Study of genomic rearrangements of the T-cell receptor loci in T-cell acute lymphoblastic leukemia

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List of abbreviations

aa	amino acid
ABL	abelson kinase
AF10	ALL1 fused gene from chromosome 10
AF6	ALL1 fused gene from chromosome 6
AGM	aorto-gonads-mesonephros
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
ATM	ataxia telangiectasia
bHLH	basic helix-loop-helix
BHLHB1	basic helix-loop-helix class B 1
CALM	clathrin assembly protein
CC	coiled-coil
CCND2	cyclin D2
CD	cluster of differentiation
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
cDNA	complementary DNA
CFU-GEMM	colony forming unit-granulocytic-erythroid-myelo-monocytic
CFU-GM	colony forming unit-granulocytic-myelo-monocytic
CGH	comparative genomic hybridization
CLL	chronic lymphoid leukemia
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
EML1	echinoderm microtubule-associated protein-like-1
EMCN	endomucin
ENL	eleven nineteen leukemia
ENTH	espin N-terminal homologous
ETS	erythroblast-transformation-specific domain
ETV6	ETS variant gene 6
FISH	fluorescent in situ hybridization
FLT3	FMS like tyrosine kinase
GEF	guanine-nucleotide-exchange factor
HDAC	histone deacetylase
HOX	homeobox
HPC	hematopoietic progenitor cell

HSC	hematopoietic stem cell
IG	immunoglobulin
IL-7	interleukin 7
ISP	intermediary single positive
JAK	Janus tyrosine kinase
kb	kilobase (1000 nucleotides)
LCK	lymphocyte specific protein tyrosine kinase
LDB1	LIM domain binding-1
LIM	transcriptional regulator, cysteine rich
LMO1	LIM domain only-1 alias rhombotin 1 (RBTN1)
LMO2	LIM domain only-2 alias rhombotin 2 (RBTN2)
LOH	loss of heterozygosity
LYL1	leukemia lymphoid 1
Mb	megabase (1 million nucleotides)
MDS	myelodysplastic syndrome
MEIS1	myeloid ecotropic viral integration site 1 homolog
MGUS	monoclonal gammopathy of undetermined significance
miRNA	micro RNA
MLL	mixed lineage leukemia
NUP	nucleoporin
PBX1	pre-B-cell leukemia transcription factor 1
PcG	polycomb genes
PCM1	human autoantigen pericentriolar material 1
PCR	polymerase chain reaction
PREP1	PBX-regulating protein 1
RAG	recombinase activating gene regulator
RNA	ribonucleic acid
RSS	recombination signal sequences
SCF	stem cell factor
SCL	stem cell leukemia
SNP	single nucleotide polymorphism
SP	single positive
Src	proto-oncogene tyrosine-protein kinase
	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
TAL1	T-cell acute lymphocytic leukemia 1 alias SCL, TCL5
TALE	three amino-acid-loop-extension
TCL1	T cell leukemia/lymphoma-1
TCR	T cell receptor
TF	transcription factor
TGF	transforming growth factor

Trx	trithorax group of proteins
UPD	uniparental disomy
USP33	ubiquitin specific protease 33
VDJ	Variety, Diversity and Joining gene segments

CHAPTER I: INTRODUCTION and RESEARCH OBJECTIVES

Chapter I : Introduction and research objectives

1. Role of cytogenetic analysis in hematological malignancies

History of cytogenetic analysis

The importance of chromosomal alterations in the etiology of cancer was first suggested by Boveri in 1914 [1] who proposed the concept of chromosomal instability as a cause of abnormal growth and cancer. However, it was not until 1956 when improved cell culture and slide preparation techniques made it possible to accurately enumerate the number of human chromosomes as 46 [2]. At that time, in the pre-banding era, the identification of chromosomes was based on size and centromere position and chromosomes fell into 7 groups (A-G). The introduction of chromosome banding [3] allowed a much more detailed examination of the tumor karyotype and led to the identification of many recurrent structural chromosomal abnormalities particularly in leukemia and lymphoma [4]. These chromosomal abnormalities assumed particular importance when some were recognized to occur regularly in certain types of cancer. Such regularity implies that genes affected by these rearrangements might be the first hit in the respective tumors and might serve as a target for therapy. The identification of the Philadelphia chromosome as the product of t(9;22)(q34;q11) in 1973 was a landmark in our understanding of the genetic basis of cancer [5].

Importance of cytogenetics in hematological malignancies

During the last 20 years, many studies have shown that chromosomal abnormalities found in leukemia patients were useful diagnostic and prognostic indicators. Several publications document the prognostic significance of recurrent chromosomal abnormalities in the different types of hematological malignancies (AML, ALL, MDS, CLL) [6-8]. This information is important for the stratification of patients for therapy. Patients with poor prognostic features may be treated more intensively, whereas patients with good prognostic features could benefit from a less intensive and less toxic treatment. Moreover, cytogenetic analysis is often the first step towards the identification of leukemia-associated genes and often leads to the identification of candidate genes. For some translocations, like t(15;17)(q22;q21) and t(9;22)(q34;q11), the biological networks that are disturbed have been largely unraveled allowing patients with these aberrations to benefit from a rational targeted therapeutic approach. In the current era of risk-adapted and molecular targeted therapy, accurate assessment of the genetic status is of high importance.

Evolution of cytogenetic techniques

Cytogenetics has been used for many years for clinical diagnostics as well as for basic genomic research. The techniques used however, have evolved largely during the last 25 years which were initially aimed at increasing the resolution of conventional chromosome analysis. Nowadays, fluorescence in situ hybridization (FISH) has become an important additional technique to conventional chromosome analysis and overcomes the need for dividing cells and subsequent metaphase chromosomes. However, FISH can not replace conventional karyotyping since

metaphases are still needed for unraveling unknown chromosomal rearrangements. Both techniques however are still based on microscopic analysis, have a limited sensitivity and are labor intensive. Although automated karyotyping systems became available, analyzing metaphase spreads remains time-consuming. Polymerase chain reaction (PCR) analysis has the advantage to be more sensitive and to screen for a specific chromosome aberration without the need for dividing cells. However, the molecular analyses are limited to known chromosomal alterations and do not allow the screening of the whole genome for other abnormalities. Besides, PCR analysis is very useful in searching for aberrant gene expression which can point to an underlying genomic aberration. Recent novel technologies were developed that permit high-throughput applications, provide whole genome coverage and have become important components of molecular diagnostics. Several array-based technologies have been developed which are based on hybridization of patient DNA/cDNA with control/reference DNA/cDNA. In array-based comparative genomic hybridization (array-CGH), metaphase chromosomes are replaced as the target by large numbers of mapped FISH clones that are spotted onto a standard glass slide. The test and reference DNA are differentially labeled and co-hybridized to a microarray. The flexibility of array design has allowed the development of specialized arrays for applications such as telomeric screening or for specific diseases (i.e. B-CLL [9]). Array-CGH has been applied to some hematological disorders and detected unknown regions of amplification/duplications or deletion which possibly harbor proto-oncogenes or tumor suppressor genes respectively [10, 11]. Besides amplification and microdeletion screening, array-CGH can be used to define breakpoints in unbalanced and even in balanced translocations. In unbalanced translocations, loss or gain of genomic material can be detected on the array-CGH profile of the derivative chromosome [12]. A more advanced technique that can be used to exactly identify breakpoint positions in balanced chromosomal translocations using array-CGH is the flow sorting or microdissection of the derivative chromosomes, linear DNA amplification and subsequent hybridization on array-CGH slides [13, 14]. Instead of FISH mapped clones, DNA sequences have also been used for array-CGH, called cDNA arrays. An advantage of cDNA arrays is that expression analysis can be carried out using the same platform; a drawback however is the uneven coverage of the genome (lack of intronic sequences) and lower sensitivity to small copy number changes than arrays produced from genomic clones. cDNA microarray technology not only allows the distinction between disease subclasses but also offers a possibility to identify new genes involved in leukemogenesis. To this purpose, cDNA libraries from normal and leukemic bone marrow were generated and sequenced. cDNA clones identifying unique transcripts from leukemic bone marrow were used to enrich an existing cDNA microarray platform. Subsequent hybridization of leukemic samples eventually leads to the detection of over –or under-expression of transcripts with previously unknown function in oncogenesis i.e. *EMCN* (endomucin) and *USP33* (ubiquitin specific protease 33) that appear to be over-expressed in B-ALL compared to T-ALL [15]. While CGH can give information on DNA copy number, it provides no information on the parental origin of chromosomes. In some cancer associated genetic diseases, a single parental origin, uniparental disomy (UPD), has been associated with imprinted genes in the homozygous regions [16]. Oligonucleotide arrays which allow genotyping of thousands of single nucleotide polymorphisms (SNPs), have been applied to the

analysis of loss of heterozygosity (LOH) in normal and tumor samples. Single nucleotide polymorphism (SNP) arrays are high-density oligonucleotide-based arrays that can identify both LOH at individual nucleotides and copy number alterations. The SNP array probes comprise 25-mer oligonucleotides and each SNP is represented by both a sense and an antisense strand. The probe intensities that correspond to the two possible alleles of the SNP reveal which of the three expected genotypes (AA, BB or AB) is present. SNP analysis in childhood acute lymphoblastic leukemia revealed LOH for chromosome 9p in the majority of patients [17]. Similarly, SNP analysis revealed large regions of homozygosity in 20% of normal karyotype AML cases that could not be accounted for by chromosomal loss or gain. One explanation is somatic recombination resulting in large regions of UPD. The challenging effect of this acquired UPD could be the unmasking of mutations of leukemia associated genes [18]. These different techniques however have their own advantages and drawbacks in the detection of chromosomal abnormalities which are summarized in Fig.1.

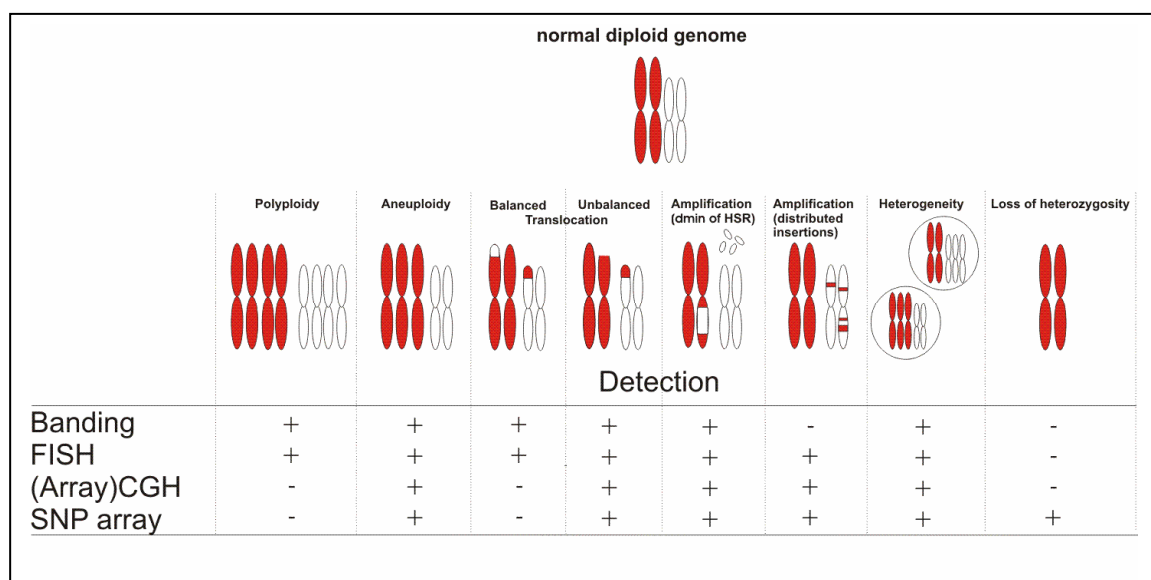| normal diploid genome | | | | | | | |
Detection	Polyploidy	Aneuploidy	Balanced Translocation	Unbalanced	Amplification (dmin of HSR)	Amplification (distributed insertions)	Heterogeneity	Loss of heterozygosity
Banding	+	+	+	+	+	-	+	-
FISH	+	+	+	+	+	+	+	-
(Array)CGH	-	+	-	+	+	+	+	-
SNP array	-	+	-	+	+	+	+	+

Fig. 1 : Overview of different genome wide screening techniques and their applicability to detect different types of chromosomal aberrations. FISH: fluorescence in situ hybridization, CGH: comparative genomic hybridization, SNP: single nucleotide polymorphism. (Adapted from Speicher et al., Nat. Rev. Genet. 2005)

2. Normal and malignant hematopoiesis

Hematopoiesis is a complex process during which hematopoietic stem cells (HSC) proliferate and differentiate to constitute both the myeloid and lymphoid branches of the hematopoietic system (Fig.2). This process takes place in successive organs beginning in the yolk sac and aorto-gonads-mesonephros (AGM) region, and then migrates from the AGM region to the fetal liver and subsequently to the bone marrow [19, 20]. HSCs only comprise between 0.01% and 0.05% of the total marrow population and are responsible for the production and maintenance of specific types of blood cells which have a limited life-span. Therefore, the ancestral pluripotent stem cells must ensure its own survival by proliferation before differentiating into all lineages of blood cells. HSC possess two characteristic features: 1) self-renewal or the ability to generate daughter cells with equivalent developmental possibilities and 2) differentiation potential to produce highly specialized mature cell types (erythrocytes, neutrophils, eosinophils, basophils, platelets, monocytes/macrophages, osteoclasts and T and B lymphocytes, NK cells, dendritic cells). Upon differentiation, cells progressively lose the ability for self-renewal and have a limited life time in the peripheral blood where they subsequently undergo apoptosis. This tightly regulated process of hematopoietic lineage determination is driven both extrinsically (through growth factors, cytokines or other external influences) and intrinsically (pattern of genes expressed by the cell). These patterns of gene expression are determined by the positive and negative regulation of transcription factors. In the absence of expression of some important transcription factors like *SCL/TAL1*, mice do not generate definitive hematopoietic cells [21], suggesting that intrinsic mechanisms are important in lineage commitment whereas extrinsic signals have been suggested to support survival and development of committed cells [22]. Abnormalities in the normal developmental program for blood cell formation result in hematological diseases. These abnormalities are due to modifications in gene expression or protein function, resulting from structural or numerical chromosome aberrations or other mutations as well as by epigenetic modifications.

Depending on the affected cell type or lineage, the maturation point and the predominant functional defect, hematological malignancies are discerned. Leukemia is a clonal proliferation of immature, functionally aberrant white blood cells which results from a maturation arrest and/or deficiency of apoptosis. Based on the affected lineage, leukemia can be divided in lymphoid and myeloid leukemia. Since T and B lymphopoiesis predominantly occurs in the thymus (T-cells) and lymph nodes/spleen (B-cells), lymphoid leukemias frequently involve these organs besides the bone marrow whereas myeloid leukemia is mostly confined to the bone marrow (and peripheral blood). Furthermore, leukemias are classified into acute and chronic leukemia based upon their clinical course without treatment: acute leukemia leads to death within weeks, chronic leukemia within years after diagnosis. Depending on the predominant functional defect (proliferation or ineffective hematopoiesis), hematological malignancies can be divided into myelo -or lymphoproliferative and myelodysplastic disorders. The myeloproliferative disorders are consequent on the proliferation of a clone of myeloid cells derived from a neoplastic precursor. Furthermore and in contrast to overt leukemia, maturation of neoplastic cells is relatively normal but proliferation is abundant. These

myeloproliferative disorders include polycythemia rubra vera, idiopathic myelofibrosis, essential thrombocytosis, chronic myeloid leukemia, chronic eosinophilic leukemia and chronic neutrophilic leukemia based on the cell type predominantly affected and on the presence/absence of characteristic chromosomal defects. The myelodysplastic syndromes (MDS) are a group of clonal hematopoietic stem cell diseases characterized by dysplasia and ineffective hematopoiesis in one or more of the major myeloid cell lineages. The dysplasia may be accompanied by an increase in myeloblasts but the number is less than 20%, which is the requisite threshold recommended for the diagnosis of acute myeloid leukemia. Different subtypes of MDS are recognized depending on the percentage of blasts, the number of cell lines (red blood cells, white blood cells, platelets) affected, the presence of ringed sideroblasts and the presence of characteristic cytogenetic findings e.g. the 5q- syndrome. Lymphoproliferative disorders include clonal proliferations of lymphoid cells, either as acute or chronic lymphoid leukemia, Hodgkin's disease and non-Hodgkin's lymphomas. the Plasma cell dyscrasias are characterized by proliferation of a neoplastic clone closely related to plasma cells and include multiple myeloma, monoclonal gammopathy of undetermined significance (MGUS) and Waldenström's macroglobulinemia. Given the focus of my research on T-cell acute lymphoblastic leukemia, this disease will be discussed in more detail below.
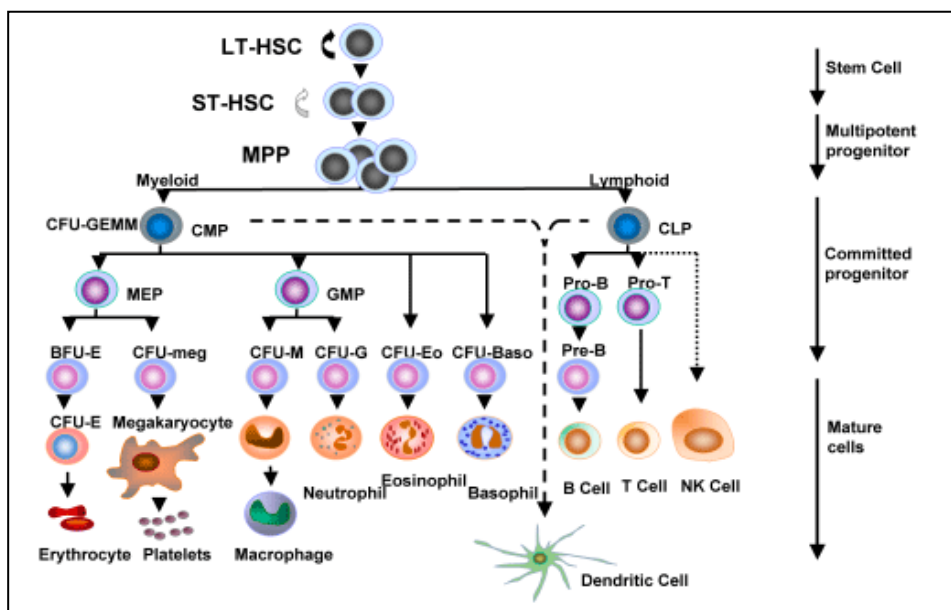

Fig.2 : The hematopoietic system: from pluripotent stem cell to mature blood cells. HSC: hematopoietic stem cell, MPP: multipotent progenitor cell, CLP: common lymphoid progenitor, CMP: common myeloid progenitor, CFU-GEMM :colony forming unit-granulocytic-erythroid-myelo-monocytic. (Adapted from www.molmed.lu.se)

3. Normal T cell development and T-ALL

3.1. Normal T cell development

Human T lymphocytes are derived from pluripotent hematopoietic progenitors that migrate throughout life from the bone marrow to the thymus, where the majority of T-cell development occurs. Immature prothymocytes enter the thymus where they interact with stromal cells that produce lineage specific cytokines such as interleukine 7 (IL-7) [23, 24], stem cell factor (SCF) [25], FLT3 ligand [26] and transforming growth factor-ß (TGFß) [27, 28]. Thymocytes are divided as being double negative (DN), double positive (DP) or single positive (SP) based on the expression of the CD4 and CD8 antigens. The DN stage is heterogeneous and can be subdivided in three stages: a CD34+CD38-CD1a-, CD34+CD38+CD1a- and a CD34+CD38+CD1a+. Most immature human thymocytes have the CD34+CD38- stem cell phenotype but rapidly acquire CD38 expression and subsequently also CD1, a marker for T cell lineage commitment [29]. These human DN thymocytes pass through an immature single positive (ISP CD4+) stage towards a DP stage into CD4+ or CD8+ SP T cells that express functional T cell receptors (TCR) [30, 31] (Fig.3). A hallmark of T cell development is the generation of T cells that express a functional TCR, TCRαß or TCRγδ. During T cell development, the variable domains of *TCRa, TCRß, TCRγ* and *TCRδ* genes are assembled following somatic rearrangement of variable (V), diversity (D) and joining (J) gene segments by a process called VDJ recombination [32]. This process is responsible for the diversity of an adaptive immune response in both TCR and immunoglobulin (IG) genes and uses the recombinase activating genes (*RAGs*) *RAG1* and *RAG2* enzymes that target recombination signal sequences (RSS) that flank V, D and J segments.

