en een extra kopie van het fusiegen *TEL-AML1* (10%). In 20% bleek er sprake te zijn van een combinatie van meerdere van deze afwijkingen. De patiënten met een extra fusiegen van *TEL-AML1* waren ongevoeliger voor prednisolon, maar niet voor L-Asparaginase. Bij uitgebreidere statistische analyse bleek dat alleen de ongevoeligheid voor prednisolon van belang is voor de overlevingskans van deze kinderen en niet zozeer de additionele genetische afwijking.

Chromosomen bevatten de bouwstenen van het genetisch materiaal, DNA. Het DNA bevindt zich in de kern van de cel. De eigenlijke aanmaak van eiwitten, de functionele producten van het DNA, vindt plaats in het cytoplasma van de cel, buiten de kern. Er moet dus overdracht van de informatie op het DNA plaatsvinden. Dit gebeurt door de functionele coderende stukken DNA om te zetten in RNA. RNA kan naar het cytoplasma vervoerd worden en met de vertaling van de RNA-informatie vindt de vorming van eiwitten plaats. In **hoofdstuk 3** werd op RNA niveau de expressie van de *TEL* en *AML1* alsmede de fusiegenen *TEL-AML1* en *AML1-TEL* bestudeerd ten aanzien van de overlevingskans en cytostatica gevoeligheid. Er werd geen relatie gevonden tussen de expressie van deze genen en de gevoeligheid voor cytostatica. Wel bleek dat hoge expressie van het fusiegen *AML1-TEL* gerelateerd is aan een slechte overlevingskans in TEL-AML1 positieve ALL.

In deel B werden mogelijke oorzaken voor de verschillen in gevoeligheid voor L-Asparaginase in zowel TEL-AML1 positieve als negatieve ALL patiënten bestudeerd. L-Asparaginase zorgt ervoor dat een aantal aminozuren (de bouwstenen van eiwitten) zoals asparagine en glutamine gedepleteerd worden. Een lage activiteit van het enzym asparagine synthetase, dat weer asparagine kan vormen, zou een verklaring kunnen zijn waarom leukemie cellen gevoelig zijn voor L-Asparaginase. Bovendien zou asparagine synthetase mogelijk aangestuurd kunnen worden door AML1 en daarom dus tegengewerkt kunnen worden door TEL-AML1. In **hoofdstuk 4** werd dan ook bestudeerd of de mate van asparagine synthetase expressie gerelateerd was aan L-Asparaginase gevoeligheid in TEL-AML1 positieve ALL cellen. Dit bleek niet het geval te zijn. Er werd zelfs een zeer hoge expressie van asparagine synthetase gevonden ten opzichte van TEL-AML1 negatieve ALL patiënten. Dit kon dus niet verklaren waarom TEL-AML1 positieve ALL cellen zoveel gevoeliger zijn voor L-Asparaginase in tegenstelling tot wat altijd gedacht werd.

Het TEL-AML1 fusieproduct werkt de normale functie van AML1 tegen. AML1 is een van de vele factoren die de celdeling regelen. De celdeling is opgedeeld in 4 stadia.

TEL-AML1 positieve ALL cellen blijven in een bepaald stadium in de celdeling steken, waarschijnlijk doordat de normale functie van AML1 wordt tegengewerkt. Aangezien de leukemiecellen in dit stadium blijven steken, probeert de cel via een andere weg de celdeling alsnog voort te zetten. Een van de factoren die daarbij gestimuleerd kan worden is de aanmaak van asparagine synthetase. Zoals beschreven in **hoofdstuk 5** is de lagere neiging tot celdeling van TEL-AML1 positieve leukemiecellen gerelateerd aan een hogere expressie van asparagine synthetase ten opzichte van TEL-AML1 negatieve ALL cellen.