Many studies investigating TCR gene rearrangements during normal T cell development suggest that recombinations in TCR genes are sequential between the different genes (*TCRδ > TCRγ > TCRß > TCRa*) as well as within a particular gene [31, 33]. The CD34+CD1a- cells start to rearrange their *TCRδ* genes, but mostly have their *TCRγ* and *TCRß* loci in germline configuration, whereas expression of CD1a is accompanied by rearrangements of *TCRδ* (V to DJ), *TCRγ* and *TCRß* (D to J) loci. The immature single positive cells (ISP) have lost CD34 expression and contain mature *TCR* (V to DJ) rearrangements. When the thymocytes have developed into ISP cells, the functionality of the TCRß chain is tested by expression on the cell surface together with a pre-TCR alpha (pTα) chain, a process called ß selection. Signals emanating from this pre-TCR complex induce the cells first to proliferate and subsequently to initiate *TCRa* gene rearrangements. Productive *TCRa* rearrangements lead to the expression of a *TCRaß* complex on the cell surface, which is then tested for the recognition of self-MHC molecules (positive selection) and absence of reactivity against self-antigens (negative selection). The surviving cells become SP thymocytes that mature into either CD4+ T helper cells or CD8+ cytotoxic T cells and migrate to the periphery as naïve circulating T lymphocytes. Studies in T-ALL have demonstrated that they largely reflect physiologic T-lymphoid development [33].
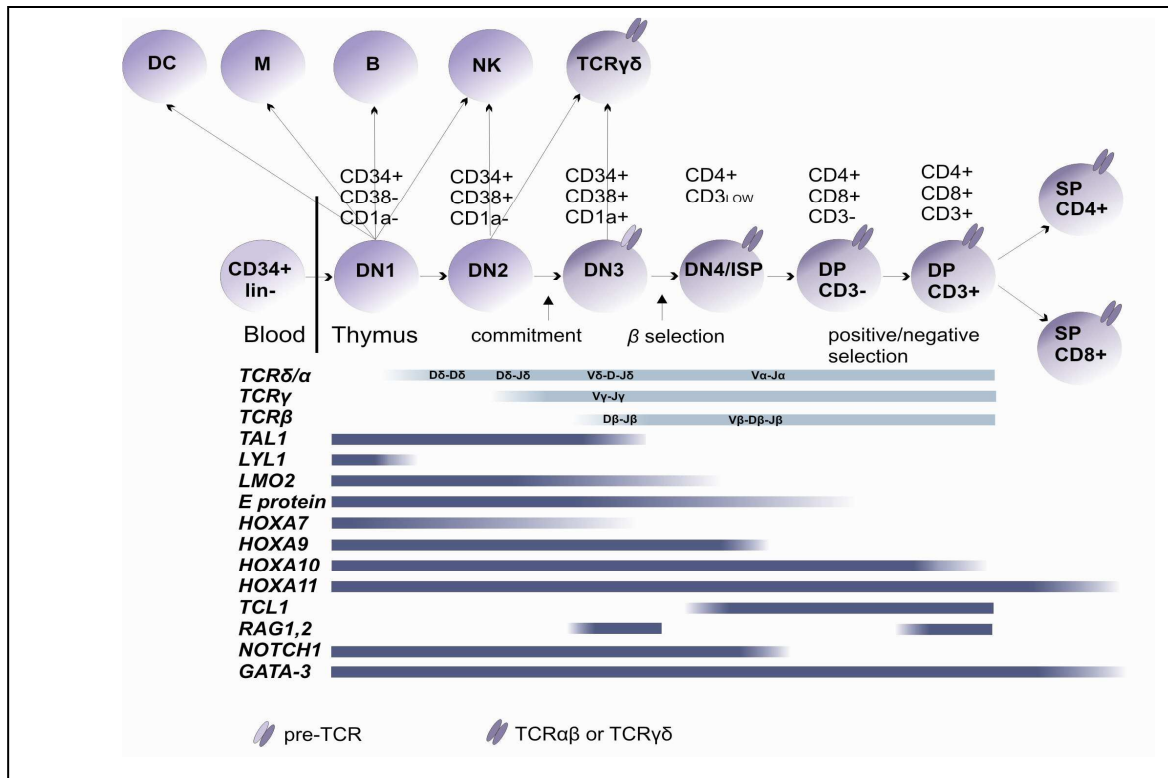
Fig.3 : Schematic representation of human T cell development; ▦ : timepoint of TCR rearrangements during T-cell development; ■ : expression of different genes involved in T cell development. DN: double negative, ISP: immature single positive, DP: double positive, SP: single positive, DC: dentritic cell, M: myeloid cell.

Many cytokine receptors (especially IL-7), the *NOTCH* family receptors and transcription factor genes (basic helix-loop-helix genes, LIM-domain genes, *GATA*, homeobox genes) are involved in regulating this complex process. Most of these genes have been identified through clonal chromosomal abnormalities in blast cells from T-ALL patients and had a profound impact on the understanding of the molecular changes involved in leukemogenesis. Some of these are specific for T cell development whereas others are involved in general hematopoiesis.

The basic helix-loop-helix family of transcription factors (bHLH) share the bHLH motif (60aa) allowing homo- or heterodimerization through the HLH domain and DNA binding through the basic domain [34]. Class A bHLH includes the E proteins (E2A and HEB) which bind DNA at specific E-Box sites in the enhancers of many T-cell specific regulatory genes like CD4 and pTα [35]. E2A proteins regulate VDJ recombination, the expression of *RAG* and pTα genes required for the formation of pre-TCR [36]. Besides, E2A proteins act as negative regulators of cell proliferation in thymic precursors [36]. Class B bHLH proteins form heterodimers with class A bHLH and are weakly (*LYL1, TAL1*) or not (*TAL2*) expressed in most primitive thymocytes [37]. The *TAL-1/SCL* gene (stem cell leukemia) was originally identified through its translocation in acute T-cell lymphoblastic leukemia [38] and is expressed in hematopoietic cells, vascular endothelium and the developing brain [39-41]. In the absence of *SCL/TAL-1*, hematopoiesis is undetectable [21]. *SCL/TAL1* is expressed in the early DN

stages of normal T cell development [42, 43] and normally gets downregulated upon differentiation towards mature T cells, suggesting a role in early T cell development. Another bHLH protein which is closely related to *SCL* in terms of amino acid identity is the *LYL-1* gene. Despite this high sequence homology these two transcription factors display a distinct expression pattern in hematopoietic cells with *LYL-1* being expressed mainly in immature hematopoietic and in B cells [44] but only weakly in T cell progenitors. *TAL2* and *BHLH1* are two bHLH proteins which were initially identified through involvement in rare translocations t(7;9)(q34;q32) and t(14;21)(q11;q22) in T-ALL whereas they are not normally expressed in T cells but are both involved in CNS development [45, 46].

SCL/TAL1 collaborating genes *LMO1* (alias *RBTN1*) and *LMO2* (alias *RBTN2*) belong to the LIM domain only genes which mediate specific protein interactions, but have no direct DNA binding capacity. *SCL* and *LMO* (especially *LMO2*) proteins seem to collaborate in normal hematopoiesis since both *SCL* and *LMO2* show overlapping expression patterns in early hemogenic sites and in adult hematopoietic lineages. In addition, *LMO2-/-* mice exhibit a complete absence of hematopoietic cells and defective yolk sac angiogenesis, a phenotype that is highly similar to that observed in *SCL-/-* mice [47, 48]. Little is known about the function of *LMO1* in normal hematopoiesis whereas *LMO2* is important in embryonic stem cell development [49]. In addition to its association with *SCL*, *LMO2* has been demonstrated to interact with hematopoietic *GATA* family members (*GATA 1-2-3*).

Transcription factor *GATA-3* is expressed in a T cell specific context and evidence for the necessity of *GATA-3* in T cell development came from several studies [50, 51]. In contrast, *GATA-1* is highly expressed in erythroid, megakaryocytic, eosinophils and mast cells whereas *GATA-2* is expressed in early hematopoietic cells and in megakaryocytic lineages [52].

NOTCH1 is a transmembrane receptor that is an important regulator of stem cell maintenance [53] and required for the commitment of pluripotent progenitors to T cell fate [54] namely the T versus B cell lineage choice in early hematopoietic progenitors [55]. Moreover, *NOTCH1* is important for the subsequent assembly of pre-T cell receptor complexes in immature thymocytes [56, 57] besides involvement in mechanisms of cell interaction. *NOTCH1* signalling affects cell differentiation, proliferation and apoptotic programs essential for various tissues and organs [58]. Inactivation of *NOTCH1* signaling in bone marrow precursors results in the generation of B cells and prevents the development of T cells [59, 60]. Several homeobox genes have also been implemented in normal T cell development. Involvement of class I homeobox genes (*HOX* genes) in thymopoiesis was suggested by generation of a *HOXA3* knock-out mice model which lacked thymus development and it was thought that *HOXA3* acts through interaction with *PAX1* and *Thy-1*, which are known to be involved in the maturation of thymocytes [61-63]. Similarly, *HOXA9* knock-out mice exhibit disturbed differentiation of myeloid cells and severely affected early T cell development [64]. Further evidence for involvement of *HOXA* cluster genes during normal T cell development came from a study from Taghon et al. who demonstrated a drastic change in *HOXA* gene expression from hematopoietic precursor cells upon entry into the thymus [65]. There was a progressive downregulation of 3'*HOXA* genes upon differentiation towards mature thymocytes while 5' *HOXA* genes were present in more mature cell types suggesting that sequential up and down regulation of these *HOXA* cluster genes plays an important role in normal T cell development. Besides these homeobox genes, lymphocyte-

specific protein tyrosine kinase (*LCK*) belonging to the Src family kinases (sarcoma viral oncogene homolog) also plays an important role in normal T cell development (TCR signaling) and T cell activation [66]. An important role for *TCL1* in normal T and B cell development was demonstrated in *TCL1* deficient mice, where a significant reduction of lymphocytes was found in bone marrow, thymus and spleen. *TCL1* expression was detected in normal lymphocytes in the DN4 and DP stages but not in mature T cells. Upon knock out, T cell lymphopoiesis was impaired at the double positive stage (CD4+CD8+) [67]. Another effector in pre-TCR signaling, cyclin D3, is crucial for the proliferative burst of DN thymocytes. Besides its role in normal T cell development, cyclin D3 also cooperates with *NOTCH1* in leukemogenesis [68].

3.2. Molecular pathogenesis of T-cell acute lymphoblastic leukemia

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of T cell precursors that develops mainly in children and adolescents (10-15% of childhood ALL) but also in adults (25% of adult ALLs)[6]. This aggressive tumor is characterized by high peripheral white blood cell counts, frequent mediastinal masses, pleural effusion and involvement of the central nervous system and chromosomal features that are distinct from those of B-lineage ALL. Over the past 50 years, treatment success rates for pediatric ALL have markedly improved with overall survival rates of nearly 80%. However, less success has been achieved in the treatment of adults with T-ALL, with long term survival rates of 30-40% among patients < 60 years of age and decreasing to 10% in patients over 60 years of age [69, 70].

Similar to other types of leukemia, T-ALL is caused by multistep mutagenesis involving various genetic alterations that shift normal cells into uncontrolled growth and clonal expansion. These genetic alterations can affect cell-cycle control, stem cell maintenance and proliferation, differentiation and survival [71]. Chromosomal translocations affecting transcription factor genes are the most common alterations in acute leukemia and functionally impair differentiation and provide a proliferative advantage of the leukemic cells. Activation of transcription factor genes by chromosomal translocations take two main forms. In T or B lymphoid leukemias, these genes are often translocated to the vicinity of genes encoding chains of the T-cell receptor (*TCR*) or immunoglobulin (IG) molecules, resulting in inappropriate expression of the translocated proto-oncogenes. In other types of leukemia, the coding exons of genes disrupted by a reciprocal translocation are incorporated into a single "fusion" gene which generates a chimeric protein with unique properties. Besides these chromosomal aberrations, T-ALLs frequently show interstitial deletions of 9p21, causing cell cycle defects. Recently, *NOTCH1* signaling has been identified as an important regulator of stem cell maintenance and was also implicated in the pathogenesis of T-ALL, suggesting that *NOTCH1* defects may serve the leukemic clone with self-renewal capacity. It is well illustrated that these different classes of functional defects cooperate with each other in the generation of an overt leukemia and that one genetic defect can lead to more than one functional effect.
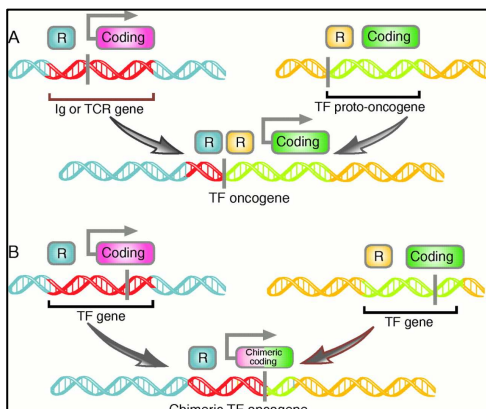

Fig.4: A) TF (transcription factor) are activated when placed under control of potent enhancer elements within the regulatory region of a gene that is normally tightly expressed or B) Chromosomal breakpoints occur within introns and produce a fusion gene that encodes a chimeric transcription factor with altered function. R: regulatory region.
(Adapted from Look et al., Science, 1997).

3.2.1. T- cell oncogenes in T-ALL

Cytogenetic analyses and molecular cloning of chromosomal translocation breakpoints led to the discovery of transcriptional regulatory proteins that are aberrantly expressed in T-ALL owing to the juxtapositioning of their respective genes next to strong T-cell receptor gene enhancers and promotors. However, recent studies have shown that several of these T cell oncogenes (*HOX11, TAL1, LYL1, LMO1* and *LMO2*) are often expressed in the absence of a genomic rearrangement, supporting other mechanisms of transcriptional activation like alterations in cis-acting or trans-acting regulatory sequences without further functional consequences. Indeed, based on similarities in gene expression signatures compared to normal T cell developmental stages, these subgroups were indicative of leukemic arrest at specific stages of normal thymocyte development and have prognostic importance [43](Fig.5). Although these translocated proteins have important functions during normal embryogenesis, many are not essential for the development of normal thymocytes such as *MYC* (8q24), *HOX11/TLX1*(10q24), *HOX11L2/TLX3* (5q35), *TAL2* (9q34), *LMO1/RBTN1*(11p15) and *LMO2/RBTN2* (11p13)(Table 1). Despite absence of hematopoiesis and embryonic lethality 10 days after inception in *LMO2* -/- mice, no effect on T-cell development could be detected when *LMO2* was selectively deleted in lymphoid progenitor cells [72].

Prominent among T cell oncogenes are members of the class B bHLH family of transcription factors : *TAL1, TAL2, LYL1* and *BHLHB1*. Rearrangements of the *TAL1* gene locus are found in up to 25% of all T-ALL cases, but only 3% involve TCR loci in a t(1;14)(p32;q11) whereas the remainder have a deletion that places *TAL1* under the control of the *SIL* gene promoter leading to *TAL1* transcriptional activation. Because the *TAL1* protein forms a pentameric DNA-binding complex with *E2A*, *LMO2*, *GATA3*, *LDB*-1 its ectopic expression in T cells might activate specific target genes that are normally quiescent in T-cell progenitors. Alternatively, *TAL1* might be leukemogenic via a dominant-negative effect since overexpression of *TAL1* can lead to a functional inactivation of *E2A* homodimers by sequestering *E2A* in a pentameric complex. This model is supported by the observations that *E2A*-deficient mice develop T-ALL [73, 74]. Moreover, it has been shown that *TAL1* tumors undergo apoptosis after administration of histone deacetylase inhibitors (HDAC) suggesting that aberrant expression of *TAL1* causes gene silencing [75]. Ectopic expression of *TAL1* in the absence of the cytogenetic aberration was found in 50% [76] of childhood T-ALLs and only 5% of adult T-ALL [77] and reflects the stage of maturation arrest in the late cortical stage of T cell development (Fig.5). The clinical relevance of *TAL1* rearrangements remains unclear, although a trend for a favorable outcome have been described [78]. The *LYL1* gene (19p13) was initially identified through molecular characterization of the t(7;19)(q35;p13) associated with T-ALL which results in the constitutive expression of *LYL1*. Ectopic expression of *LYL1* in the absence of the corresponding genetic defect is associated with maturation arrest of thymocytes in the double negative stage [43] (Fig.5). However, recent studies show that *LYL1* expression is not limited to immature cases and is expressed in almost all T-ALL cases, especially in *CALM-AF10* positive cases [78]. The *TAL2* (9q32) and *BHLHB1* (21q22) genes were identified as a consequence of t(7;9)(q34;q32) and t(14;21)(q11;q22) and share

many properties with *TAL1*, supporting the idea that these proteins promote T-ALL by a common mechanism [79].

The genes encoding the LIM-domain only genes *LMO1* (11p15) and *LMO2* (11p13) are often translocated in T-ALL but are primarily expressed in the central nervous system and only minimally expressed or absent in T cell progenitors. Both *LMO1* and *LMO2* possess zinc-finger-like structures in their LIM domains but lack the DNA-binding domains common to other transcription factors in this family, suggesting that the LIM domain functions in protein-protein rather than protein-DNA interactions. Most common translocations are the t(11;14)(p15;q11) and t(11;14)(p13;q11) juxtaposing *LMO1* or *LMO2* to the *TCRa* or *TCRδ* loci respectively [49, 80, 81]. Studies in mice show that *LMO2* overexpression induces leukemia with long latency [82]. Interestingly, ectopic expression of these genes also occurred in the absence of the chromosomal rearrangements and was frequently found in T-ALL cases that already have deregulated *TAL1 (LMO1* and *LMO2)* or *LYL1 (LMO2)* expression. The observations of co-deregulation of both *TAL1, LMO1, LMO2* and *LYL1* were consistent with animal model studies showing that *LMO* proteins form heterocomplexes and act in concert with *TAL1* and *LYL1*. As a consequence, ectopic expression of *LMO1* and *LMO2* cannot be shown independently in Fig.5 but is overlapping with *TAL1* and *LYL1* expression. In a recent study [83], a novel *LMO2* activation mechanism was suggested i.e. a cryptic deletion on chromosome 11, del(11)(p12p13), with loss of a negative regulatory region upstream of *LMO2*.

Class I homeobox genes, *HOXA* cluster genes in particular, have only recently been associated with T-ALL due to genomic rearrangements with *TCR* loci (*TCRδ* and *TCRB*). The unraveling of these new genomic rearrangements was the result of the present study and is described in detail in chapter II.

Class II homeobox genes *HOX11* and *HOX11L2* are frequently involved in T-ALL whereas both have no specific role in normal T cell development [43, 84] but in spleen morphogenesis (*HOX11*) and in CNS development (*HOX11L2*). Ectopic expression of *HOX11* was found in 3% of childhood and 33% of adult T-ALL and was associated with chromosomal translocation to the *TCRδ* or *TCRB* locus in only half of these cases. These findings suggest that other trans-acting mechanisms can lead to this aberrant gene expression, probably by disrupting gene silencing mechanisms that operate during normal T cell development. Microarray analysis revealed that *HOX11* expressing T-ALLs show a gene expression pattern resembling that of early cortical thymocytes and the favorable clinical outcome of these patients [85] may be explained by their high expression of genes associated with increased cell proliferation and lack of expression of anti-apoptotic genes at this stage of thymocyte development [43]. Enforced expression of *HOX11* in CD34+ progenitor cells block T cell differentiation prior to the DP thymocyte stage [86] and lead to immortalization of hematopoietic precursor cells [87]. Approximately 25-30 % of childhood and 13% of adult T-ALL cases are characterized by ectopic *HOX11L2* expression [88-90] and in most cases, this ectopic expression is caused by the cryptic t(5;14)(q35;q32) juxtaposing *HOX11L2* to the distal region of *BCL11B*, a gene highly expressed during T cell differentiation. Variant translocations have been described involving both the *HOX11L2* and

the *BCL11B* genes. The *CDK6* gene on 7q21 was involved in a t(5;7)(q35;q21) with *HOX11L2*, whereas a rearrangement of the *NKX2-5*(5q35) gene and the *BCL11B* gene is described in a t(5;14)(q35;q32). Although *HOX11* and *HOX11L2* expression profiles predominantly occur at the early cortical stages of thymocyte development, these two subgroups seem to have a different clinical outcome. Whereas *HOX11* expression was associated with a favorable outcome, conflicting results exist for patients with ectopic *HOX11L2* expression [78, 88, 90]. The majority of *HOX11* positive T-ALL express CD1a, a marker of cortical thymocytes, whereas this expression is heterogeneous in *HOX11L2* positive cases. This indicates that these subgroups are arrested at slightly different developmental stages and may have different responses to therapy [78] since CD1a expression is a good prognostic marker. Indeed, Asnafi et al. [77] have shown that *HOX11L2* rearranged T-ALL comprises not only the early cortical developmental stage, but also a more immature phenotype, intermediate between the αß and γδ TCR lineages. The differences in prognostic relevance of *HOX11L2* expression between several studies may also depend on the presence of other genetic aberrations like *NUP214-ABL1* episomal amplification [78].

Recent studies showed first evidence for involvement of cyclin D2 in T cell oncogenesis due to a recurrent chromosomal translocation affecting the *TCRaδ* locus and the *CCND2* locus at 12p13. This was the first example of a T-cell neoplasm with a targeted deregulation of a member of a cyclin-encoding gene family [91, 92].

Table 1 : Genomic alterations in T-cell acute lymphoblastic leukemia

Type of protein	Oncogene	Chromosomal translocation	% of T-ALL carrying the chromosomal translocation	% of T-ALL with ectopic expression of oncogene	Genes affected
Homeodomain	HOX11 (10q24)	t(7;10)(q34;q24) t(10;14)(q24;q11)	7 % [c] 14% [a]	10% [c] 33% [a]	TCRB (7q34) TCRδ (14q11)
Homeodomain	HOX11L2 (5q35)	t(5;14)(q35;q32) t(5;14)(q35;q11) t(5;7)(q35;q21)	25-30% [c] 13% [a]	25-30% [c] 13% [a]	BCL11B(14q32) TCRδ(14q11) CDK6(7q21)
Homeodomain	HOXA (7p15)	inv(7)(p15q34) t(7;7)(p15;q34) t(7;14)(p15;q11)	< 5% < 1%		TCRB (7q34) TCRαδ(14q11)
bHLH	LYL1 (19p13)	t(7;19)(q34;p13)	<1%	35%	TCRB (7q34)
bHLH	TAL1 (1p32)	t(1;14)(p32;q11)	3% 22% SIL-TAL1 fusion	50% [c] 5% [a]	TCRαδ (14q11)
bHLH	TAL2 (9q34)	t(7;9)(q34;q34)	<1-2%		TCRB (7q34)
bHLH	MYC (8q24)	t(8;14)(q24;q11)	2%		TCRαδ (14q11)
bHLH	BHLHB1 (21q22)	t(14;21)(q11;q22)	<1%		TCRαδ (14q11)
LIM domain	LMO1 (11p15)	t(11;14)(p15;q11)	5%	8% [c+a]	TCRαδ (14q11)
LIM domain	LMO2 (11p13)	t(11;14)(p13;q11) t(7;11)(q34;p13)	5-10%	31%[c+a]	TCRαδ(14q11) TCRB (7q34)
Tyrosine kinase	LCK (1p34)	t(1;7)(p34;q34)	<1%		TCRB (7q34)
Cyclin	CCND2 (12p13)	t(12;14)(p13;q11)	<1%		TCRαδ (14q11)
CC-domain-Tyrosine kinase	EML1-ABL1	t(9;14)(q34;q32)	<1%		Gene fusion
ETS-domain-Tyrosine kinase	ETV6-JAK2	t(9;12)(p24;p13)	<1%		Gene fusion
ETS-domain-Tyrosine kinase	ETV6-ABL1	t(9;12)(q34;p13)	<1%		Gene fusion
Nucleoporin-GEF	NUP98-RAP1GDS1	t(4;11)(q21;p15)	2%		Gene fusion
Nucleoporin-Tyrosine kinase	NUP214-ABL1	Episomal	6%		Gene fusion
ENTH-domain-AT-hook	CALM-AF10	t(10;11)(p13;q21)	5-10%		Gene fusion
	MLL-ENL	t(11;19)(q23;p13)	4%		Gene fusion
	MLL-AF6	t(6;11)(q27;q23)	<1%		Gene fusion
	PCM1-JAK2	t(8;9)(p22;p24)	<1%		Gene fusion
Notch receptor	NOTCH1	t(7;9)(q34;q34)	50%		truncation; activation
Tyrosine kinase	FLT3		<1%		ITD/point mutation; activation
Cyclin dependent kinase	CDKN2A-CDKN2B	del(9)(p21)	40-80%		gene inactivation

For abbreviations of oncogenes, see list p. 5-7
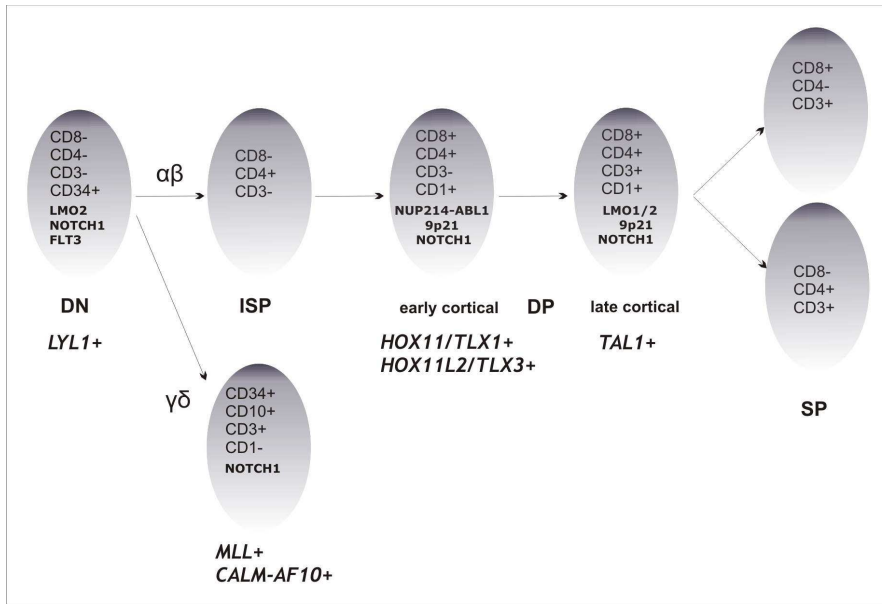[a]: adult : [c]: childhood

Fig.5 : Correlation between gene-expression profiles and stages of thymocyte differentiation. DN: double negative, ISP: immature single positive, DP: double positive, SP: single positive.

3.2.2. Fusion genes in T-ALL

A second class of chromosomal translocations that have been identified in T-ALL result in fusion proteins. *MLL*(mixed lineage leukemia)-*ENL* fusions result from t(11;19)(q23;p13) and are associated with AML, B cell precursor ALL and T-ALL. In T-ALL, this fusion gene is associated with an early thymocyte differentiation arrest leading to increased expression of *HOXA9*, *HOXA10*, *HOXC6* and *MEIS1* [43]. Another *MLL* fusion rarely described in T-ALL is the *MLL-AF6* fusion resulting from the t(6;11)(q27;q23) [93]. The *CALM-AF10* fusion resulting from the t(10;11)(p13-14;q14-21) was identified in 10% of T-ALL but has also been described in lymphomas and acute leukemias of several lineages and has to be considered nonlineage specific [94-98]. Within the T-ALL however, this fusion is specific for the TCRγδ lineage [99] and is associated with upregulation of *HOXA5*, *HOXA9*, *HOXA10* and *BMI-1* [100]. Unexpectedly, several *ABL1* fusions have recently been detected in T-ALL: *ETV6-ABL1*, *EML1-ABL1* and *NUP214-ABL1* [101-103]. *NUP214-ABL1* is found on episomal elements in about 6% of T-ALL patients and is associated with increased expression of *HOX11* and *HOX11L2*. These *ABL1* fusion kinases are constitutively phosphorylated resulting in excessive activation of survival and proliferation, which can be inhibited upon addition of imatinib, a selective inhibitor of *ABL1* kinase activity. *NUP98-RAP1GDS1* resulting from a t(4;11)(q21;p15) is a recurrent but infrequent translocation in T-ALL (<5%) and was the first fusion gene involving *NUP98* in lymphoblastic leukemia [104]. The rare *ETV6-JAK2* fusion was originally identified in pediatric T-ALL [105] and has been shown to induce T cell leukemia in a transgenic mouse model [106]. Very recently, a second gene fusion involving *JAK2* and *PCM1* has been detected in an adult patient with T-cell lymphoma [107].

3.2.3. Other aberrations involving tumor suppressor genes or receptor proteins

A common genetic defect in 40 to 60 % of childhood and adult T-ALL is the inactivation of the tumor suppressor genes *CDKN2A* and to a lesser extent *CDKN2B* located at chromosome 9p21 by mono- or biallelic deletion, promoter hypermethylation or by mutation [108, 109]. The *CDKN2A* gene encodes the tumor suppressor proteins p14INK4 and p16INK4 whereas *CDKN2B* encodes p15INK4, which inhibit the activity of cyclin-CDK complexes involved in cell cycle progression. Inactivation of *CDKN2A* and *CDKN2B* by homozygous deletion has been described in 65% and 23% whereas hemizygous deletions are less frequent (10% and 15%). Ferrando et al. found 9p21 deletions in most T-ALL cases also expressing *HOX11* or *TAL1* [43]. Several reports have showed an association of homozygous 9p deletions and adverse prognosis both in pediatric B- and T-ALL [110] which could not be confirmed in recent studies in pediatric B-ALL cases [111, 112]. Disagreement exists about the correlation of 9p21 deletions with prognosis in adult ALL [113].

Recent findings point to a central role for aberrant *NOTCH1* signaling in the pathogenesis of T-ALL. Activating *NOTCH1* mutations were found in 50% of primary T-ALL samples [114], including most molecular subtypes of T-ALL (*HOX11, HOX11L2, TAL1, CALM-AF10*) suggesting that these defects predominantly affect immature T cells or uncommitted pluripotent progenitor cells. *NOTCH1* has been reported as an important regulator of stem cell maintenance [53], which implicates that these mutations could provide the leukemic cells with self-renewal capacity. Enforced *NOTCH1* signaling is a potent inducer of T-ALL in the mouse [115, 116]. Most interestingly the identification of *NOTCH1* mutations has therapeutic implications, as most mutant forms of *NOTCH1* still require gamma-secretase activity to generate downstream signals. Gamma-secretase inhibitors have been developed for treatment of Alzheimer's disease so these drugs could provide a rational, molecular targeted therapy in T-ALL patients carrying these *NOTCH1* mutations [117].

The *FLT3* gene encodes a receptor tyrosine kinase playing an important role in the development of HSC. Activating mutations of *FLT3* are the most common genetic abnormality in AML but are quite rare in T-ALL and seem to be restricted to CD117/Kit+ T-ALL lymphoblasts with high expression of *LYL1* and *LMO2* [118]. The finding of *FLT3* activating mutations was confirmed in another study but failed to show any link with CD117+ T-ALLs [119]. Similar to AML, the mutations in T-ALL are internal tandem duplications (ITD) in the juxtamembrane domain of the receptor and point mutations in the activation loop of the kinase domain, leading to constitutive kinase activity, suggesting *FLT3* inhibitors as a valuable therapeutic option in this subgroup of patients. However, one report [119] claims that the *FLT3* mutated subclone was eradicated by classical chemotherapy, destroying the need for additional therapeutic agents in T-ALL patients carrying *FLT3* activating mutations.

4. Roles of *HOX* and *HOX* cofactors in normal and malignant hematopoiesis

In the last decade, it has become clear that homeobox containing genes not only play a significant role in embryogenesis (especially determining the developmental fate of a cell) but also contribute to organization and regulation of hematopoiesis. A number of studies have indicated that homeobox gene expression can be detected in specific cell lineages and differentiation stages during hematopoiesis [120-124]. Furthermore, homeobox genes have been involved in translocation events in certain leukemic cells, suggesting that mutant forms of these genes may be important in oncogenesis. The term homeobox gene arose from an earlier genetic term, the so-called homeotic mutation, a mutation in which the identity of one body segment is converted to that of another. Such mutations were first described in *Drosophila* [125] and are exemplified by a mutation in which flies have legs in stead of their antennae. The cloning of the gene, called *Antennapedia*, represented the identification of the first homeobox gene. These genes contain a highly conserved 183 bp homeobox encoding a DNA binding motif, the homeodomain. Homeodomain proteins are transcription factors which play a role in specifying relative positions and tissue fate in the embryo, particularly along various body axes, including the anterior-posterior axis and the axes of the developing limb bud. The three dimensional structure of the homeobox is a helix-turn-helix motif, consisting of four helices. Mammalian homeobox genes are categorized in two classes.

The class I homeobox genes are highly homologous (>80%) to the first discovered homeobox gene of *Drosophila*, *Antennapedia* and consists of 39 genes, organized in four clusters (*HOXA, HOXB, HOXC, HOXD*) as the result of an ancestral quadruplication of a single gene cluster. These four clusters each contain 8-11 genes and are localized on different chromosomes (*HOXA* on 7p15, *HOXB* on 17q21, *HOXC* on 12q13 and *HOXD* on 2q31) (Fig.6). Based on the strong vertical conservation within the homeobox domains, these four clusters can further be divided in 13 paralogs (Fig.6). The order of genes within each cluster is also highly conserved throughout evolution, suggesting that the physical organization of *HOX* genes is essential for their expression. In human hematopoietic cells, it was demonstrated that *HOX* genes are coordinately switched on or off in blocks following a wave of gene expression starting at the 3' end of the *HOX* locus and proceeding to the 5' end along with maturation of the cells [126].

Class II homeobox genes (non-*HOX* genes) are dispersed throughout the genome, show < 50% homology to *Antennapedia* and some have been shown to act as cofactors for *HOX* proteins. These *HOX* cofactors which enhance DNA-binding specificity and avidity include the three-amino-acid-loop-extension (*TALE)* proteins such as *PBX1* (pre-B-cell leukemia transcription factor 1)*, MEIS1* (myeloid ecotropic viral integration site 1 homolog), and *PREP1* (PBX-regulating protein 1) and are co-synthesized during embryonic development. Products of the 3' *HOX* genes (paralog groups 1-8) preferentially associate with *PBX1*, whereas products of the 5' *HOX* genes (paralog groups 11-13) associate with *MEIS1* [127, 128]. Other non-HOX genes like *HOX11* are more restricted in their patterns of expression and are involved in organogenesis or differentiation of specific cell types.
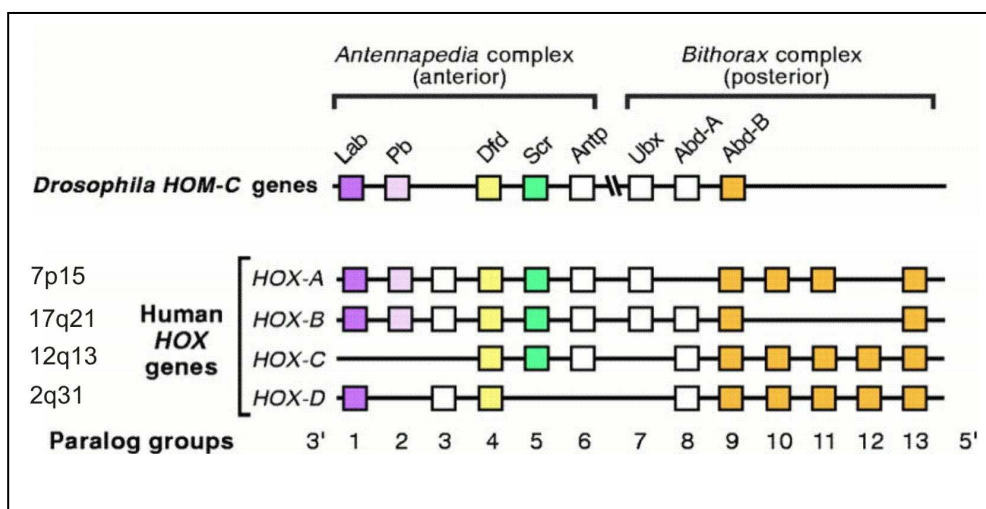
Fig.6: *Drosophila HOX* genes and their human homologs. *Drosophila HOM-C* genes at the 3' end comprise the *Antennapedia* complex (Lab, Pb, Dfd, Scr and Antp) that controle the formation of anterior structures. Posterior segments are controlled by genes of the *Bithorax* complex (Ubx, Abd-A, Abd-B). Human counterparts of drosophila *HOM-C* genes are designated *HOX-A* through *HOX-D* and are arranged on four different chromosomes. Genes within the *HOM-C* and *HOX* clusters show striking structural and functional conservation as indicated by the color coding. This vertical conservation lead to the division in 13 paralog groups for each *HOX* cluster.

Individual *HOX* genes and *HOX* cofactors have been implicated in normal and malignant hematopoiesis. First evidence of *HOX* gene expression in unfractionated normal human bone marrow cells came from RNase protection assays that demonstrated expression of *HOXB2*, *HOXB6* and *HOXA10* [129]. Furthermore, RT-PCR analysis on purified subpopulations of CD34+ progenitor cells showed that the number of *HOX* genes expressed was the highest for the *HOXA* cluster, and decreased for genes of the *HOXB* and *HOXC* clusters whereas no expression of *HOXD* clusters was found [126, 130, 131]. Not only the number of *HOXA* genes, but also the expression level of these genes was higher than that of *HOXB* and *HOXC* genes in CD34+ populations. In addition, genes in the 3' region of the *HOXA* and *HOXB* clusters are downregulated as CD34+ cells progress to the stage of committed erythroid and myeloid progenitors, while 5' genes remain active into the committed progenitor stage and are inactivated as cells leave the CD34+ compartment [126]. Inversely, expression of *HOX* regulatory genes like Polycomb (PcG) genes seem to show progressive upregulation upon differentiation of CD34+ cells which possibly induces formation of condensed chromatin resulting in 3' to 5' closure of the *HOX* cluster [132]. Whereas some *HOX* genes show broad expression in cells of different cell phenotypes, several appear to have lineage-restricted patterns of expression. Expression of all *HOXB* cluster genes is mainly associated with cell lines with erythroid potential [133-135] except for *HOXB4* and *HOXB7* which are also expressed in T and B cell lines, and for *HOXB1* which is not expressed in the hematopoietic department. *HOXC4* expression is limited to lymphoid leukemias [122] while *HOXA10* is strongly expressed in myeloid leukemias [129] and until the results presented in this thesis, unnoticed in lymphoid leukemia.

Early evidence for involvement of *HOX* genes in human leukemia came from the identification of translocations involving the nucleoporin gene *NUP98* and *HOXA9* in rare cases of AML and subsequently a number of *NUP98* fusions with other *HOX* genes have been described [136-139]. Although translocations involving *HOX* genes are uncommon, other studies demonstrated that *HOXA* and *HOXB* genes are frequently overexpressed in human AML [140-143] and may portend a worse prognosis [140, 144]. Further evidence for *HOX* gene function in malignant hematopoiesis came from knock-out mouse models and enforced expression studies in hematopoietic stem cells from murine fetal liver and bone marrow and in human cord blood progenitors. Forced expression of certain *HOX* genes such as *HOXB3, HOXA9, HOXB8, HOXB6* and *HOXA10* lead to myeloid leukemia after several months [145-149]. This latency period can be shortened dramatically if the *HOX* cofactors like *PBX1* and *MEIS1* are co-overexpressed with their *HOX* partner. Conversely ectopic expression of *HOXB4* leads to hematopoietic stem cell expansion without induction of leukemia. Knock-out animal models have been described for the *HOXA9* gene and exhibited disturbed differentiation of myeloid and megakaryocytic cells, while early T cell development was seriously diminished [64, 150]. Similarly *HOXA3* mutant mice have a spectrum of abnormalities in the pharyngeal tissues derived from the mesenchymal neural crest. Amongst these were defects in the generation of the thymus and thyroid glands leading to defective thymocyte development. Satokata et al. [151] generated a *HOXA10* knock-out mouse which show an anterior homeotic transformation of lumbar vertebrae and had severe fertility defects in both genders. Likewise, targeted disruption of *HOXA11* was shown to result in both male and female sterility [152] and the mutant males show a partial homeotic transformation of the vas deferens to an epididymis. Besides these defects, disruption of *HOXA10* or *HOXA11* established limb malformations to varying degrees [153]. The hematopoietic consequences of *HOXA10* and *HOXA11* knock-out were apparently not lethal but in fact have not been studied in these mouse models yet. The only *HOXA* gene mutation associated with a non-neoplastic hematological disorder thus far, is the germline *HOXA11* mutation described in the amegakaryocytic thrombocytopenia with radio-ulnar synostosis [154]. Thus *HOX* and *HOX* cofactors are critical components for normal hematopoiesis and their cis-deregulation may be the underlying mechanisms for different hematological disorders.

Several mechanisms have been identified that regulate expression of *HOX* genes. The identification of TAAT sequences [155, 156] and retinoic acid response elements in upstream regions allows the binding and activation or repression by homeoproteins (autoregulation) and activation of expression by retinoic acid [157, 158]. Also the maintenance of *HOX* gene expression is guided by the Polycomb [159-160] (PcG) and Trithorax genes (Trx) where PcG genes repress *HOX* gene transcription while Trx genes contribute to strong expression [161]. Following their discovery in *Drosophila*, PcG genes have also been identified in humans where they appear to have a similar role, while the *MLL* (mixed lineage leukemia) gene and its homologs ares the only known human Trx homologs [162, 163]. Recently, a miRNA-mediated mechanism for the posttranscriptional restriction of *HOXB8* gene expression during vertebrate development has been suggested for miR-196 [164]. miR-196 directs the

cleavage of *HOXB8* messenger RNA during mouse development, and other evidence suggests that miR-196 can regulate the translation of other *HOX* genes in the mouse *HOX* cluster.

5. Research objectives

The aim of this thesis was the identification of new partner genes in chromosomal rearrangements affecting the T cell receptor (TCR) loci in T-ALL. This work was triggered by previous work on childhood ALL from our group which resulted in the detection of a cryptic chromosomal rearrangement between the *TCRß* locus (7q34) and an unknown partner gene on chromosome 11q24 in a childhood T-ALL patient.

Study of cryptic *TCRß* rearrangements in T-ALL

In a first step we decided to screen for additional cases of t(7;11)(q34;q24) in a cohort of 94 T-ALL cases. To this purpose, FISH was used with BAC clones flanking the *TCRß* locus (7q34). This lead to the detection of a new recurrent inv(7)(p15q34) involving the *TCRß* and the *HOXA* cluster genes (7p15) (paper 1).

Study of *TCR* chromosomal rearrangements in T-ALL

Given the unexpected high incidence of *TCRß* chromosomal rearrangements in T-ALL, we decided to perform a comprehensive screening for rearrangements of all three *TCR* loci (*TCRß, TCRaδ* and *TCRγ*) in an extend cohort of 126 T-ALLs. This provided the first complete and unbiased assessment of *TCR* chromosomal rearrangements in T-ALL (paper 2). This study also lead to the identification of the first *HOXA* chromosomal rearrangement with the *TCRδ* locus in a T-ALL patient already carrying a *CALM-AF10* translocation (paper 4).

Molecular and cytogenetic profile of patients carrying a t(7;7)(p15;q34) or inv(7)(p15q34)

In order to get a comprehensive molecular and cytogenetic picture of the patients carrying the new *TCRß-HOXA* chromosomal aberration and in search for additional patients, a large series of T-ALL patients (n= 424) from Belgium and France was investigated which yielded 5 additional cases. Subsequently, all 14 cases carrying a t(7;7)(p15;q34) or inv(7)(p15q34) were analysed in detail in order to determine the clinical and biological profile of this new T-ALL subentity (paper 3).

Identification of new partner regions involved in *TCR* rearrangements in T-ALL

Using the TCR screening strategy, we could detect three new TCR partner regions located at chromosome bands 6q22, 11q24 and 20q12 (paper 2). Further investigation of these regions could possibly identify unknown genes involved in T cell oncogenesis.

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CHAPTER II:

STUDY OF GENOMIC REARRANGEMENTS OF THE T-CELL RECEPTOR LOCI IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA.

CHAPTER II : Study of genomic rearrangements of the T-cell receptor loci in T-cell acute lymphoblastic leukemia

Paper 1:   47

> **A new recurrent inversion, inv(7)(p15q34) leads to transcriptional activation of *HOXA10* and *HOXA11* in a subset of T-cell acute lymphoblastic leukemias.**
>
> F. Speleman*, B. Cauwelier*, N. Dastugue, J. Cools, B. Verhasselt, B. Poppe, N. Van Roy, J. Vandesompele, C. Graux, A. Uyttebroeck, M. Boogaerts, B. De Moerloose, Y. Benoit, D. Selleslag, J. Billiet, A. Robert, F. Huguet, P. Vandenberghe, A. De Paepe, P. Marynen, A. Hagemeijer. * Both authors contributed equally.
>
> Leukemia, 2005, Mar 19(3):358-366.