Uit onze studies naar mogelijke oorzaken van ongevoeligheid voor L-asparaginase bleek dat er grote verschillen waren tussen patienten met en zonder deze TEL-AML1 fusie. In tegenstelling tot TEL-AML1 positieve ALL cellen, blijkt uit ons onderzoek dat een hoge asparagine synthetase expressie in TEL-AML1 negatieve ALL cellen wel gerelateerd is aan ongevoeligheid voor L-Asparaginase (**hoofdstuk 6**). Om mogelijke oorzaken van L-Asparaginase ongevoeligheid in TEL-AML1 positieve ALL patiënten te vinden, werd een gen expressie onderzoek verricht zoals beschreven in **hoofdstuk 7**. Zo'n gen expressie onderzoek vindt plaats met behulp van micro-arrays. Dit zijn microchips waarmee men kan meten welke genen wel en welke genen niet tot expressie komen voor iedere afzonderlijke patiënt. Door nu groepen van patiënten te vergelijken die gevoelig en ongevoelig voor L-Asparaginase zijn, werden 52 genen geïdentificeerd die goed onderscheid maakten tussen L-Asparaginase gevoelige en -ongevoelige TEL-AML1 positieve ALL patiënten. Deze genen waren met name betrokken bij de vet stofwisseling en het celdood mechanisme. Een relatie van deze genen met L-Asparaginase gevoeligheid is niet eerder beschreven in de literatuur. Sommige resistentie genen werden zowel in TEL-AML1 positieve als negatieve leukemie patienten gevonden. Echter, ongevoeligheid voor L-asparaginase bij TEL-AML1 positieve leukemie ging samen met een hogere expressie van deze genen terwijl dit bij TEL-AML1 negatieve leukemie juist andersom was (en vice versa). Deze resultaten benadrukken nog een keer dat beide typen leukemie verschillende oorzaken van L-asparaginase ongevoeligheid hebben. Verder functioneel onderzoek zal uit moeten wijzen of deze genen inderdaad betrokken zijn bij het mechanisme van L-Asparaginase (on)gevoeligheid.

In deel C werd het effect bestudeerd van een mogelijk nieuw middel dat gericht tegen het TEL-AML1 fusiegen gebruikt zou kunnen worden in de behandeling van TEL-AML1 positieve ALL. Zoals eerder aangegeven werkt TEL-AML1 de normale functie van AML1 tegen. Dit gebeurt doordat het fusieproduct TEL-AML1 bepaalde stoffen aantrekt, histon deacetylases (HDACs) genoemd. Deze HDACs zorgen ervoor dat de chromosomen zeer compact in elkaar gaan zitten zodat het bijna niet mogelijk is om de DNA code af te lezen. Een remmer van deze HDACs, FK228 genaamd, zou dit effect tegen kunnen gaan. In **hoofdstuk 8** wordt ons onderzoek beschreven naar het effect van

blootstelling van leukemiecellen aan FK228. Het blijkt dat ALL cellen veel gevoeliger zijn voor FK228 dan normale beenmerg cellen. Bovendien zorgt FK228 ervoor dat de uitrijping van de leukemie cellen gestimuleerd wordt en dat de leukemie cellen dood gaan. Dit effect was echter niet selectief voor de TEL-AML1 positieve ALL cellen, maar werd ook gezien bij de TEL-AML1 negatieve ALL cellen. Bovendien zorgde FK228 in combinatie met L-Asparaginase ervoor dat de ALL cellen al bij een lage dosering van L-asparaginase doodgingen Mogelijk brengt aanvullend onderzoek ons zover dat in de toekomst bijwerkingen kunnen worden tegengegaan door lagere doseringen cytostatica toe te dienen tesamen met HDAC remmers zonder dat het uiteindelijke effect van de behandeling vermindert.

Samenvattend worden in dit proefschrift nieuwe genetische en biologische kenmerken van TEL-AML1 positieve ALL patiënten beschreven. Dit nieuwe inzicht in deze kenmerken biedt belangrijke aanknopingspunten voor vervolgonderzoek naar de efficiëntie en toxiciteit van de chemotherapie bij deze patienten. Aanpassingen in de therapie van TEL-AML1 positieve ALL zijn nodig om de overlevingskans te verbeteren van die kinderen die momenteel nog niet voldoende reageren op therapie alsmede om de bijwerkingen van therapie te kunnen verminderen.

About the author

CURRICULUM VITAE

Wendy Stams was born on December 17, 1973 in Oisterwijk, the Netherlands. She graduated in 1992 at "Gymnasium Beekvliet" in Sint Michielsgestel. In 1999, she obtained her medical degree cum laude at the Erasmus University in Rotterdam. Subsequently, she worked as a resident in Pediatrics for one year at the Beatrix Hospital in Gorinchem and for five months at the Medical Center Rijnmond Zuid in Rotterdam. In September 2000, she started the work described in this thesis at the department of Pediatric Oncology/Hematology of the Erasmus MC-Sophia Children's Hospital in Rotterdam in collaboration with the department of Clinical Genetics of the Erasmus MC-University Medical Center Rotterdam. She started her specialist training in Clinical Genetics at the Academic Hospital of Maastricht in December 2004.

She is married to Ron van Zelst and together they have two beautiful daughters, Lieke and Sanne.

DANKWOORD

Het zit erop!!!

Vele mensen hebben me tijdens de afgelopen jaren bijgestaan en geholpen en wil ik hierbij graag bedanken.

Allereerst de patiënten met ALL en hun ouders, die toestemming gaven voor wetenschappelijk onderzoek op een van de moeilijkste momenten in hun leven. Mijn grote bewondering en dank hiervoor. Extra persoonlijk werd het onderzoek toen mijn nabije vriendenkring met TEL-AML1 positieve ALL te maken kreeg. Hidde, dank je wel dat ik jouw mooie tekening mocht gebruiken als kaft voor mijn proefschrift. AnneMarie, ik vind het een eer dat je een voorwoord wilde schrijven, zoals alleen een moeder kan doen die zoiets heeft meegemaakt. Ik wens jullie en de rest van het gezin een zeer gezonde en gelukkige toekomst toe.

Mijn promotor, Prof.dr. R. Pieters, wil ik bedanken. Beste Rob, ik heb bewondering voor jouw visie op onderzoek. Jij weet altijd op een heldere en simpele manier de rode draad zichtbaar te houden en wetenschappelijk onderzoek te vertalen naar de kliniek. Ik hoop dat ik er in de toekomst mijn voordeel mee kan doen.

Tevens een woord van dank aan mijn copromotoren Dr. M.L. den Boer en Dr. H.B. Beverloo. Beste Monique, hartelijk dank dat je me persoonlijk informeerde toen er de mogelijkheid was dit onderzoek te starten. Ik heb veel geleerd van jouw manier van begeleiden en vind het best een eer dat ik jouw eerste promovendus ben. Beste Berna, onder jouw begeleiding en enthousiasme heb ik ruim negen jaar geleden voor het eerst kennis gemaakt met een genetisch laboratorium tijdens mijn studie geneeskunde. Hiervoor wil ik je heel hartelijk danken. De liefde voor de genetica was er altijd al, maar werd door jou destijds versterkt. Bovendien heb je me bij Rob geïntroduceerd met dit proefschrift tot gevolg.

Het onderzoek dat in dit proefschrift wordt beschreven zou niet mogelijk zijn geweest zonder de steun van de SKION en de COALL. Dr. E.R. van Wering, beste Elisabeth, dank je wel voor het verzamelen van de benodigde patiëntendata en je behulpzaamheid tijdens het onderzoek. Dear Prof. dr. G.E. Janka-Schaub, thank you for sending the patient materials of the COALL study group en your fast review of the manuscripts.

Ook alle andere co-auteurs wil ik hartelijk danken voor hun intellectuele inbreng en vervolmaking van de manuscripten. Een aantal wil ik persoonlijk noemen. Ellen, hartelijk dank dat je Rolinda en mij wegwijs hebt gemaakt in de wereld die FISH heet. Rolinda wil ik heel, heel hartelijk danken voor het werk dat zij voor mij heeft verricht. Dankzij jou liep het onderzoek door tijdens mijn zwangerschappen en kon ik, evenals

jij, vele uren in het donker rode en groene puntjes tellen. Ook alle andere mensen betrokken bij het cytogenetica lab, hartelijk dank dat Rolinda en ik met vragen bij jullie terecht konden. Jules, ik vergeet nooit meer het telefoontje van jou toen je me vertelde dat ik aangenomen was op dit project. Enorm bedankt dat je me op weg hebt geholpen met alle ins and outs van de Taqman. Het proefschrift bestaat grotendeels uit data verkregen met de Taqman. Karin, dank je voor jouw hulp bij de flowcytometer. Zonder jou was dat onderdeel van het onderzoek niet zo snel en soepel verlopen. Renee, thank you very much for your support in the statistical array analysis. Inge, ik hoop dat jij het werkingsmechanisme van asparaginase kan ontrafelen.