Paper 2:   57

> **Cytogenetic study of 126 unselected T-ALL cases and 19 T-ALL cell lines reveals unexpected high incidence of cryptic rearrangements of the *TCRβ* locus with at least 2 new recurrent partner genes.**
>
> B. Cauwelier, N. Dastugue, J. Cools, B. Poppe, C. Herens, A. De Paepe , A. Hagemeijer, F. Speleman.
>
> Leukemia, 2006, Jul 20(7):1238-1244.

Paper 3:   65

> **Clinical, cytogenetic and molecular characteristics of 14 T-ALL patients carrying the *TCRB-HOXA* rearrangement: a study of the Groupe Francophone de Cytogénétique Hématologique (GFCH).**
>
> B. Cauwelier, H. Cavé, C. Gervais , M. Lessard , C. Barin, C. Perot , J. Van den Akker, F. Mugneret , C. Charrin, M.P. Pagès, M.J. Grégoire, P. Jonveaux , M. Lafage-Pochitaloff, M. J. Mozzicconacci, C. Terré, I. Luquet, P. Cornillet-Lefebvre, B. Laurence, G. Plessis, C. Lefebvre, D. Leroux, H. Antoine-Poirel, C. Graux, L. Mauvieux, P. Heimann, C. Chalas, E. Clappier, B. Verhasselt, Y. Benoit, B. De Moerloose, B. Poppe, N. Van Roy, K. De Keersmaecker, J. Cools, F. Sigaux, J. Soulier, A. Hagemeijer, A. De Paepe, N. Dastugue, R. Berger, F. Speleman.
>
> Leukemia, 2006, in press.

Paper 4:   75

> ***HOXA* cluster deregulation by a *TCRδ-HOXA* chromosomal translocation in a *CALM-AF10+* T-ALL.**
>
> J. Bergeron, E. Clappier, B. Cauwelier, N. Dastugue, C. Millien, E. Delabesse, K. Beldjord, F. Speleman, J. Soulier, E. Macintyre, V. Asnafi.
>
> Leukemia, 2006, Jun 20(6):1184-1187.

CHAPTER II:

Paper 1:

A new recurrent inversion, inv(7)(p15q34) leads to transcriptional activation of *HOXA10* and *HOXA11* in a subset of T-cell acute lymphoblastic leukemias.

F. Speleman*, B. Cauwelier*, N. Dastugue, J. Cools, B. Verhasselt, B. Poppe, N. Van Roy, J. Vandesompele, C. Graux, A. Uyttebroeck, M. Boogaerts, B. De Moerloose, Y. Benoit, D. Selleslag, J. Billiet, A. Robert, F. Huguet, P. Vandenberghe, A. De Paepe, P. Marynen, A. Hagemeijer. * Both authors contributed equally.

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A new recurrent inversion, inv(7)(p15q34), leads to transcriptional activation of *HOXA10* and *HOXA11* in a subset of T-cell acute lymphoblastic leukemias

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Chromosomal translocations with breakpoints in T-cell receptor (*TCR*) genes are recurrent in T-cell malignancies. These translocations involve the *TCRαδ* gene (14q11), the *TCRβ* gene (7q34) and to a lesser extent the *TCRγ* gene at chromosomal band 7p14 and juxtapose T-cell oncogenes next to *TCR* regulatory sequences leading to deregulated expression of those oncogenes. Here, we describe a new recurrent chromosomal inversion of chromosome 7, inv(7)(p15q34), in a subset of patients with T-cell acute lymphoblastic leukemia characterized by CD2 negative and CD4 positive, CD8 negative blasts. This rearrangement juxtaposes the distal part of the *HOXA* gene cluster on 7p15 to the *TCRβ* locus on 7q34. Real time quantitative PCR analysis for all *HOXA* genes revealed high levels of *HOXA10* and *HOXA11* expression in all inv(7) positive cases. This is the first report of a recurrent chromosome rearrangement targeting the *HOXA* gene cluster in T-cell malignancies resulting in deregulated *HOXA* gene expression (particularly *HOXA10* and *HOXA11*) and is in keeping with a previous report suggesting *HOXA* deregulation in *MLL*-rearranged T- and B cell lymphoblastic leukemia as the key factor in leukaemic transformation. Finally, our observation also supports the previous suggested role of *HOXA10* and *HOXA11* in normal thymocyte development.
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Introduction

T-cell acute lymphoblastic leukemia (T-ALL) represents 10–15% of childhood and 25% of adult ALLs, and are associated with an intermediate prognosis within the total group of ALLs.[1] In T-ALL, chromosomal rearrangements affecting the T-cell receptor loci *(TCR)* were among the first to be detected. Similar to translocations involving the *IGH* locus, these chromosomal changes cause ectopic expression of target genes due to juxtaposition to *TCR* gene enhancers. These pivotal studies showed that T-ALLs were genetically very heterogeneous with the most frequently detected translocation, the t(11;14)(p15;q11), being found in less than 10% of cases.[2] One particular rare translocation, t(1;14)(p32;q11), lead to the discovery of the *TAL1(SCL)* gene that turned out to be of crucial importance in normal T-cell development. Most interestingly, *TAL1(SCL)* was later shown to be transcriptionally activated due to a cryptic 90 kb interstitial deletion in as much as 20% of T-ALLs, thus representing the most frequent genetic abnormality in this disease.[3] Similarly, other T-cell leukemia specific genes (*HOX11, LYL1, LMO1, LMO2, HOX11L2*), initially identified through rare chromosomal rearrangements involving *TCR* loci, were also shown to be transcriptionally activated in T-ALLs without evidence for particular translocations. Also, recent studies showed biallelic overexpression of some of these genes thus further supporting the notion for a broader implication in T-ALL through mechanisms other than the known translocation events.[4] Furthermore, the unexpected finding of amplification of a *NUP214/ABL1* fusion gene in a minority of T-ALL patients,[5,6] further illustrated the diversity of genetic events involved in T-ALLs. Likewise, the *NOTCH1* gene, involved in the t(7;9)(q34;q34) in less than 1% of T-ALLs, was shown to harbor activating mutations in more than 50% of T-ALLs.[7]

Although involvement of each of the three TCR loci in recurrent chromosomal changes in T-ALL is well recognized,[8] both the incidence and the exact contribution of each of these loci in recurrent chromosome abnormalities have not yet been assessed in detail. The finding of a previously unreported t(7;11)(q34;q24) in a child with T-ALL, as the result of a multicolor fluorescence *in situ* hybridization (M-FISH) study on T-ALLs, prompted us to screen a large cohort of 94 patients with T-ALL. This resulted in the finding of a new recurrent chromosomal rearrangement, that is, a pericentric inversion of chromosome 7, inv(7)(p15q34), in a subset of T-ALL patients. This inversion was associated with an elevated expression of *HOXA10* and *HOXA11*, most probably due to juxtaposition near strong enhancers embedded within the *TCRβ* locus. This is the second report providing evidence for involvement of class I *HOXA* genes in the development of T-ALL.

Materials and methods

Patients and thymocytes

Diagnostic bone marrow, peripheral blood or pleural fluid samples from 49 adults and 45 children with T-ALL were collected from three institutes, retrospectively. For 32 T-ALL cases (16 children and 16 adults) frozen cells were available for expression analysis. These selected cases contained all cases positive for *TCRβ* chromosomal rearrangement and 13 cases

Table 1 Biological characteristics and cytogenetic findings of TCRβ positive (case n° 1–19; with identified partner gene) and negative T-ALL patients (case n° 20–32)

Case no.	Age	Sex	Karyotype	TCRβ partner gene	Revised karyotype
1	4	M	46,XY, t(1;14)(p32;q11) [20]	Unknown	46,XY, t(1;14)(p32;q11), t(6;10)(q25;q24), t(7;11)(q34;q24)[a]
2	19	M	46, XY, t(1;7)(p32;q34), del(6)(q12q16) [14]/46, XY [1]	TAL1 (1p32)	46, XY, t(1;7)(p32;q34), del(6)(q12q16) [14]/46, XY [1]
3	45	M	46,XY [9]	Unknown	46,XY, t(7;?)(q34;?) [9]
4	6	M	47,XY,+8,del(9)(p21) [3]/46,XY [8]	Unknown	47,XY, t(7;?)(q34;?),+8, del(9)(p21) [3] /46,XY [8]
5	12	F	46, XX,del(6)(q23q26),add(7)(q31),del(11)(q13), −14,+mar mar = ?der(1) i(1)(q10)add(1)(q32) [18]/92, idem x 2 [2]	Unknown	46, XX,del(6)(q23q26), t(7;?)(q34;?),del(11)(q13),−14,+mar mar = ?der(1) i(1)(q10)add(1)(q32) [18]/92, idem x 2 [2]
6	6	M	46,XY,del(7)(q21),?inv(14)(q11q32) [14]/46,XY [6]	Unknown	46,XY,t(7;?)(q34;?),?inv(14)(q11q32) [14]/46,XY [6]
7	15	F	46,XX [20]	RBTN2 (11p13)	46,XX, t(7;11)(q34;p13) [20]
8	10	M	46,XY [20]	Unknown	46,XY, t(7;?)(q34;?) [20]
9	9	F	46,XX,del(7)(q34),t(10;14)(q22;q32) [11]	RBTN1 (11p15)	46,XX,t(7;11)(q34;p15),t(10;14)(q22;q32) [11]
10	9	M	46,XY,t(8;14)(q24;q11) [8]/46,XY [9]	RBTN1 (11p15)	46,XY,t(8;14)(q24;q11), t(7;11)(q34;p15) [8]/46,XY [9]
11	26	M	46,XY[20]	Unknown	46,XY, t(7;?)(q34;?) [20]
12	15	M	47,XY,t(7;9)(q34;q34),+20 [2]/47, idem, add(11)(q24) [9]	TAN1 (9q34)	47,XY,t(7;9)(q34;q34),+20 [2]/47, idem, add(11)(q24) [9]
13	30	M	46–47,XY,+mar1, +mar2, [cp5]	Unknown	46–47,XY, t(7;?)(q34;?), +mar1, [cp5]
14	23	M	42–48,XY,t(3;11)(p12;p15),t(7;10)(q35;q24), t(8;10)(q21;q?24), +11, +12 [5]/46,XY [4]	HOX11 (10q24)	42–48,XY,t(3;11)(p12;p15),t(7;10)(q35;q24), t(8;10)(q21;q?24), +11, +12 [5]/46,XY [4]
15	35	F	46,XX [4] failure	HOXA (7p15)	46,XX, inv(7)(p15q34) [4]
16	27	F	46, XX, add(5)(q31) [11]/ 46, XX [9]	HOXA (7p15)	46, XX, add(5)(q31), inv(7)(p15q34) [11]/ 46, XX [9]
17	15	M	46,XY [20]	HOXA (7p15)	46,XY, inv(7)(p15q34) [20]
18	34	M	46,XY [20]	HOXA (7p15)	46,XY, inv(7)(p15q34) [20]
19	49	M	47,XY,del(6)(q14),del(7)(p?),del(9)(p21),+mar1 [4]/46,XY [6]	HOXA (7p15)	47,XY,del(6)(q14),inv(7)(p15q34),del(9)(p21), +mar1 [4]/46,XY [6]
20	35	M	46, XY, t(9;20)(p21;q12), t(10;14)(q24;q11), del(12)(p12) [13]/46, XY [12]	Negative	
21	26	M	46,XY,t(6;11)(q21–22;p15),add(12)(p13) [17]/46,XY [3]	Negative	
22	35	M	46, XY [20]	Negative	
23	27	M	47, XY, t(7;14)(p12;q24), del(9)(q21q31), +10 [16]/46, XY [4]	Negative	
24	35	M	46, XY [25]	Negative	
25	24	M	46, XY [25]	Negative	
26	45	M	47, XY, +8 [2]/ 46, XY[20]	Negative	
27	5	M	47,XY,del(6)(q12),+8, del(10)(q24)[5]/ 46,XY [18]	Negative	47,XY,del(6)(q12),+8, del(10)(q24), t(5;14)(q35;q32)* [5]/ 46,XY [18]
28	9	M	46,XY [20]	Negative	
29	9	M	46,XY [20]	Negative	
30	3	M	46,XY,add(19)(p13.3),del(6)(q15q24) [6]/ 46, XY [13]	Negative	
31	15	M	46,XY,add(14)(q32) [9]/46,XY [18]	Negative	
32	16	F	46,XX,del(1)(q32),del(5)(q22q34),−7,+mar [5]/46,XX [7]	Negative	

[a]Including M-FISH analysis.

without TCRβ rearrangement, which were investigated by FISH (see below). Table 1 lists biological and cytogenetic findings for these patients. Total child thymocyte suspension (CT) and purified CD34^{+} thymus cells (CD34 + T) were isolated as described before.[9]

Immunophenotyping

Immunophenotypic studies were carried out in the respective centers according to established protocols. Cases were classified using the European Group for the Immunological Characterization of Leukemias (EGIL) recommendations.[10] Briefly, EGIL T1 (pro-T ALL) was defined by the presence of only CD7, T2 (pre-T

ALL) by CD2 and/or CD5 and/or CD8 positivity, T3 (cortical T-ALL) by CD1a positivity and T4 (mature T-ALL) by the presence of surface CD3 and lack of CD1a.

Cytogenetic studies

The different diagnostic specimens (bone marrow, blood and pleural fluid) were cultured and harvested for cytogenetic analysis according to established methods. Chromosome slides were G-, Q- or R-banded. Chromosome aberrations are described according to guidelines of an International System for Human Cytogenetic Nomenclature (ISCN 1995).[11]

Fluorescence in situ *hybridization (FISH)*

Cytogenetic cell suspensions (methanol/acetic acid fixed cells) or unstained slides were available for all 94 patients. For FISH the following *TCRβ* (7q34) and *HOXA* (7p15) flanking BAC clones were selected: RP11-1220K2 (located centromeric to *TCRβ*) and RP11-556I13 (located telomeric to *TCRβ*); RP1-167F23 (containing telomeric *HOXA* genes *HOXA1, HOXA2* and part of *HOXA3*) and RP5-1103I5 (located centromeric to *HOXA*); RP11-1036C18, RP11-163M21, RP11-1132K14 and RP11-1025G19 clones represent a contig spanning the entire *HOXA* gene cluster (Figure 2). For further delineation of the *TCRβ* breakpoint, following gene covering clones were selected: RP11-785K24 (centromeric) and RP11-701D14 (telomeric) (Figure 3).

RPCI-11 (Human BAC Library) clones were provided by the Welcome Trust Sanger Institute (Cambridge, UK) and Invitrogen (Paisley, Scotland). Clone DNA isolation, labeling and FISH were performed as previously described,[12] using biotin-16-dUTP and digoxigenin-11-dUTP (Roche Diagnostics Belgium, Vilvoorde, Belgium) as haptens.

Disruption of the *TCRβ* locus was assessed by dual color FISH with *TCRβ* flanking probes (RP11-1220K2 and RP11-556I13). Cases carrying *TCRβ* chromosomal rearrangement (identified by a split signal of the flanking probes) were further analyzed to confirm the presumed *TCRβ* partner genes using flanking probes for the respective partner loci (*RBTN1* on 11p15, *RBTN2* on 11p13, *HOX11* on 10q24, *TAN1* on 9q34, *TAL1(SCL)* on 1p32). For those cases, which showed inversion of the distal *TCRβ* flanking probe to 7p, dual color FISH using *HOXA* flanking probes was performed.

Hybridization signals were evaluated by two independent observers and interpreted at the interphase (100 nuclei) and metaphase level (when available).

RNA isolation, cDNA synthesis and quantitative real-time RT-PCR

Patients were selected retrospectively on the basis of the availability of frozen material at diagnosis. All human samples were obtained according to the guidelines of the local ethical committees. These included 13 *TCRβ* positive cases including five inv(7) positive and 13 *TCRβ* negative cases. Cells obtained from total child thymus (CT), and purified CD34$^+$ thymocytes (CD34 + T) were used as a reference for *HOX* gene expression in normal developing T-cells.[13] RNA was isolated using Trizol (Invitrogen, Merelbeke, Belgium) and RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNase treatment, cDNA synthesis, primer design and SYBR Green I quantitative real-time RT-PCR were performed as previously described.[14] Reactions were performed on an ABI Prism 5700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR data analysis and expression normalization were performed using three internal control genes (*RPL13A, UBC, YWHAZ*), as described[15] and correlated to the mean expression level of each gene. Primers for *HOXA1, HOXA4, HOXA5, HOXA7, HOXA11* were designed according to Thompson *et al.*[16] Primers for the other *HOXA* genes (*HOXA2, HOXA3, HOXA6, HOXA9, HOXA10, HOXA13*) and for *HOXB3* and *HOXC4* expression analysis were designed and developed according to Wang *et al*[17] at the Primer Bank. Primer sequences for the tested genes are deposited in RTPrimerDB, a public database for real-time PCR primers (http://medgen.ugent.be/rtprimerdb) (RTPrimerDB).[18]

Data analysis

Statistical analysis was performed using SPSS Software (SPSS Inc., Chicago, IL, USA) version 12.0. The nonparametric Mann–Whitney *U* test (two-tailed) was used to evaluate the significance of difference in mean expression levels between the patients subgroups (inv(7) positive *vs* inv(7) negative patients). The Fisher exact test was used to evaluate the relationship between the specific immunophenotype and the presence of the inv(7).

Results

Frequency of chromosomal rearrangements affecting the TCRβ locus in T-ALL

A previously conducted M-FISH analysis in one T-ALL patient (case no. 1) with a known t(1;14)(p32;q11) and *TAL1(SCL)* overexpression, uncovered a cryptic translocation t(7;11)(q34;q24) affecting the *TCRβ* locus. No known T-cell oncogene is located on distal 11q and breakpoint analysis is currently ongoing. FISH screening for *TCRβ* chromosomal rearrangements in a large series of 93 T-ALLs yielded split signals for the *TCRβ* locus in 18 cases, which brings the total of detected *TCRβ* alterations to 20% (19/94). In six of these 19 cases, the distal probe for *TCRβ* was translocated to recurrent *TCRβ* partner genes: *TAL1* (case no. 2), *RBTN2* (case no. 7), *RBTN1* (cases no. 9–10), *TAN1* (case no. 12), *HOX11* (case no. 14) as confirmed by FISH with the appropriate probes (Table 1). In eight of the 19 cases showing *TCRβ* rearrangement, involvement of known partner genes was excluded. Interestingly, in five other cases with split signals for the *TCRβ* flanking probes, the telomeric BAC clone for *TCRβ* moved to the distal end of the short arm of chromosome 7, thus revealing the presence of a pericentric inversion with an unknown partner gene (Figure 1a).

Biological characteristics, immunophenotyping and cytogenetics

Biological characteristics for all *TCRβ* positive cases (cases no. 1–19) and selected *TCRβ* negative T-ALLs (case no. 20–32) are summarized in Table 1. *TCRβ* positive cases with inv(7), aged 15–49 years (median 32 years), showed an M/F ratio: 1.5:1.0. *TCRβ* positive cases without inv(7) showed a lower age of onset compared to inv(7) positive cases: median 16.6 years (range 4–45 years) and a pronounced male predominance (M/F ratio 3.6:1.0). Compared to inv(7) positive cases, *TCRβ* negative cases also showed a lower age of onset with a median of 21.8 years (range 3–45 years) and a striking male predominance (M/F ratio 12:1).

Immunophenotypic findings from all but two cases are listed in Table 2. Inv(7) positive cases were classified as either T3 (*n* = 1) or T4 (*n* = 4) T-ALL immunophenotypes but with a distinct pattern of CD2 and CD8 negativity and CD1a, CD4, CD5, CD7 and CD10 positivity. Four inv(7) positive cases were analyzed for CD13 and CD33 expression and were found to be negative.

Cytogenetic analysis showed abnormal karyotypes in 11 out of 19 *TCRβ* positive cases (Table 1). Translocations affecting the 7q34 locus were detected upon banding analysis in three of 19 cases and partial deletions of chromosome 7q or 7p in three cases; one case showed additional material on 7q. Altogether, *TCRβ* genomic rearrangement was unsuspected from
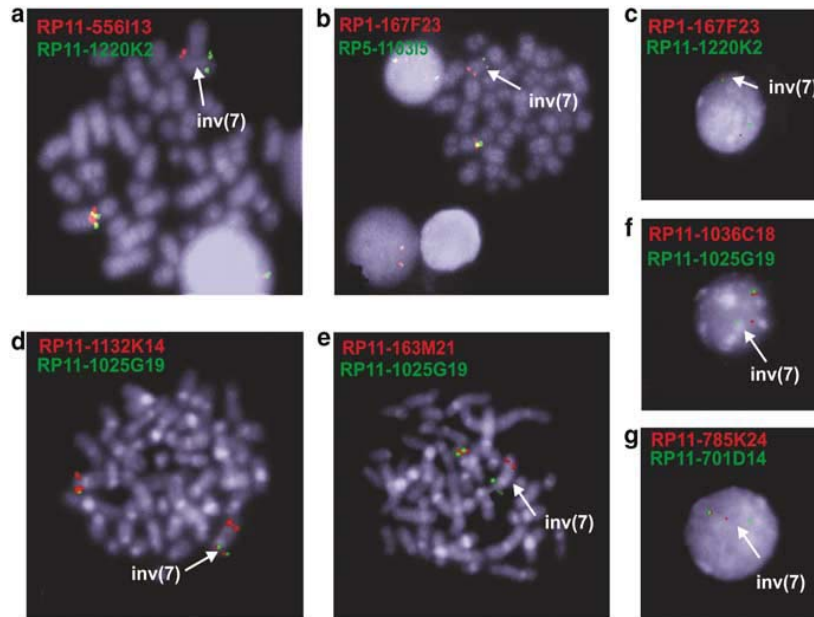
Figure 1 Dual colour FISH results using (a) *TCRβ* flanking probes RP11-1220K2 (green) and RP11-556I13 (red) demonstrating disruption of the *TCRβ* locus due to the inv(7) (case no. 18); (b) *HOXA* flanking centromeric probe RP5-1103I5 (green) with the telomeric and partly covering probe RP1-167F23 (red) demonstrating disruption of the *HOXA* gene cluster due to the inv(7) (case no. 18); (c) centromeric flanking *TCRβ* probe RP11-1220K2 (green) with the telomeric *HOXA* flanking probe RP1-167F23 (red), which yielded the expected fusion signals on the inverted chromosome 7 in interphase nuclei (case no. 17); (d) *HOXA* covering probes RP11-1132K14 (red) and RP11-1025G19 (green) revealing two fusion signals and an extra red signal (case no. 16); (e) *HOXA* covering probes RP11-163M21(red) and RP11-1025G19 (green) yielding one fusion and one split signal (case no. 16); (f) *HOXA* covering probes RP11-1036C19(red) and RP11-1025G16(green) yielding one fusion and one split signal(case no. 16); (g) *TCRβ* covering clones RP11-785K24 (red) and RP11-701D14 (green) gave the expected split signals in inv(7) (case no. 17).