Collega's van het lab kinderoncologie/hematologie: Bedankt voor alles wat nodig is om te promoveren. Ook hier wil ik een aantal mensen persoonlijk noemen. Als eerste natuurlijk mijn kamergenoten en mede AIO's van het eerste uur. Ronald, Amy en Barbara enorm bedankt voor alle steun, die we allemaal om de beurt nodig hadden om het onderzoek door te zetten en succes met jullie laatste loodjes. Ik zal onze kamer en onze gezellige etentjes enorm missen. Ronald, veel geluk en succes met je baan als postdoc op het lab. Hopelijk brengt het je wat je verwacht. Amy, ik vind het erg stoer van je dat je het hebt aangedurfd om alsnog geneeskunde te gaan studeren. Veel succes en ik vind het erg leuk dat ik je in de toekomst weer collega mag noemen. Bar, vriendin door dik en dun! Ik ben erg blij dat jij mijn paranimf bent. Ik weet zeker dat je een geweldig kinderarts wordt en die promotie komt er ook wel. Henne, ik vond het een enorm moedige beslissing van je om elders onderzoek te gaan doen. Jammer dat we daardoor je gezelligheid moesten missen, maar gelukkig zien we je nog af en toe op onze etentjes. Wim en Inge, succes met de afronding van jullie proefschriften en bedankt voor jullie gezelligheid. Paul, John en Marcel, bedankt voor de computerondersteuning. Ingrid L. bedankt voor jouw steun en medeleven. Alle andere medewerkers van het lab kindergeneeskunde: Thanks! Ook de medewerkers van het specieel hematologisch lab beneden: heel hartelijk bedankt.

Mijn geweldige vriendinnen en paranimfen: Gysèle, Aagje en Barbara. Vriendinnen vanaf het eerste moment dat we elkaar ontmoetten. Ik vond het geweldig dat we met z'n allen in hetzelfde promotieschuitje zaten. Ik mis onze lunches al enorm. Lieve Gysèle, jij was de eerste die de klus klaarde, ik ben de tweede. Zo gaat het al sinds onze coschappen en ik ben trots op zo'n voorbeeld! Lieve Aag, zo sterk als jij bent, ook dit ga jij ondanks alle tegenslagen die je nu hebt redden. Lieve Bar, dank je voor je luisterend oor. Je hebt de gave mensen op te vrolijken, gewoon door aanwezig te zijn. Ook al hebben we het nu allemaal erg druk met onze opleiding, ik weet zeker dat we toch de tijd zullen vinden om elkaar te blijven zien en spreken. Lang leve de email!

Om alle werkstress te vergeten, heb ik gelukkig een zeer hechte en grote vriendenkring. Lieve KV, met jullie is er altijd zoveel gezelligheid en plezier dat ik de promotiestress spontaan kon vergeten. Dank daarvoor! Ook alle lieve meiden van mijn jaarclub Baloe, wil ik danken. Het wordt weer eens tijd voor een clubweekend, vinden jullie ook niet?

Lieve pap, mam, Maykel, Dymphie, Dirk en Colin, jullie zijn voor mij onmisbaar. Zonder jullie onvoorwaardelijke liefde en steun zou ik niet geworden zijn wie ik nu ben. Het is super om te weten dat jullie altijd trots op me zijn, wat ik ook doe. Ik ben ook reuze trots op jullie. En dan heb ik ook nog eens een geweldige schoonfamilie: Jan, Els, Meja, Richard, Marnix en Kirsten! Allemaal enorm bedankt voor alle steun.

Ook nog eens hartelijk dank, lieve oma's, opa's, tante's en ooms, omdat jullie zo geweldig voor onze dochters en ondersteuning thuis zorgen. Werkende ouders kunnen natuurlijk niet zonder zo'n enorm geweldig sociaal vangnet!

Lieve Ron, al meer dan de helft van mijn leven ben jij mijn steun en toeverlaat. Bedankt voor jouw nuchterheid, rust, vriendschap en liefde. Het laatste jaar was wel erg bewogen. Hopelijk komt er nu weer wat rust. Hoewel, met jou blijft het altijd spannend. Ik hou van je!

Als laatste onze mooie, lieve dochters Lieke en Sanne. Sinds jullie er zijn weet ik waar het in het leven allemaal om draait en is mijn geluk compleet! Wat zullen jullie blij zijn dat mama klaar is.

En nu is het tijd voor een feestje!!!

Wendy