Table 2 Immunophenotype of 30 T-ALL patients

Case no.	1	2	4	5	6	7	8	9	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
CD34	60	2	0	0	1	0	0	54	nd	0	0	56	3	0	0	0	nd	0	0	0	88	1	0	0	3	0	3	31	0	82
cyCD3	90	99	99	nd	99	49	82	100	nd	95	nd	92	98	94	86	nd	nd	24	91	98	90	59	99	98	76	99	nd	89	88	37
CD2	86	99	100	73	99	96	50	91	0	100	100	79	2	6	0	26w	9	96	99	17	1	97	99	99	75	0	nd	92	99	13
CD7	86	99	99	70	99	95	99	87	99	97	97	95	96	73	98	99	72	96	99	97	98	94	99	99	88	99	nd	92	98	92
CD5	88	99	99	76	15	96	97	92	96	98	99	96	96	74	91	98	94	95	99	87	86	96	99	99	43	80	nd	92	98	72
CD1a	38	24	98	56	88	0	0	5	50w	98	97	40	75	41	43	98	5	94	0	0	0	2	21	99	14w	60	nd	29	0	0
CD3m	80	95	99	98	95	23	98	96	59	1	2	1	3	73	85w	98	73	3	0	97	0	21	98	0	50w	83	3	60	96	0
CD4	22	56	99	13	1	36	95	3	50w	0	98	20	93	58	43	75	48	95	0	1	0	92	80	100	54	76	0	0	69	0
CD8	12	96	99	76	96	74	96	5	84	99	34	91	1	15	0	1	3	50	0	1	0	95	90	100	15	15	95	60	86	0
CD10	0	2	99	74	99	16	0	0	97	96	1	94	95	69	73	70	92	50	99	0	11	9	20	99	0	37	98	73	34	77
TCRgd	0	0	0	2	1	0	0	0	57w	0	nd	nd	2	56	0	nd	1	0	0	87	0	1	0	0	0	45	nd	2	0	0
TCRab	0	93	49	3	1	0	0	99	0	0	nd	nd	0	5	0	nd	6	3	0	3	0	20	97	99	0	0	nd	8	0	0
EGIL	T4	T4	T4	T4	T4	T4	T4	T4	T4	T3	T3	T3	T3	T4	T4	T4	T4	T3	T2	T4	T2	T4	T4	T3	T4	T4	?	T4	T4	T2
TCRβ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−

Data represent % of blast cells positive for the respective antigen (CD); nd: not done; w: weak.
TCRβ genomic rearrangement: +: present; −: absent.
Case numbers correspond to those in table n°1.
Antigens are arranged in order in which they appear during T-cell differentiation.

cytogenetic analysis in 16/19 cases, partly due to inferior quality of metaphase chromosomes in T-ALL. Details on cytogenetic findings for all 94 cases including additional FISH analysis for the other *TCR* loci will be reported elsewhere (Cauwelier *et al*, in preparation).

Inv(7)(p15q34) is a new recurrent rearrangement in T-ALL

Since the *HOXA* gene cluster is known to be involved in normal human T-cell development,[13] this locus was considered as candidate partner gene for rearrangement with the *TCRβ* locus.

Therefore, dual color hybridization was performed on the inv(7) positive cases using probes flanking the *HOXA* gene cluster located at 7p15 (RP1-167F23 telomeric and RP5-1103I5 centromeric clones). In all five inv(7) positive cases, a split for the *HOXA* flanking probes was observed, with the telomeric clone being inverted to distal 7q (Figure 1b). To confirm these results, we combined the centromeric flanking *TCRβ* probe RP11-1220K2 with the telomeric *HOXA* flanking probe RP1-167F23, which yielded the expected fusion signals on the inverted chromosome 7 in metaphases as well as in interphase nuclei (Figure 1c).
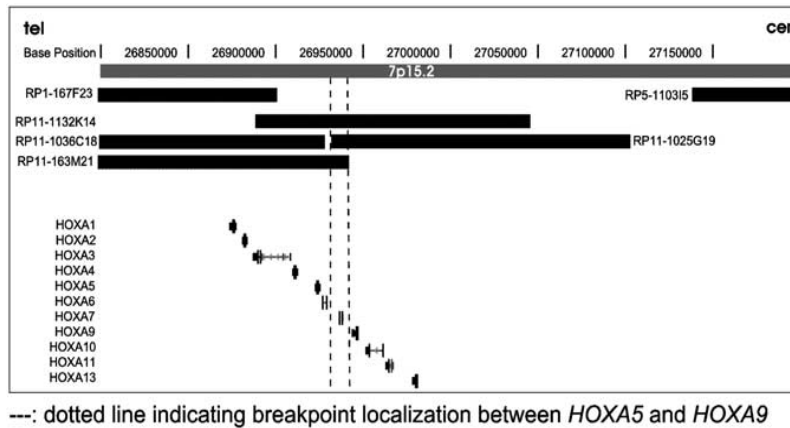
---: dotted line indicating breakpoint localization between *HOXA5* and *HOXA9*

Figure 2 Base position of *HOXA* flanking (RP11-167F23 telomeric and RP11-1103I5 centromeric) and *HOXA* covering clones (RP11-1132K14, RP11-1036C18, RP11-163M21 and RP11-1025G19), according to UCSC Genome Browser May 2004 Freeze.
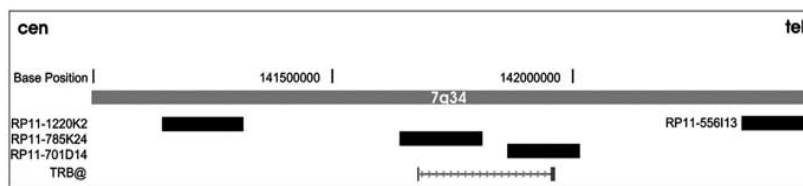

Figure 3 Base position of *TCRβ* flanking (RP11-1220K2 centromeric and RP11-556I13 telomeric) and covering clones (RP11-785K24, RP11-701D14), according to UCSC Genome Browser May 2004 Freeze.

Further characterization of the chromosome 7p breakpoint using three different probe combinations covering the *HOXA* locus (Figure 2) yielded similar hybridization patterns in all five inv(7) positive cases. For each of the three probe combinations (centromeric clone RP11-1025G19 combined with more telomeric located clones RP11-1036C18, RP11-163M21 or RP11-1132K14, respectively) split signals of the combined probes and complete (RP11-1036C18, RP11-163M21) or partial (RP11-1132K14) inversion of the more telomeric located probes were observed (Figure 1d–f). Consequently, these experiments further confirmed the localization of the inversion 7p15 breakpoint within the *HOXA* gene cluster and more specifically allowed us to assign the breakpoint to the region covered by BAC clone RP11-1132K14. Given the relative position of the four BAC clones used in these experiments and absence of disruption of RP11-1036C18, RP11-163M21 or RP11-1025G19, we assume that the most probable position of the 7p15 breakpoint is within a ∼20 kb segment located between *HOXA5* and *HOXA9* (Figure 2).

Two clones spanning most of the *TCRβ* locus (RP11-785K24 centromeric and RP11-701D14 telomeric) revealed split signals in all inv(7) positive cases (Figure 1g), and thus confirmed the involvement of the *TCRβ* gene in the inv(7) positive cases (Figure 3). Moreover, in two inv(7) positive cases a partial deletion of the centromeric *TCRβ* probe in 95% (case no. 15) and 96% of cells (case no. 18) carrying the inversion was observed.

Real time quantitation of gene expression for HOXA, HOXB3 *and* HOXC4 *genes*

Expression level measurements of all eleven members of the *HOXA* gene cluster (*HOXA1, HOXA2, HOXA3, HOXA4, HOXA5, HOXA6, HOXA7, HOXA9, HOXA10, HOXA11 and*

HOXA13) were performed on a total of 26 patient samples, total CT and CD34 + purified child thymocytes (CD34 + T). Patient samples included five inv(7) positive cases (group A), eight *TCRβ* positive without inv(7) (group B) and 13 *TCRβ* negative selected cases (group C). As no significant differences in the observed expression pattern was noted for group B as compared to group C (Student's *t*-test: *P* > 0.05), both patient groups were joined and inv(7) positive cases were compared to inv (7) negative cases. A high *HOXA10* and *HOXA11* expression was noted for all inv(7) positive cases whereas two inv(7) negative T-ALLs showed similar increased levels of *HOXA10* and *HOXA11* transcripts (cases no. 21 and 22) (Figure 4A, B). Levels of *HOXA10* and *HOXA11* expression in total CT and CD34 + purified child thymocytes were comparable but at least 10-fold lower than the mean expression of these genes in inv(7) positive T-ALL samples. *HOXA11* expression levels were statistically significant increased in the inv(7) positive cases *vs* inv(7) negative cases (*P* = 4.5 × 10E-4 and *P* = 8.8 × 10E-4, respectively), the latter showing only marginal expression for *HOXA10* and *HOXA11*. *HOXA9* expression was slightly increased in inv(7) positive patients as compared to the inv(7) negative samples (*P* = 1.8 × 10E-3). Remarkably, in one inv(7) positive case (no. 15), all other *HOXA* genes also showed increased expression (Figure 4C(a) and (b)). Likewise, one inv(7) negative case (no. 28) showed elevated expression for all *HOXA* genes except for *HOXA11* (Figure 4C(a) and (b)). For these three cases showing elevated expression for *HOXA10* and/or *HOXA11* (cases no. 21, no. 22, no. 28), dual color FISH using the *HOXA* flanking probes was performed to evaluate disruption of the *HOXA* gene cluster but no evidence for any disruption of the *HOXA* gene cluster was found.

In addition to *HOXA* gene expression, we tested the inv(7) positive patients, total CT cells and CD34 + purified child thymocytes (CD34 + T) for expression of *HOXB3* and *HOXC4*
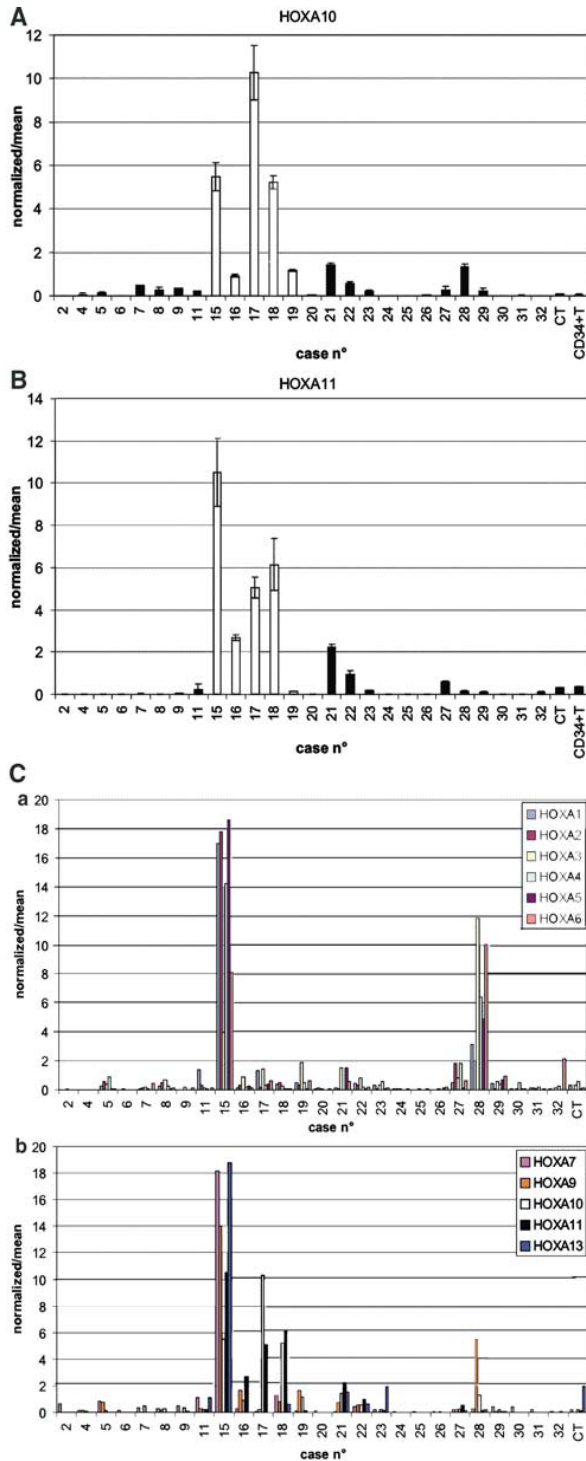
A HOXA10

B HOXA11

C
a

b

Figure 4 (A) Geometric averages of normalized expression of *HOXA10* in inv(7) positive T-ALL cases (white boxes), inv(7) negative T-ALL cases (black boxes), total child thymocytes (CT) and CD34 + sorted stem cells (CD34 + T). (B) Geometric averages of normalized expression of *HOXA11* in inv(7) positive T-ALL cases (white boxes), inv(7) negative T-ALL cases (black boxes), total child thymocytes (CT) and CD34 + sorted stem cells (CD34 + T). (C (a and b)) Global overview of normalized expression of all class I *HOX* genes tested in inv(7) positive T-ALLs (cases no. 15–19), inv(7) negative T-ALLs (cases no. 2–11 and no. 20–32) and total child thymocytes (CT).
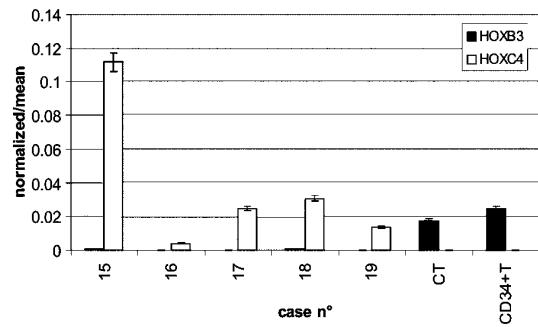

Figure 5 Geometric averages of normalized expression of *HOXB3* and *HOXC4* in inv(7) positive T-ALL cases (no. 15–19), total child thymocytes (CT) and CD34 + sorted stem cells (CD34 + T).

gene expression since, according to Taghon *et al*,[13] these were the only homeobox genes apart from *HOXA7, HOXA9, HOXA10* and *HOXA11* that were expressed at all major stages of human thymopoiesis. In comparison with child thymocytes and CD34 + purified child thymocytes, *HOXB3* was only weakly expressed in the inv(7) positive patients whereas *HOXC4* expression was clearly detected in all inv(7) positive patients and slightly higher than expression levels of total child thymocytes and CD34 purified child thymocytes (Figure 5).

Discussion

In this study, we describe a new recurrent chromosomal alteration, a pericentric inversion of chromosome 7, inv(7)(p15q34), in five patients with T-ALL leading to disruption of the *HOXA* gene cluster and juxtaposition to sequences within the *TCRβ* locus. As a consequence of this rearrangement, increased *HOXA10* and *HOXA11* expression was evident in all inv(7)(p15q34) positive cases as compared to most inv(7) negative T-ALLs.

A number of findings support our assumption that this inv(7)(p15q34) indeed represents a new recurrent chromosomal abnormality in T-ALL. First of all, FISH results with BAC clones covering the *HOXA* gene cluster at 7p15 were similar in all cases and were in keeping with a breakpoint cluster within the ~107 kb genomic region containing all *HOXA* genes, most probably distal to *HOXA5* and proximal to *HOXA10*. Second, gene expression analysis showed consistent increased expression of *HOXA10* and *HOXA11* in all inv(7) positive cases. Thirdly, all inv(7) positive T-ALLs were classified as mature T-ALLs (T3–4 group according to the EGIL recommendations[10]) but with characteristic CD2 negative CD4 positive, CD8 negative blast cells.

The present study was initially triggered by the finding of a cryptic t(7;11)(q34;q24) in an M-FISH study of childhood ALLs with complex karyotypic rearrangements. Based upon this observation, we anticipated that the distal chromosomal localization of the *TCRβ* locus might also predispose to the formation of other undetected rearrangements, which indeed turned out to be the case as demonstrated by the finding of the recurrent inv(7)(p15q34). In addition to this new discovery, the finding of chromosomal rearrangements affecting the *TCRβ* locus in as much as 20% in an unselected group of T-ALLs is in itself remarkable. Although the study by Gesk *et al*[8] showed a relatively high proportion of *TCRβ* rearrangements in a series of T-ALLs with cytogenetically detectable rearrangements affecting

the *TCR* loci, no systematic study on unselected cases investigating *TCRβ* rearrangements has been reported thus far. The high percentage of *TCRβ* rearrangements is only partly explained by the presence of the five inv(7) cases. Indeed, for 8/19 *TCRβ* positive cases, FISH screening for known *TCRβ* partners was negative and further testing for partner identification is currently ongoing. In addition, some chromosomal disruptions of the *TCRβ* locus were found in patients with apparent normal karyotypes and representing partly cryptic rearrangements with known T-cell oncogenes such as *RBTN2* and *RBTN1* with subtelomeric localization.

Homeobox genes are a particular class of transcription factors that play an important role in regulating aspects of morphogenesis and cell differentiation during normal embryonic development. Class I *HOX* genes are located in tightly linked physical clusters, whereas class II *HOX* genes are dispersed throughout the genome. In humans, a total of 39 class I *HOX* genes are located in four distinct gene clusters on 7p15.3 (*HOXA*), 17q21.3 (*HOXB*), 12q13.3 (*HOXC*) and 2q31.1 (*HOXD*).[19] They encode a complex network of transcription regulatory proteins whose precise targets remain poorly understood. *HOX* genes were shown to play a significant role not only in regulating body formation but also in organization and regulation of hematopoiesis[20] and leukemogenesis.[21–23] Direct effects of class I *HOX* gene function in hematopoiesis have been shown in various studies where *HOX* gene expression has been modulated by antisense oligonucleotides,[24,25] gene disruption by homologous recombination[26,27] or by overexpression studies.[28–31] In brief, forced expression of *HOXA10* in murine[31,32] and cord blood[28,29] hematopoietic stem cells resulted in increased proliferation and impaired myeloid differentiation besides a profound reduction in B and T cell differentiation. *HOXA9* overexpression studies showed similar effects in myeloid lineages but without effect on T lymphoid development.[33] Both overexpression studies of *HOXA10* and *HOXA9* however failed to produce any lymphoid neoplasms. In myeloid leukemia, several class I *HOX* genes have been implicated predominantly due to formation of fusion transcripts with *NUP98*.[34] A role for class I homeobox genes in T-ALL was first suggested by Ferrando *et al*,[35] who demonstrated consistently increased expression levels of a subset of *HOX* genes (*HOXA9, HOXA10, HOXC6* and *MEIS1*) in *MLL* rearranged T- and B-ALL, while myeloid lineage genes were only expressed in *MLL* rearranged B-ALL and not in T-ALL cases. These findings suggested *HOX* gene deregulation rather than myeloid gene deregulation being the key factor in leukaemic transformation mediated by *MLL* fusion proteins.[35] Further support for a crucial role of *HOXA* genes came from a study of *HOXA* gene expression in developing thymocytes[13] where high *HOXA10* expression was found in the earliest T-cell precursors in human thymus, which showed subsequent downregulation in CD4 and CD8 single positive mature thymocytes. This observation suggested a role for *HOXA10* in T-cell maturation, which was in keeping with their previous studies that showed impairment of final T-cell differentiation upon enforced *HOXA10* expression in cord blood cells.[29] In contrast to *HOXA* genes, expression of the other *HOX* cluster genes was less prominent or absent except for *HOXB3* and *HOXC4*. *HOXA11* was expressed at all major stages of T-cell development also underlining the putative important role of this particular *HOX* gene in thymocyte maturation. Taken together, the present findings and published data on *HOXA* expression in T-cell precursors and maturing thymocytes strongly suggest that the *HOXA* gene cluster and *HOXA10* and *HOXA11* genes, in particular, are of crucial importance in normal T-cell development.

In contrast to class I homeobox genes, two particular class II homeobox genes has been extensively studied in T-ALL, that is *HOX11*, initially discovered through the t(10;14)(q24;q11),[36] and *HOX11L2* more recently shown to be implicated in the cryptic t(5;14)(q35;q32).[37]

Chromosomal rearrangements disrupting *HOX* gene clusters might also shed light onto the mechanisms controlling *HOX* gene expression, that is, locally cis-acting control sequences, global enhancer sequences located outside the gene clusters.[38,39] Here, we propose two possible oncogenetic mechanisms resulting from the inv(7). First, the disruption of the *HOXA* gene cluster could interfere with the normal scheduled program of sequential up and downregulation following the 3′ to 5′ localization of the *HOXA* genes, that is, 3′ region *HOXA* genes being expressed in the more primitive cells and 5′ genes in more differentiated cells. The physical disruption of the cluster could therefore block downregulation of *HOXA10* and *HOXA11* required in order to allow the cells to complete their further differentiation and maturation. The second and more classical hypothesis is that enhancers embedded within the *TCRβ* locus lead to sustained high expression levels of *HOXA10* and *HOXA11*. Based upon the 10-fold higher expression levels observed for *HOXA10* and *HOXA11* in the inv(7) positive cases as compared to normal developing thymocytes, we favor the latter hypothesis.

Immunophenotypically, the five inv(7) patients showed a distinct pattern of CD2 negativity and CD4 single positivity within the T3–4 subgroup of T-ALLs. Obviously, lack of CD2 expression on their leukaemic blasts is the most striking feature as this pan T-cell marker is present on the leukaemic blasts of 85% of T-ALL.[40] CD4 single positivity is another intriguing finding within the T3–4 group as most cases either show CD4/CD8 double positivity or CD8 single positivity. Taken together, the phenotypic combination of CD2−, CD4+ and CD8− showed significant correlation with the presence of the inv(7) genomic rearrangement ($P = 5 \times 10E\text{-}5$).

In addition to the inv(7)(p15q34) positive cases, our study indicates that other mechanisms can lead to abnormal *HOXA* gene expression as two inv(7) negative cases also showed an elevated *HOXA10* and *HOXA11* expression. Moreover, a third case was noted, which like one of the inv(7) positive cases, showed upregulated expression of all *HOXA* genes. This observation is intriguing and somewhat reminiscent to the observed enhanced expression of certain T-cell oncogenes (*HOX11, LYL1, TAL1, LMO1* and *LMO2*) in the absence of any detectable chromosomal rearrangement affecting these loci.[41] Further study of these remarkable cases might increase our understanding of the mechanisms governing the tight control of expression of these developmentally important genes.

In conclusion, the present report describes a new recurrent cryptic chromosomal inversion inv(7)(p15q34) in T-ALL,which leads to elevated expression of *HOXA10* and *HOXA11* in five cases and increased expression of all *HOXA* genes in one case and seems to delineate a specific subset of CD2 negative CD4 single positive T-ALLs. This observation for the second time implicates class I *HOXA* genes, and in particular *HOXA10* and *HOXA11*, in T-cell oncogenesis and strongly supports the previously proposed role for *HOXA* genes in thymocyte development. We are currently collecting additional cases of inv(7) patients in order to determine in more detail the clinical and biological profile of this patient subgroup and, most importantly, in order to assess the prognostic importance of inv(7)(p15q34) within a large cohort of uniformly treated patients. Finally, we anticipate that this study might trigger a search for compounds interfering with T-cell differentiation

pathways controlled by *HOXA* genes and will also stimulate new investigations focused at determining the role of *HOXA* genes in T-cell development.

Acknowledgements

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CHAPTER II:

Paper 2:

Cytogenetic study of 126 unselected T-ALL cases and 19 T-ALL cell lines reveals unexpected high incidence of cryptic rearrangements of the *TCRβ* locus with at least 2 new recurrent partner genes.

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ORIGINAL ARTICLE

Molecular cytogenetic study of 126 unselected T-ALL cases reveals high incidence of *TCRβ* locus rearrangements and putative new T-cell oncogenes

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Chromosomal aberrations of T-cell receptor (*TCR*) gene loci often involve the *TCRαδ* (14q11) locus and affect various known T-cell oncogenes. A systematic fluorescent *in situ* hybridization (FISH) screening for the detection of chromosomal aberrations involving the *TCR* loci, *TCRαδ* (14q11), *TCRβ* (7q34) and *TCRγ* (7p14), has not been conducted so far. Therefore, we initiated a screening of 126 T-cell acute lymphoblastic leukemia (T-ALL) and T-cell lymphoblastic lymphoma cases and 19 T-ALL cell lines using FISH break-apart assays for the different *TCR* loci. Genomic rearrangements of the *TCRβ* locus were detected in 24/126 cases (19%), most of which (58.3%) were not detected upon banding analysis. Breakpoints in the *TCRαδ* locus were detected in 22/126 cases (17.4%), whereas standard cytogenetics only detected 14 of these 22 cases. Cryptic *TCRαδ/TCRβ* chromosome aberrations were thus observed in 22 of 126 cases (17.4%). Some of these chromosome aberrations target new putative T-cell oncogenes at chromosome 11q24, 20p12 and 6q22. Five patients and one cell line carried chromosomal rearrangements affecting both *TCRβ* and *TCRαδ* loci. In conclusion, this study presents the first inventory of chromosomal rearrangements of *TCR* loci in T-ALL, revealing an unexpected high number of cryptic chromosomal rearrangements of the *TCRβ* locus and further broadening the spectrum of genes putatively implicated in T-cell oncogenesis.
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Keywords: T-ALL; cytogenetics; *TCR* rearrangements; *HOXA*

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LL) are lymphoid malignancies representing a heterogeneous group of diseases that vary with respect to morphological, cytogenetic and immunologic features of the T-lymphoblasts.[1] The discovery of chromosomal rearrangements in these disorders has been pivotal in the identification of the genes involved in T-ALL development and normal thymocyte differentiation.[2,3] In most instances, these chromosomal aberrations are translocations that preferentially involve the T-cell receptor *TCRαδ* locus (14q11) and to a much lesser extent the *TCRβ* locus (7q34) and affect a wide array of genes with oncogenic properties 1p32(*TAL1*), 1p34(*LCK*), 8q24(*MYC*), 9q34(*TAL2*), 9q34(*TAN1/NOTCH1*), 10q24 (*HOX11*), 11p13 (*RBTN2/LMO2*), 11p15(*RBTN1/LMO1*), 14q32(*TCL1*), 19p13

(*LYL1*), 21q22(*BHLHB1*) and Xq28 (*MTCP1*).[3–6] Translocations affecting *TCR* genes largely result in deregulated expression of proto-oncogenes by juxtaposing promoter and enhancer elements of *TCR* genes in the proximity of these developmentally important genes.[7] Interestingly, further molecular studies revealed that some of these genes (*TAL1, HOX11, NOTCH1*) were functionally activated in a much higher frequency than expected from a cytogenetic point of view,[8–10] further underlining the importance of the original cytogenetic investigations. Also, biallelic expression has been reported in about half of the cases with *TAL1, LMO2* and *HOX11* expression pointing at disturbance of an upstream regulatory control mechanism.[11] In contrast to B-ALL, in which predominantly chimeric transcription factor proteins are generated, gene fusions occur in a much lower rate in T-ALL (predominantly *MLL/ENL* and *CALM/AF10*).[12–14]

Recently, the spectrum of chromosomal abnormalities in T-ALL has been further widened by the finding of new recurrent but cryptic alterations. First, a cytogenetically undetectable translocation t(5;14)(q35;q32) was found in about 20% of childhood T-ALL juxtaposing the *HOX11L2* gene (5q35) to the distal region of the *BCL11B* gene.[5] A further remarkable finding was the extrachromosomal (episomal) amplification of *NUP214-ABL1* fusion genes in 6% of T-ALL cases leading to constitutively phosphorylated tyrosine kinase activity, which can be inhibited upon addition of imatinib, a selective inhibitor of *ABL1* kinase activity.[10,15,16] Recently, we observed yet another recurrent chromosomal rearrangement, that is, an inv(7)(p15q34) in a subset of T-ALLs. This rearrangement juxtaposes the distal part of the *HOXA* gene cluster on 7p15 to the *TCRβ* locus on 7q34 and causes increased *HOXA10* and *HOXA11* expression levels.[17] The occurrence of this inv(7) as well as a t(7;7)(p15;q34) was subsequently also demonstrated by Soulier *et al.*[18] Interestingly, upregulated *HOXA* gene expression was also found in *MLL-ENL* and *CALM-AF10*-positive T-ALLs, thus pointing at a more general role of *HOXA* genes in T-cell oncogenesis.[19,20]

These observations thus indicate that a plethora of genes can be implicated in the development of T-ALL as a result of various recurrent chromosomal changes, many of which remained undetected upon cytogenetic investigation until recently. In particular, the finding of a cryptic t(7;11)-(q34;q24) involving the *TCRβ* locus (7q34) in a childhood T-ALL case[21] and the new recurrent inv(7)(p15q34) triggered the screening for additional cryptic *TCR* rearrangements in a cohort of 126 T-ALL patients and 19 T-ALL cell lines. Our findings show that the majority of *TCRβ* chromosomal rearrangements remained undetected upon routine karyotyping and that new T-cell oncogenes may be implicated in some of these cases (Figure 1).
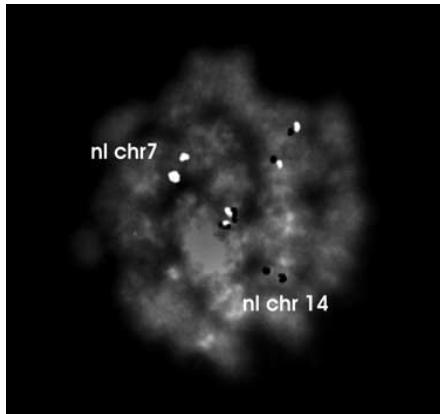
Figure 1 Fluorescent *in situ* hybridization analysis in patient no. 23 carrying a reciprocal *TCRβ-TCRαδ* chromosomal rearrangement. T-cell receptorβ flanking BACs are labeled in Spectrum Green (white) and *TCRαδ* flanking BACs in Spectrum Orange (black).

Table 1 Flanking bacterial artificial chromosomes clones used for FISH analysis in the present study

Locus	Centromeric	Telomeric
TCRβ (7q34)	RP11-1220K2	RP11-556I13
TCRαδ (14q11)	RP11-242H9	RP11-447G18
TCRγ (7p15)	RP11-273L18	RP11-243E12
LCK (1p34)	RP11-22K3	RP11-68H10
TAL1 (1p32)	RP11-332M15	RP11-346M5
TAL2 (9q32)	RP11-287A8	RP11-576E23
NOTCH1 (9q34)	RP11-83N9	RP11-251M1
HOX11 (10q24)	RP11-108L7	RP11-107I14
LMO2 (11p13)	RP11-313G13	RP11-60G13
LMO1 (11p15)	RP11-782G4	RP11-1065L8
TCL1 (14q32)	RP11-952P19	RP11-760A15
LYL1 (19p13)	RP11-1078F11	RP11-963I8
CDKN2A (9p21)	RP11-478M20	RP11-454D15
HOXA (7p14)	RP5-1103I5	RP1-167F23
OLIG2 (21q22)	RP11-94I18	RP11-76I24
CDKN2A (9p21)	RP11-478M20	RP11-454D15
MTCP1 (Xq28)	RP11-402H20	

Abbreviations: FISH, fluorescent *in situ* hybridization; TCR, T-cell receptor.

Materials and methods

Patients and controls

Cytogenetic cell suspensions or unstained slides from 126 diagnostic T-ALL ($n=109$) and T-LL ($n=17$) samples were collected retrospectively from cytogenetic centers between 1988 and 2005. Diagnosis of T-ALL/LL was made according to the morphological and cytochemical criteria of the French–American–British classification[22] and by imunophenotyping. The selection of cases was based on the availability of fixed cell suspension or unstained slides, which permit fluorescent *in situ* hybridization (FISH) investigation. Molecular data and immunophenotype were available only for most recent cases and are not shown. This cohort of T-ALL and T-LL cases included 80 children and 46 adults.

The 19 T-ALL cell lines were purchased from DSMZ (http://www.dsmz.de, Braunschweig, Germany). Peripheral blood lymphocytes from healthy donors with normal karyotypes served as negative controls for validation and cutoff level determination of the different FISH assays. Cell suspensions from T-ALL cases or cell lines with cytogenetically proven breakpoints at the different known T-cell oncogenes (*LCK, MYC, HOX11, LMO2, LMO1, NOTCH1*) were used as positive controls.

Methods
Cytogenetic analysis. Diagnostic specimens (bone marrow, blood and pleural fluid) and cell lines were cultured and harvested for cytogenetic analysis according to established methods. Chromosome slides were G or R banded. Chromosome aberrations are described according to guidelines of an International System for Human Cytogenetic Nomenclature (ISCN 1995).[23]

Clone selection and validation. RPCI-11 (Human BAC Library) clones were selected using the bioinformatics resources available at National Center for Biological Information (http://genome.ucsc.edu) and Ensembl Genome Browser (http://www.ensembl.org/). Clones were provided by the Welcome Trust Sanger Institute (Cambridge, UK) and Invitrogen (Paisley, Scotland).

Disruption of the *TCR* loci was assessed by dual color FISH with *TCR* flanking probes. Clones for the *TCRαδ*, *TCRβ* and *TCRγ* applied in the present study are listed in Table 1. Additional FISH probes used to confirm the involvement of *TCR* partner genes in cases carrying *TCRβ* and/or *TCRαδ* chromosomal rearrangement (identified by a split signal of the flanking probes) are listed in Table 1, with the exception of *MYC* for which we applied the LSI MYC Dual Color, Break Apart Rearrangement Probe (Vysis, Abbott, Ottignies, Belgium). DNA isolation of bacterial artificial chromosome (BAC) clones and FISH was performed as described previously.[24] Subsequently, large-scale DNA amplification was performed using the GenomiPhi Amplification Kit (Amersham Biosciences, Roosendaal, The Netherlands), which utilizes bacteriophage Phi-29DNA polymerase and exponentially amplifies single- or double-stranded linear DNA templates during an isothermal (30°C), strand displacement reaction.[25] Phi-amplified DNA was labeled as described previously[24] using spectrum green- and spectrum orange-dUTP (SG-dUTP and SO-dUTP, Vysis, Abbott).

Validation of BAC clone genomic positions of *TCRαδ*, *TCRβ* and *TCRγ* flanking clones was performed using STS (sequence tagged site) PCR with two STS genomic markers per BAC clone.

Using this approach, the genomic positions of the different *TCR* gene loci provided by the bioinformatics resources were confirmed.

Determination of cutoff levels. Determination of cutoff levels of these new probe sets was performed by counting 200 nuclei in five negative controls (peripheral blood lymphocytes from healthy donors) for each probe set.

Based on the results in negative controls, a split was defined as a spatial separation of the flanking probes of more than three times the estimated signal diameter.[26]

Using these criteria, the cutoff levels of the different probe combinations were 6% for *TCRαδ*, 6.5% for *TCRβ* and 2% for *TCRγ*.

Results

A total of 126 T-ALL/LL cases and 19 T-ALL cell lines were investigated. Karyotypic analysis was successful in 119 cases,

whereas seven cases did not yield metaphases. Clonal abnormalities were present in a high percentage of cases (74.7%:89/119) (data not shown), which can be biased by the selection of patient samples with possibly higher white blood cell counts at presentation. Fluorescent *in situ* hybridization screening for *TCR* rearrangements using dual color break apart assays showed *TCRαδ* rearrangements in 22/126 patients (17.4%) in keeping with data from the literature, a surprisingly similar high number of patients (24/126, 19%) with *TCRβ* aberrations and no *TCRγ* rearrangements. Split signals with the specific probe sets were observed in 15–95% of cells for *TCRαδ* and in 20–98% for *TCRβ*. Simultaneous rearrangements targeting both the *TCRβ* and *TCRαδ* genes were observed in six patients and in one T-ALL cell line (SUP-TI).

Translocations affecting the TCRβ locus in T-cell acute lymphoblastic leukemia

Fluorescent *in situ* hybridization screening for *TCRβ* chromosomal rearrangements yielded split signals for the *TCRβ* locus in 24 cases (19%: 24/126) (Tables 2 and 4); these included three T-LL and 21 T-ALL. In 19 of these 24 positive cases, involvement of a known recurrent *TCRβ* partner gene could be confirmed by FISH with the appropriate probes (Table 1): *TAL1* (case no. 2), *HOXA* (case nos. 15–22), *HOX11* (case nos. 3,4–8–14–20), *LMO2* (case no. 7), *LMO1* (cases nos. 9 and 10), *TCRαδ* (case nos. 13–23) and *NOTCH1* (case no. 12). *HOXA* gene expression levels were reported previously for case nos. 15–19. In the additional cases 21 and 22 detected by FISH screening, a similar pattern of increased *HOXA10* and *HOXA11* was observed (details will be published elsewhere). Owing to poor quality and/or paucity of metaphases, the partner chromosome remained undetermined in the remaining five cases showing *TCRβ* rearrangement. These rearrangements were slightly more frequent in adults compared to children, 14/80 children (17.5%) versus 10/46 adults (21.7%). One patient (case no. 1) showed rearrangement of the *TCRβ* locus with an as yet unidentified partner gene on chromosome 11q24. Further analysis to identify this new partner gene is ongoing and will be reported elsewhere.

Abnormal karyotypes were found in 16 out of 24 *TCRβ*-positive cases (Table 2), whereas translocations affecting the 7q34 locus were detected in only four of 24 cases. Partial deletions of chromosome 7q or 7p were detected in three cases and two cases showed additional material on 7q. Taken together, *TCRβ* genomic rearrangement was unsuspected from cytogenetic analysis in as much as 14 of 24 (58.3%) cases when excluding failures. Two out of 19 cell lines screened with the *TCRβ* flanking probes showed genomic rearrangement of this locus with *LCK* (T-ALL cell line HSB2) and *NOTCH1* (T-ALL cell line SUP-TI) as partner genes (Table 2).

Translocations affecting the TCRαδ locus in T-cell acute lymphoblastic leukemia

Translocations involving the *TCRαδ* locus were detected in approximately the same number of patients as for *TCRβ*, that is, 22/126 (17.4%) cases (Tables 2 and 4) and mostly included T-ALL (*n* = 21). However, involvement of known T-cell oncogenes was confirmed in only 12 of 22 rearranged cases. Unexpectedly, one *TCRαδ* rearranged case (no. 23) showed a balanced rearrangement with the *TCRβ* locus, which brings the total number of identified *TCRαδ* partner genes to 13 out of 22. These findings suggest that many of these rearrangements targeted unknown T-cell oncogenes. Within the *TCRαδ* rearranged group involving known T-cell oncogenes, *HOX11* was most frequently

involved followed by *LMO2, TAL1* and *MYC*, which involved two cases each. *TCL1, HOXA* and *TCRβ* were detected in single cases. For the remaining nine cases carrying a *TCRαδ* rearrangement, partner genes could not be identified. Interestingly, *TCRαδ* rearrangements showed involvement of an unknown partner gene on chromosome 6q22 (case no. 40) and 20p12 (case no. 41) (Table 2). Of the 19 cell lines screened with *TCRαδ* flanking clones, five showed rearrangements with partner genes *LMO1* (RPMI 8402), *LMO2* (TALL 104), *MYC* (MOLT16 and KE-37), *HOX11* (ALL-SIL) and an unidentified gene (SUP-TI). In contrast to *TCRβ* rearrangements, *TCRαδ* rearrangements were more frequent in children (16/80; 20%) versus adult T-ALL patients (6/46: 13%).

In this group of *TCRαδ* rearranged cases (*n* = 22), abnormal karyotypes were present in 19 patients (Table 2). Of these, translocations involving the *TCRαδ* locus were obvious from banding analysis in only 12 of these 19 patients. In seven cases with clonal karyotypes, no 14q11 rearrangement was apparent, indicating that at least 30% (7/22) of *TCRαδ*-positive cases in this series was cryptic. This could be explained in one of these cases through the presence of complex abnormalities. This case (no. 28) showed two subclones carrying rearrangement of chromosome 14 at different chromosome bands. One subclone carried a rearrangement of chromosome 14 at the ~14q32 band (IgH locus) and was translocated to the long arm of chromosome 20,[24] revealing a t(14;20)(q32;q12). In another subclone, chromosome 14 showed disruption of the *TCRαδ* locus (14q11) with inversion of the distal probe to the short arm of chromosome 14 thus revealing a new inv(14)(p?q11).

Simultaneous occurrence of TCRβ and TCRαδ genomic rearrangements

Five T-ALL patients (cases nos. 1, 6, 10, 13, 23) and one T-ALL cell line (SUP-TI) carried genomic rearrangements of both *TCRβ* and *TCRαδ* loci. In four of these patients, both *TCR* genes targeted different T-cell oncogenes (Table 2) as confirmed by FISH with the appropriate probes. Unexpectedly, patient nos. 13 and 23 showed a rearrangement between the *TCRβ* and *TCRαδ* gene loci, unbalanced in case no. 13 and balanced in patient no. 23. Five of these patients were children, possibly reflecting the higher susceptibility to genomic rearrangements involving *TCR* loci during childhood.

Previously unreported chromosome aberrations in T-cell acute lymphoblastic leukemia

Chromosome aberrations found in this series, which have not been reported previously in T-ALL, are listed in Table 3. Fluorescent *in situ* hybridization investigation for oncogenes known to be involved in leukemogenesis *(LMO1, LMO2, HOX11, HOXA, ETV6, NUP98)* were applied and in most cases turned out to be negative, indicating that a large set of putative T-cell oncogenes still remains undiscovered.

Discussion

Here we report the results of a comprehensive FISH screening performed in 109 T-ALL, 17 T-LL and 19 T-ALL cell lines using *TCR* flanking FISH probes to determine the incidence of chromosomal rearrangements involving the *TCR* genomic loci *TCRαδ* (14q11), *TCRβ* (7q34) and *TCRγ* (7p15). This approach allowed us to demonstrate for the first time that *TCRβ* rearrangements occur in a similarly high frequency as *TCRαδ*

Table 2 Biological characteristics and cytogenetic findings of TCRβ- and/or TCRαδ-positive T-ALL patients and cell lines with identified partner gene and revised abnormal karyotypes

Case no	Age	Sex	Karyotype	TCRαδ parmter gene	TCRβ partner gene	Revised abnormal karyotype
1	4	M	46,XY,t(1;14)(p32;q11)[20]	TAL1 (1p32)	Unknown (11q24)	46,XY,t(1;14)(p32;q11), t(6;10)(q25;q24), **t(7;11)(q34;q24)**
2	19	M	46,XY,t(1;7)(p32;q34), del(6)(q12q16)[14]/46, XY[1]	Negative	TAL1 (1p32)	46, XY,t(1;7)(p32;q34), del(6)(q12q16)
3	45	M	46,XY[9] failure	Negative	HOX11(10q24)	46,XY,t(7;10)(q34;q24)
4	6	M	47,XY,+8,del(9)(p21)[3]/46,XY[8]	Negative	HOX11(10q24)	47,XY,t(7;10)(q34;q24),+8, del(9)(p21)
5	12	F	46,XX,del(6)(q23q26),add(7)(q31),del(11)(q13),−14,+mar mar = ?der(1) i(1)(q10)add(1)(q32)	Negative	Unknown	46,X,del(6)(q23q26),t(7;?)(q34;?),del(11)(q13),−14,+mar mar = ?der(1) i(1)(q10)add(1)(q32)
6	6	M	46,XY,del(7)(q21),?inv(14)(q11q32)[14]/46,XY[6]	TCL1 (14q32)	Unknown	46,XY,t(7;?)(q34;?),inv(14)(q11q32)
7	15	F	46,XX[20]	Negative	LMO2 (11p13)	46,XX,**t(7;11)(q34;p13)**
8	10	M	46,XY[20]	Negative	HOX11(10q24)	46,XY,**t(7;10)(q34;q24)**
9	9	F	46,XX,del(7)(q34),t(10;14)(q22;q32)[11]	Negative	LMO1 (11p15)	46,XX,t(7;11)(q34;p15),t(10;14)(q22;q32)
10	9	M	46,XY,t(8;14)(q24;q11)[8]/46,XY[9]	MYC(8q24)	LMO1 (11p15)	46,XY,t(8;14)(q24;q11), **t(7;11)(q34;p15)**
11	26	M	46,XY[20]	Negative	Unknown	46,XY,**t(7;?)(q34;?)**
12	15	M	47,XY,t(7;9)(q34;q34),+20[2]/47, idem, add(11)(q24)[9]	Negative	NOTCH1 (9q34)	47,XY,t(7;9)(q34;q34),+20
13	30	M	46−47,XY,+mar1, +mar2 [cp5]	Unknown	TCRδ (14q11)	46−47,XY, +der(14)t(7;14)(q34;q11), +mar1
14	23	M	42−48,XY,t(3;11)(p12;p15),t(7;10)(q34;q24),+11, +12[5]/46,XY[4]	Negative	HOX11 (10q24)	42−48,XY,t(3;11)(p12;p15),t(7;10)(q34;q24),t(8;10)(q21;q?24), +11, +12
15	35	F	46,XX[4] failure	Negative	HOXA (7p15)	46,XX, inv(7)(p15q34)
16	27	F	46,XX, add(5)(q31)[11]/46, XX[9]	Negative	HOXA (7p15)	46, XX, add(5)(q31), **inv(7)(p15q34)**
17	15	M	46,XY[20]	Negative	HOXA (7p15)	46,XY, **inv(7)(p15q34)**
18	34	M	46,XY[20]	Negative	HOXA (7p15)	46,XY, **inv(7)(p15q34)**
19	49	M	47,XY,del(6)(q14),del(7)(p?),del(9)(p21),+mar1[4]/46,XY[6]	Negative	HOXA (7p15)	47,XY,del(6)(q14),inv(7)(p15q34),del(9)(p21),+mar1
20	8	M	47,XY,+8, t(7;10)(q34;q24),del(6)(q12)[5] /46,XY[18], t(5;14) (FISH)	Negative	HOX11 (10q24)	47,XY,+8, t(7;10)(q34;q24),del(6)(q12), t(5;14) (FISH)
21	21	M	46,XY[4] failure	Negative	HOXA (7p15)	46,XY, inv(7)(p15q34) or t(7;7)(p15;q34)
22	6	F	46,XX, +21[20]	Negative	HOXA (7p15)	46,XY, +21, inv(7)(p15q34) or t(7;7)(p15;q34)
23	5	M	45,X,der(X)?, add(7q35), −9,−9, del(11)(p11.2), del(12)(p?13), mar[15]/ 46,XX[1]	TCRβ (7q34)	TCRδ (14q11)	45,X,der(X)?, **t(7;14)(q34;q11)**, −9,−9, del(11)(p11.2), del(12)(p?13), mar
24	8	M	46,XY, add(22)(q12)[3]/46,XY[1]	Negative	Unknown	45,X,der(X)?, **t(7;?)(q34;?)**, add(22)(q12)
25	35	M	46,XY, t(9;20)(p21;q12), t(10;14)(q24;q11), del(12)(p12)[13]/46, XY[12]	HOX11 (10q24)	Negative	46,XY,t(9;20)(p21;q12), t(10;14)(q24;q11), del(12)(p12)
26	3	F	46,XX[20]	LMO2 (11p13)	Negative	46,XX,t(8;14)(q24;q11)
27	9	M	46,XY,t(11;14)(p13−15;q11)[20]	Unknown (14p?)	Negative	46,XY,t(11;14)(p13;q11)
28	7	F	46,XX,del(6)(q16q27), t(14;20)(q32;q12)[3]/46,XX[7] FISH t(5;14)	HOX11 (10q24)	Negative	46,XX,del(6)(q16q27), t(14;20)(q32;q12), inv(14)(p?q11)
29	36	F	46,X,t(10;14)(q24;q11)[6]/46,XX[27]	HOX11 (10q24)	Negative	46,X,t(10;14)(q24;q11)
30	32	M	46,XY,t(7;14)(p15;q11),t(10;11)(p14;q21),add(18)(q23)[11]/46,XY[9]	HOXA (7p15)	Negative	46,XY,t(7;14)(p15;q11),t(10;11)(p14;q21),add(18)(q23)
31	34	M	46,XY,t(10;14)(q24;q11)[19]/46,XY[6]	HOX11 (10q24)	Negative	46,XY,t(10;14)(q24;q11)
32	4	M	46,XY, add(9p21)[16]/92, idemx2 [5]/46,XY[9]	Unknown	Negative	46,XY, add(9p21), t(?;14)(?;q11)
33	8	M	47,XY,+19[20]/46,XY[2] FISH t(5;14)	Unknown	Negative	47,XY,+19, t(?;14)(?;q11)
34	6	M	46,XY,del(6)(q13q23), t(11;14)(p13;q11)[28]/46,XY[8]	LMO2 (11p13)	Negative	46,XY,del(6)(q13q23), t(11;14)(p13;q11)
35	14	M	46,XY, add(5)(p15)[8]/46,XY[20]	Unknown	Negative	46,XY, add(5)(p15), t(?;14)(?;q11)
36	8	M	Failure	HOX11 (10q24)	Negative	46,XY,t(10;14)(q24;q11)
37	3	F	46,XX,t(9;11)(p22;q23)[1]/46,XX [48]	Unknown	Negative	46,XX,t(9;11)(p22;q23), t(?;14)(?;q11)
38	9	F	Failure	Unknown	Negative	46,XX,t(?;14)(?;q11)
39	4	M	46,XY,t(1;14)(p32;q11)[7]/46,XY[4]	TAL1 (1p32)	Negative	46,XY,t(1;14)(p32;q11)
40	43	F	47,XX,t(3;6)(q23;q26), −4, −5, t(6;14)(q22;q11), −8, del(10)(q23q25), t(10;11)(q23;q14), der(12)(t(8;12)(q21;q24), ins(13;?), add(15)(p13), +3 mar1, +mar2[6]/46,XX[2]	Unknown (6q22?)	Negative	47,XX,t(3;6)(q23;q26), −4, −5, t(6;14)(q22;q11), −8, del(10)(q23q25), t(10;11)(q23;q14), der(12)(t(5;12)(q22;p12), der(12)(t(8;12)(q21;q24), ins(13;?), add(15)(p13), +3 mar1, + mar2
41	15	F	92,XXXX, del (6)(q14q16), t(14;20)(q11,p12)[17]/46,XY[7]	Unknown (20p12 ?)	Negative	92,XXXX, del (6)(q14q16), del(9)(p11), t(14;20)(q11,p12)
T-ALL cell lines						
RPMI 8402			79−91, XXX, −X, der(1)t(1:9)(p35;q11),+3, +3, dup(4)(q13q23)x2, del(6)(q14q22)x2, −10, t(11;14)(p15;q11)x2, −13, add(13)(q34), −14, +15, add(15)(p13), −18, −20, +mar1, +mar2	LMO1(11p15)	Negative	79−91, XXX, −X, der(1)t(1;9)(p35/36;q11),+3, +3, dup(4)(q13q23)x2, del(6)(q14q22)x2, −10, t(11;14)(p15;q11)x2, −13, add(13)(q34), −14, +15, add(15)(p13), −18, −20, +mar1, +mar2
ALL-SIL			90−95, XX/XXYY, t(1;13)(p32;q32)x2, +6, del(6)(q25)x2, +8, +8, del(9)(?p23p24)x2, t(10;14)(q24;q11.2)x2, add(17)(p11)x2	HOX11 (10q24)	Negative	90−95, XX/XXYY, t(1;13)(p32;q32)x2, +6, del(6)(q25)x2, +8, +8, del(9)(?p23p24)x2, t(10;14)(q24;q11.2)x2, add(17)(p11)x2
TALL-104			46,XY,t(11;14)(p13;q11)	LMO2 (11p13)	Negative	46,XY,t(11;14)(p13;q11)
HSB2			42−46, XY, t(1;7)(p34;q34)	Negative	LCK (1p34)	42 −46, XY, t(1;7)(p34;q34)
KE-37			86−92, XXYY, t(7;12)(q32−33;p12−13)x2, +8, der(8)t(8;14)(q24;q11)x4, −14, −14, der(14)t(8;14)(q24;q11)x2	MYC (8q24)	Negative	86−92, XXYY, t(7;12)(q32−33;p12−13)x2, +8, der(8)t(8;14)(q24;q11)x4, −14, −14, der(14)t(8;14)(q24;q11)x2
MOLT-16			43−47,XX, t(3;11)(p21;p13), t(8;14)(q24;q11)	MYC (8q24)	Negative	43−47,XX, t(3;11)(p21;p13), t(8;14)(q24;q11)
SUP-T1			85, XXX/XXXYY, inv(2)(p22q11)x2, t(2;?20)(p13;?p11), del(4)(q31q35), del(6)(q25)x2, add(7)(q32), −8, −8, −9, add(9)(q34)x2, inv(14)(q11q32)x2, −12	Unknown	NOTCH1 (9q34)	85, XXX/XXXYY, inv(2)(p22q11)x2, t(2;?20)(p13;?p11), del(4)(q31q35), del(6)(q25)x2, **t(7;9)(q34;q34)**, −8, −8, −9, inv(14)(q11q32)x2, −12

Abbreviations: T-ALL, T-cell acute lymphoblastic leukemia; TCR, T-cell receptor.
Cryptic TCRβ rearrangements are indicated in bold.
Cryptic TCRαδ rearrangements are underlined.

Table 3 Chromosome aberrations not previously reported in T-ALL

t(X;10)(p23;q23)
t(1;9)(p13;p21)
inv(3)(p11q22)
t(3;6)(q23;q26)
t(3;11)(p12;p15)
t(4;9)(q25;p21)
t(5;7)(p15;q31)
t(6;10)(q25;q24)
t(6;14)(q22;q11)
t(6;16)(p21;p12)
t(6;16)(q21;q23)
t(6;17)(p21;p13)
t(7;9)(p11;p24)
t(7;12)(p14;*p13*)
t(7;14)(p12;q24)
t(8;10)(q21;q21)
t(9;20)(p21;q12)
t(10;12)(p12;q21)
inv(11)(p12q23)
t(11;12)(q13;*p13*)
t(11;12)(q23;*p13*)
Dic(14;19)(p11;p11)
t(14;20)(q32;q12)

Abbreviation: T-ALL, T-cell acute lymphoblastic leukemia.

Table 4 Frequency of detected TCR chromosomal rearrangement using conventional karyotyping versus FISH

Locus	Conventional karyotyping (%)	FISH (%)	
TCRαδ (14q11)	9.5[a]	24.7[b]	17.4[a]
TCRβ (7q34)	3.1[a]	26.9[b]	19[a]
TCRγ (7p15)	0	0	0

Abbreviations: FISH, fluorescent *in situ* hybridization; TCR, T-cell receptor.
[a]In percentage of all T-ALL.
[b]In percentage of T-ALL abnormal karyotypes.

rearrangements, that is, in 19% of T-ALL patients. Of further importance, we showed that as much as half (14/24) of the *TCRβ* and about one-third (8/22) of the *TCRαδ* rearrangements were not detected upon karyotypic analysis. Our study thus indicates that using standard karyotyping, chromosomal rearrangements involving T-cell receptors (in particular *TCRβ*) have been significantly underestimated so far.[27] In line with previous reports, no *TCRγ* aberrations were observed in this series. Apparently, these *TCRγ* rearrangements are confined to T-cell tumors in patients with ataxia telangiectasia (ATM) where these rearrangements are frequently found.[28] The frequency and age distribution of *TCRαδ* rearrangements in our study are in line with the series reported by Heerema *et al.*,[29] whereas other larger studies of adult T-ALL found that the frequency of *TCRαδ* rearrangements was much higher in adults compared to childhood T-ALL.[30,31] For patients showing a *TCRβ* locus rearrangement, conventional karyotyping showed aberrations in only 3.1%, which is somewhat in between previous cytogenetic reports of 7q34 abnormalities in adult (7.5%)[27] and childhood (1%)[30] T-ALL. The incidence of *TCR* rearrangements (*TCRβ* and/or *TCRαδ*) was slightly higher in the T-ALL group, which was possibly biased by the much larger number of patients with T-ALL compared to T-LL. In the T-LL subgroup, 4/17 (23.5%) were positive for a *TCR* rearrangement, whereas 36/109 (33%) T-ALL showed one of these rearrangements.

The high incidence of cytogenetically undetected *TCRβ* (14 out of 24 *TCRβ*-positive cases) and *TCRαδ* (eight out of 22 *TCRαδ*-positive cases) rearrangements is remarkable. For *TCRβ* rearrangements, eight of 14 cytogenetically undetected cases showed other clonal rearrangements and re-evaluation of the karyotypes allowed us to detect the *TCRβ* aberration in two cases (nos. 3 and 4). This could be explained by the distal localization of the *TCRβ* locus (7q34) together with a distal chromosomal position of the breakpoints of the *TCRβ* partner genes, that is, t(7;11)(q34;q24), t(7;10)(q34;q24) and inv(7)(p15q34). In two cases (nos. 7 and 8) only normal metaphases were found at diagnosis and re-evaluation of karyotypes did not show the aberration, suggesting that only non-leukemic cells were cultured. This explanation is not valid for the *TCRαδ* locus rearrangements, which should be readily detectable on G-banding analysis. For *TCRαδ* rearrangements that remained undetected upon karyotyping, two were presented as a marker chromosome (case nos. 13–28), one resulted form a complex rearrangement (case no. 28) and a third (case no. 26) was not detected as probably only normal cells were karyotyped. Re-evaluation of case no. 26 remained negative. Interestingly, for the remaining *TCRαδ*-positive cases that remained undetected, chromosomal rearrangements were found in karyotypically abnormal cells (case nos. 32, 33, 35, 37) raising the question that these represent true cryptic rearrangements.

Preferential partner genes for *TCRβ* in our series include *HOXA* (7p15)[17] and *HOX11* (10q24), which were involved in seven and four cases, respectively. Recently, *HOXA* cluster genes were shown to be involved in a chromosomal rearrangement with the *TCRαδ* (14q11) locus in a T-ALL patient (case no. 30) carrying a t(7;14)(p15;q11) (unpublished observation).

The finding that in 14 *TCRαδ* or *TCRβ* chromosomal rearrangements the partner gene could not be identified after testing of all genes known to be implicated in T-ALL is of great potential importance. Also, two new translocations involving the *TCRαδ* locus were found in this series, that is, t(6;14)(q22;q11) (case no. 40) and t(14;20)(q11;p12) (case no. 41) (Table 2). Further efforts to identify these putative new partner genes are ongoing. Recently, a new translocation, that is, t(7;11)(q34;q24)[21] affecting the *TCRβ* locus and an as yet unidentified partner gene on chromosome 11q24 was described (case no. 1 in Table 3) and is analyzed further. Other chromosomal aberrations not reported previously in T-ALL (Table 2) include three cases with rearrangements of chromosome 9p21–24. This is the region harboring the *CDKN2A* (encoding p14 and p16 proteins) and *CDKN2B* (encoding p15 protein) tumor suppressor genes, which are the primary targets of 9p21 deletions in T-ALL and have been described to be present in 65% of T-ALLs.[32,33] Translocations affecting this gene locus have been reported in ALL but mostly in B-ALL.[30,34,35] Interestingly, two of these patients carrying a rearrangement of 9p also showed a homozygous deletion of p16.

An interesting observation is the presence of rearrangements of both *TCRαδ* and *TCRβ* loci in six patients and one cell line. Five of these patients were children, possibly reflecting the higher susceptibility for errors in VDJ recombination as a consequence of greater antigen exposure during childhood. In four patients, involvement of different T-cell oncogenes by both *TCRβ* and *TCRαδ* genes was confirmed using FISH with the appropriate flanking BAC clones. No preferential involvement of a particular gene seems evident from this small series, but it should be noted that four of the 12 partner genes in these cases remained unidentified. Interestingly, two patients (case nos. 13 and 23) carried a translocation between the two *TCR* loci: *TCRβ* (7q34) and *TCRαδ* (14q11); unbalanced in case no. 13 and balanced in case no. 23. So far, rearrangements between two

TCR loci, *TCRγ* (7p15) and *TCRαδ* (14q11), have been reported in a high frequency in patients with ATM.[28] However, this aberration has also been found at very low frequencies in T-lymphocytes from healthy individuals.[36] These observations raise the possibility that rearrangements affecting both *TCR* loci are not merely chromosomal aberrations associated with tumorigenesis, but could represent the capacity of the recombinase system to generate additional immune diversity.[37]

In the light of the present findings, we would recommend thorough cytogenetic and molecular cytogenetic screening for cases included in ongoing and future gene expression profiling studies in T-ALL. This information may be critically important in the data analysis and delineation of genetic subgroups. Also, it will broaden our understanding of the various genetic mechanisms that lead to unscheduled activation or sustained expression of the plethora of genes implicated in T-cell oncogenesis.

Acknowledgements

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CHAPTER II:

Paper 3:

Clinical, cytogenetic and molecular characteristics of 14 T-ALL patients carrying the *TCRB-HOXA* rearrangement: a study of the Groupe Francophone de Cytogénétique Hématologique (GFCH).

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Clinical, cytogenetic and molecular characteristics of 14 T-ALL patients carrying the *TCRβ-HOXA* rearrangement: a study of the Groupe Francophone de Cytogénétique Hématologique

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Recently, we and others described a new chromosomal rearrangement, that is, inv(7)(p15q34) and t(7;7)(p15;q34) involving the T-cell receptor beta (*TCRβ*) (7q34) and the *HOXA* gene locus (7p15) in 5% of T-cell acute lymphoblastic leukemia (T-ALL) patients leading to transcriptional activation of especially *HOXA10*. To further address the clinical, immunophenotypical and molecular genetic findings of this chromosomal aberration, we studied 330 additional T-ALLs. This revealed *TCRβ-HOXA* rearrangements in five additional patients, which brings the total to 14 cases in 424 patients (3.3%). Real-time quantitative PCR analysis for *HOXA10* gene expression was performed in 170 T-ALL patients and detected *HOXA10* overexpression in 25.2% of cases including all the cases with a *TCRβ-HOXA* rearrangement (8.2%). In contrast, expression of the short *HOXA10* transcript, *HOXA10b*, was almost exclusively found in the *TCRβ-HOXA* rearranged cases, suggesting a specific role for the *HOXA10b* short transcript in *TCRβ-HOXA*-mediated oncogenesis. Other molecular and/or cytogenetic aberrations frequently found in subtypes of T-ALL (*SIL-TAL1*, *CALM-AF10*, *HOX11*, *HOX11L2*) were not detected in the *TCRβ-HOXA* rearranged cases except for deletion 9p21 and *NOTCH1* activating mutations, which were present in 64 and 67%, respectively. In conclusion, this study defines *TCRβ-HOXA* rearranged T-ALLs as a distinct cytogenetic subgroup by clinical, immunophenotypical and molecular genetic characteristics.

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Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of immature T cells characterized by high numbers of bone marrow and circulating blast cells, enlargement of mediastinal lymph nodes and often central nervous system involvement.[1] T-ALL accounts for approximately 15% of pediatric and 25% of adult ALL cases. During the past 20 years, a large number of genes involved in the pathogenesis of T-ALL have been identified by molecular characterization of recurrent chromosomal aberrations and cryptic alterations.[2] Several oncogenes initially identified by rare genetic alterations were shown to be functionally activated in subsets of T-ALLs such as *LMO1*, *LMO2*, *LYL1*, *NOTCH1* in the absence of the corresponding genetic aberration. Recently, we[3] and others[4] described a new recurrent chromosomal aberration in a subgroup of T-ALLs affecting the *TCRβ* (7q34) and *HOXA* (7p15) loci. This group of patients showed a significant upregulation of particular *HOXA* cluster genes, under the influence of regulatory sequences embedded in the *TCRβ* locus. In this study,[3] especially *HOXA10* showed a significant higher expression level in the *TCRβ-HOXA* rearranged subgroup compared to non-*TCRβ-HOXA* rearranged T-ALLs. Ectopic expression of *HOXA* cluster genes was already described in other cytogenetic subgroups of T-ALL, that is, *MLL*[5,6] and *CALM-AF10* rearranged cases,[4,7,8] further underlining the importance of *HOXA* genes in T-cell oncogenesis. The mechanisms underlying this overexpression are however different. Human leukemias and cell lines carrying *MLL* rearrangements show upregulated expression of especially 5′ *HOXA* cluster genes, that is, *HOXA5*, *HOXA7*, *HOXA9*, *HOXA10* and *HOXA11*.[5,9,10] In contrast, 3′ *HOXA* genes showed low levels or no expression.[11] The transcriptional activation of *HOX* cluster genes by *MLL* was shown to be dependent on histone H3 methylation of *HOX* genes, with *HOXA9* and *HOXA7* proven to be the direct targets.[12–15] Similarly, T-ALLs carrying the *CALM-*

AF10 rearrangement show an elevated expression of especially *HOXA5*, *HOXA9*, *HOXA10* and *BMI1*,[7] suggesting that these two aberrations activate common oncogenic pathways. However, as no obvious DNA binding domain exists in *CALM*, the mechanism of *HOXA* gene activation must be different. Previous gene expression analysis on a large group of T-ALLs already showed the existence of a *HOXA*-expressing subgroup, consisting of *MLL*, *CALM-AF10* and *TCRβ-HOXA* rearranged cases.[4] Interestingly, these data pointed to the expression of a specific short *HOXA10b* transcript in the *TCRβ-HOXA* rearranged cases, which was absent in other T-ALLs. Given these findings and in order to make a comprehensive picture of patients carrying the *TCRβ-HOXA* rearrangements, we analyzed additional T-ALL patients using fluorescent *in situ* hybridization (FISH) and real-time quantitative reverse transcriptase-PCR (RT-PCR) for expression of the long *HOXA10* transcript. Cases showing overexpression of the long transcript of *HOXA10* were subsequently analyzed for expression of the short *HOXA10b* transcript, which was shown to be specifically expressed in *TCRβ-HOXA* rearranged cases. This study described the largest group of *TCRβ-HOXA* rearranged T-ALLs identified so far and interestingly showed one patient carrying a triplication of a *TCRβ-HOXA* on a ring chromosome 7, pointing to an additional mechanism of transcriptional activation of *HOXA* cluster genes. Moreover, all 14 *TCRβ-HOXA+* patients showed absence of additional molecular–cytogenetic alterations like *SIL-TAL1*, *HOX11*, *HOX11L2*, *CALM-AF10* and*NUP214-ABL1*, providing further evidence for a distinct cytogenetic entity. In contrast, deletions of 9p21 harboring the tumor suppressor genes *CDKN2A* and *CKDN2B* and *NOTCH1* activating mutations were present in 64 and 67% of *TCRβ-HOXA+* patients, pointing to a multistep oncogenesis in this cytogenetic T-ALL subgroup. Of further interest is the finding of the highly clustered breakpoints in three *TCRβ-HOXA*-positive cases and all three showed breakpoints in intron 1A from *HOXA*9 and JB2.7–JB2.1 segments of the *TCRβ* gene.

Materials and methods

Patients

Diagnostic bone marrow or pleural fluid samples from T-ALL patients were collected retrospectively at different cytogenetic centers. The only inclusion criterion was the diagnosis of T-ALL and the availability of fixed cells for FISH ($n = 424$) and/or RNA or frozen cells for real-time quantitative RT-PCR ($n = 170$ of 424). Besides newly diagnosed T-ALLs, these series of patient samples includes 229 cases analyzed in the *HOX11L2* study of the Groupe Francophone de Cytogénétique Hématologique (GFCH),[16] patients analyzed in the first study,[3] that is, 94 patients analyzed with FISH and 26 with real-time quantitative PCR, and patients of another study,[4] that is, 92 patients analyzed by FISH and 21 with real-time quantitative PCR. This total series of patients included 50% children and adults. Diagnosis of T-ALL was made according to the morphological and cytochemical criteria of the French–American–British classification[17] and by immunophenotyping.[18]

Methods
Immunophenotyping.
Immunophenotypical analyses were carried out in the respective centers according to established protocols. Blast cells were analyzed for forward/side scatter and fluorescence by BD FACS Calibur using monoclonal antibodies directed against CD34, CD33, CD13, CD2, CD3, CD5, CD7, CD1a, TdT, CD10, CD4, CD8, *TCRαβ*, *TCRγδ*, CD19 and CD20.

Karyotyping.
Diagnostic specimens were cultured and harvested for cytogenetic analysis according to established methods. Chromosome slides were G- or R-banded. Chromosomal aberrations are described according to the guidelines of an International System for Human Cytogenetic Nomenclature (ISCN 1995).[19]

Fluorescence *in situ* hybridization.
RPCI-11 (Human BAC Library) clones were selected using the bioinformatics resources available at NCBI (http://genome.ucsc.edu) and Ensembl Genome Browser (http://www.ensembl.org/). Clones were provided by the Welcome Trust Sanger Institute (Cambridge, UK) and Invitrogen (Paisley, Scotland).

Disruption of the *TCRβ or HOXA* gene locus was assessed in all 424 cases by dual-color FISH with *TCRβ* or *HOXA* flanking probes. Clones for the *TCRβ* and *HOXA* gene locus applied in the present study are shown in Figure 2. DNA isolation of bacterial artificial chromosome (BAC) clones, labelling and FISH were performed as described previously.[20] Amplification of *NUP214-ABL1* was investigated by the LSI BCR/ABL1 dual-color, ES probe (Vysis, Abbott, Ottignies, Belgium) in 137 samples from which cytogenetic cell suspension was left out. Disruption of the *MLL* genomic locus was assessed by FISH with the commercial LSI MLL dual-color, break-apart probe (Vysis, Abbott, Ottignies, Belgium) in all patients ($n = 43$) showing overexpression of *HOXA10*. Deletion of 9p21 was detected using the LSI p16 (9p21)/CEP 9 dual-color probe (Vysis, Abbott, Ottignies, Belgium) in all *TCRβ-HOXA* rearranged cases.

RNA isolation, cDNA synthesis and real-time quantitative PCR.
Frozen cells or RNA were available for expression analysis in 170 of 424 T-ALL patients. These included all *TCRβ-HOXA* rearranged cases ($n = 14$). All human samples were obtained according to the guidelines of the local ethical committees. RNA isolation from frozen cells was performed using TRIzol (Invitrogen, Merelbeke, Belgium) and the RNeasy mini kit (Qiagen, Hilden, Germany) or RNA Plus (Appligene, Illkirch, France) for detection of the *NUP214-ABL1* amplification at different laboratories. DNase pretreatment, cDNA synthesis and SYBR green real-time quantitative PCR were performed for *HOXA10* and *HOXA10b* expression as described previously.[3] Reactions were performed on an ABI Prism 5700 sequence detector (Applied Biosystems, Foster City, CA, USA). Real-time quantitative RT-PCR data analysis and expression normalization were performed using three internal control genes with the qBase data analysis software (Hellemans *et al.*, in preparation; medgen.ugent.be/qbase). Expression analysis for the full-length transcript of the *HOXA10* gene (Fw: 5′-GAGAG CAGCAAAGCCTCGC-3′; Rev: 5′-CCAGTGTCTGGTGCTTCG TG-3′) was performed in all 170 T-ALL patients. Cases showing 'overexpression' of this transcript were subsequently tested for the expression of the short *HOXA10b* transcript (Fw: 5′-GCAC TTCCGATCAATGTCAA-3′; Rev: 5′-AGCGAACAAAGGCCAAG TT-3′), or as described.[4] Cytogenetically proven *TCRβ-HOXA* rearranged cases were analyzed for the expression pattern of all *HOXA* cluster genes with primers used as described.[3]

The presence of a *NUP214-ABL1* fusion was assessed by quantitative real-time RT-PCR using the fluorescent TaqMan methodology. Four *NUP214* primers were used (X23, X29, X31, X34) (E Delabesse, personal communication) in combination with *ABL* primer (ENR 561) and probe (ENP541), previously designed for BCR-ABL transcript quantitation.[21] This PCR reaction can detect all *NUP214* breakpoints that have been described so far.[22]

Expression of *HOX11* was assessed using the primers designed by Ferrando *et al.*[23] in an SYBR green real-time quantitative RT-PCR as described previously.[24]

Long-distance PCR and cycle sequencing. For 10 patients showing a *TCRβ-HOXA* rearrangement (eight inv(7)(p15q34), one t(7;7)(p15;q34) and one triplication on a ring chromosome), DNA was extracted using the QIAmp DNA mini kit (Qiagen, Hilden, Germany). Long-distance PCR was performed using the iProof High-Fidelity PCR Kit (BioRad, Nazareth, Belgium), according to the manufacturer's instructions. Forward and reverse primers were selected in the *HOXA9* and *TCRβ, Dβ1* gene, respectively, as breakpoints of four patients were suggested to be located between the *HOXA9* and *HOXA10* gene on der (7p) and upstream from the *Dβ1* locus of the *TCRβ* gene on der (7q), respectively.[4] Subsequently, fragments were sequenced using the ABI Prism BigDye Terminator v3.0 Ready Reactions Cycle Sequencing Kit and analyzed on an ABI3730 XL Sequence detector.

RT-PCR detection assays. A standard RT-PCR protocol was used to detect the *CALM-AF10* fusion in two separate RT-PCR assays using one *CALM* forward primer (5′-GCAATCTTGGC ATCGGAAAT-3′) and either *AF10* reverse primer AF10AS559 (5′-CGATCATGCGGAACAGACTG-3′) or AF10AS1002 (5′-GCG CTTCAATGATCCAGATATAGAG-3′) as described.[8]

HOX11L2 was detected using a standard RT-PCR protocol with forward primer (5′-GCGCAT CGGCCACCCCTACCAGA-3′) and reverse primer (5′-CCGCTCCGCCTCCCGCTCCTC-3′), according to Bernard *et al.*[25]

Detection of the *SIL-TAL1* fusion was performed using a multiplex RT-PCR for simultaneous screening of multiple chromosomal aberrations in acute leukemia (HemaVision, BioRad). Primers used were *SIL* prim (5′-CGACCCCAACGTCCC AGAG-3′) and *TAL1* prim (5′-CGGTCATCCTGGGGC ATATT T-3′) and nested primers SIL nest (5′-CCCGCTCCTACCCTGC AAAC-3′) and *TAL1* nest (5′-AGACCGGCCCCTCTGAATAG-3′).[26]

NOTCH1 *mutation detection.* Mutation detection of the *NOTCH1* receptor was performed on genomic DNA in 12 out of 14 *TCRβ-HOXA*-positive cases from which DNA was available. PCR amplification of exons 26, 27 of the HD (heterodimerization) domain and of the PEST (proline, glutamate, serine and threonine) domain encoding region of exon 34 was performed with the following primer pairs: ex26F (5′-TGAGGGAGGGACCT GAACTTG-3′) and ex26R (5′-TGGAATGCTGCCTCTACTCC-3′); ex27F (5′-GTTGGTGGGTATCTGGGATG-3′) and ex27R (5′-CG GAGTGCCATTCAGAAAAT-3′); and ex34F (5′-CCATGGCTAC CTGTCAGACG-3′) and ex34R (5′-TGGCTCTCAGAACTTGCTT GT-3′). Subsequent sequencing of PCR products was performed with primers ex26seq1 (5′-GAGGGCCCAGGAGAGTTG-3′) and ex26seq2 (5′-CACGCTTGAAGACCACGTT-3′) for exon 26; ex27seq1 (5′- CGGGGGAGGAGGAAG-3′) and ex27seq2 (5′-C TGCAGGCAGAGCCTGTT-3′) for exon 27; and ex34F, ex34R and ex34seq1 (5′-GCTGCACAGTAGCCTTGCTG-3′) for exon 34.

Statistical analysis. Statistical analysis was performed using SPSS Software (SPSS Inc., Chicago, IL, USA) version 12.0. The non-parametric Mann–Whitney *U*-test (two-tailed) was used to evaluate the significance of difference in mean expression levels between the patients' subgroups (*TCRβ-HOXA* rearranged versus *TCRβ-HOXA* non-rearranged patients) for the different *HOXA* cluster genes. Differences in the expression level of a gene were considered statistically significant if *P*-value <0.05.

Results

FISH analysis was performed in 424 T-ALL cases and real-time quantitative RT-PCR in 170 of these patients. These series of patient samples includes the patients analyzed in the first two studies, that is, 94 patients analyzed with FISH and 26 with real-time quantitative PCR,[3] and 92 patients analyzed with FISH and 21 with real-time quantitative PCR.[4] This study also includes 229 patients studied in the *HOX11L2* study of the GFCH.[16]

Incidence and immunophenotypical and molecular features of TCRβ-HOXA *rearranged T-ALL*

The present study performed on 424 T-ALLs identified five patients carrying *TCRβ-HOXA* rearrangements in addition to the first two studies,[3,4] which brings the total to 14 cases (3.3% of T-ALLs studied): nine inv(7), two t(7;7) and one *TCRβ-HOXA* triplication on a ring chromosome. For the remaining two cases, no mitoses were available, which hampers a distinction between an inv(7) or a t(7;7). The median age at diagnosis was 25.7 years (range 9–49 years) and affected both men and women (M/F: 8/6), whereas children were less affected (*n* = 5 of 14, <18 years). All but three (*n* = 11 of 14) *TCRβ-HOXA* T-ALLs showed a typical CD2-negative immunophenotype, besides CD4 single positivity and low or lack of *TCRαβ* of *TCRγδ* surface expression, pointing to an immature stage of maturation arrest. Remarkably, none of these *TCRβ-HOXA* T-ALLs showed additional molecular–cytogenetic aberrations commonly observed in T-ALL (*HOX11*, *HOX11L2*, *CALM-AF10*, *NUP214/ABL1*, *SIL-TAL1*), except for 9p21 deletions, which were present in 64% (9/14) of *TCRβ-HOXA*-positive cases, most of which were mono-allelic deletions. *NOTCH1* activating mutations were present in 67% (8/12) of the *TCRβ-HOXA* T-ALLs, mostly affecting exon 26 of the HD domain and included deletions, insertions and missense mutations. PEST domain mutations were present in two cases, both were point mutations that created a premature stop codon, whereas exon 27 of the HD domain carried a missense mutation in a single patient. One patient showed both a PEST domain and an HD domain exon 26 mutation.

Karyotypic findings in TCRβ-HOXA-*positive T-ALL patients*

Conventional karyotyping showed clonal aberrations in seven out of 14 *TCRβ-HOXA* rearranged cases. Of these seven patients, an inv(7)(p15q34) was cytogenetically found in all metaphases in two patients (case no. 2 and no. 4; Figure 1), whereas two other patients showed only a del(7)(p15). Taken together, a chromosome 7 aberration was suggested in four out of 14 cases. Interestingly, case no. 10 harbored a monosomy 7 and a ring chromosome, which was shown to be derived from chromosome 7 upon further analysis. Only few additional chromosomal abnormalities were found in five patients and included add(6)(qter) (case no. 2), del(6)(q14) (case no. 5), add(5)(q31) (case no. 8), del(9)(p21) (case no. 5), del(9)(p12p24) (case no. 4) and +11 and +21 (case no. 3).
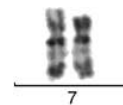

Figure 1 R-banded karyotype from patient no. 2 carrying an inv(7)(p15q34).

TCRβ-HOXA *triplication as an alternative mechanism for* HOXA *upregulation*

Based on real-time quantitative RT-PCR results for *HOXA10* and *HOXA10b* expression in 170 T-ALLs, we could identify five additional *TCRβ-HOXA* rearranged cases. Among these, there was a patient showing an interesting hybridization pattern, using FISH with *TCRβ* and *HOXA* flanking probes: the *TCRβ* (RP11-1220K2) and *HOXA* (RP5-1103I5) proximal flanking BACs showed 3–6 signals in 98% of interphase cells with deletion of both the *TCRβ* (RP11-556I13) and *HOXA* (RP1-167F23) distal flanking BACs (Figure 2). The majority of metaphases showed a triplication of these proximal flanking BACs on a ring/marker chromosome, which was already detected by karyotypic analysis (see Table 1). Further FISH analysis, using a whole chromosome paint probe, identified the ring chromosome as chromosome 7 (data not shown). Given the abnormal hybridization pattern using *TCRβ* and *HOXA* flanking FISH clones, additional FISH was performed with BACs covering the *HOXA* and *TCRβ* gene locus (Figure 2) and revealed the same hybridization pattern for clones RP11-1025G19, RP11-1132K14 (*HOXA* covering) and RP11-784K24, RP11-701D14 (*TCRβ* covering), that is, 3–6 fusion signals in both the nuclei and on the ring chromosome, whereas telomeric *HOXA* covering FISH clones showed a deletion in the majority of cells (RP11-1036C18, RP11-163M21). Finally, a FISH analysis using a combination of both proximal *TCRβ* and *HOXA* flanking FISH probes confirmed the juxtaposition and triplication of these two loci on the ring chromosome 7.

TCRβ-HOXA *rearranged T-ALL specifically express* HOXA10b

Real-time quantitative PCR for *HOXA10* expression in 170 T-ALL cases revealed an upregulated expression in 43/170 (25.2%) of cases, whereas only 14 carried the *TCRβ-HOXA* rearrangement (8.2%). Upregulation of *HOXA10* expression was defined as expression of more than the mean expression level of all samples analyzed. Given the fact that within the *TCRβ-HOXA*-positive group, there was a large fluctuation of expression levels with some samples showing very low expression (similar to thymocytes), we put the threshold for positivity sufficiently low, in order to avoid missing any true positive cases. Consequently, it can be expected that some of the cases included as positive represent normal *HOXA10* expression reflecting their stage of differentiation arrest. Five *CALM-AF10*-positive patients and two *MLL* rearranged patients, which are known to be *HOXA*-expressing T-ALL subtypes, were also included in this series. Interestingly, one patient showing *HOXA10* overexpression carried an *NUP214/ABL1* fusion. However, for the remaining 21/170 patients showing elevated *HOXA10* expression, there was a lack of evidence for one of the genetic alterations mentioned above. In 30 out of the 43 patients showing *HOXA10* overexpression, material was available to detect the presence of the *HOXA10b* short transcript. Interestingly, 16 of these 30 patients tested showed expression of *HOXA10b*, whereas expression was absent in the remaining T-ALL patients. Remarkably, these 16 *HOXA10b*-positive patients included all 14 *TCRβ-HOXA* rearranged cases and only two additional T-ALL patients lacking this rearrangement or other chromosomal defects. These data demonstrate that overexpression of this transcript is almost typically found in the *TCRβ-HOXA* rearranged cases.

HOXA *expression profiling of* TCRβ-HOXA *rearranged T-ALL*

Gene expression of the different *HOXA* cluster genes (*HOXA1*, *HOXA2*, *HOXA3*, *HOXA4*, *HOXA5*, *HOXA6*, *HOXA7*, *HOXA9*, *HOXA10*, *HOXA11* and *HOXA13*) was measured by real-time quantitative RT-PCR in 10 *TCRβ-HOXA* rearranged T-ALLs and in 21 non-*TCRβ-HOXA* rearranged
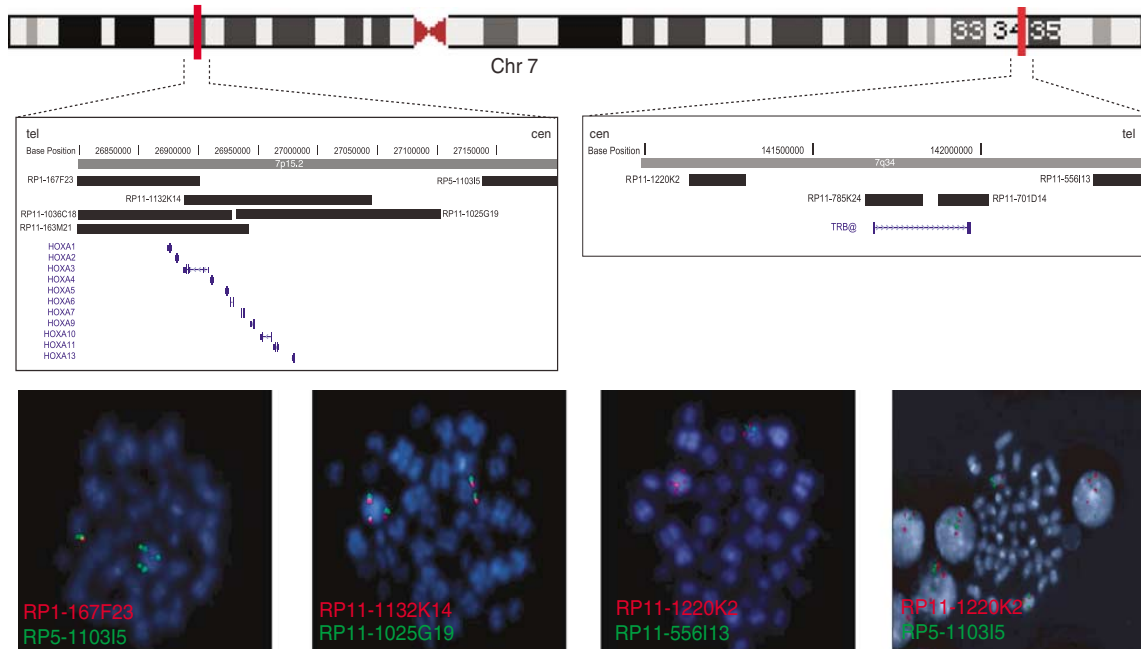

Figure 2 Genomic position of the FISH clones (black bars) flanking and covering the *TCRβ* and *HOXA* gene loci on chromosome 7; tel: telomeric; cen: centromeric. Position of *HOX* genes and *TCRβ* is indicated below clones. Lower panels: FISH results using different combinations of clones in case no. 10.

Table 1 Clinical, karyotypic and immunophenotypic findings of *TCRβ-HOXA* rearranged T-ALL

Case no.	Age	Gender	Karyotype	Revised karyotype	CD1a	CD2	CD4	CD8	TCRαβ	TCRγδ
							IF[a]			
1	35	M	46,XY, del(7)(p14p15)[4]/46,XY[16]	46,XY, t(7;7)(p15;q34)[4]/46,XY[16]	78	2	84	18	1	1
2	21	F	46,XX, add(6)(qter), inv(7)(p15q34)[20]	46,XX, add(6)(qter), inv(7)(p15q34)[20]	92	0	54	24	0	0
3	29	M	47,XY, +11[7]/47,XY, +21[4]/46,XY[1]	47,XY, +11[7]/47,XY, +21, inv(7)(p15q34)[4]/46,XY[1]	18	12	35	11	ND	ND
4	11	M	46,XX, inv(7)(p15q34), del(9)(p12p24)[29]	46,XX, inv(7)(p15q34), del(9)(p12p24)[29]	79	13	12	99	0	0
5	49	M	47,XY, del(6)(q14), del(7)(p?), del(9)(p21), +mar1[4]/46,XY[6]	47,XY, del(6)(q14), inv(7)(p15q34), del(9)(p21), + mar1[4]/46,XY[6]	5	26	48	3	6	1
6	34	M	46,XY[20]	46,XY, inv(7)(p15q34)[20]	98	0	75	1	ND	ND
7	15	M	46,XY[20]	46,XY, inv(7)(p15q34)[20]	43	0	43	0	0	0
8	27	F	46,XX, add(5)(q31)[11]/46,XX[9]	46,XX, add(5)(q31), inv(7)(p15q34)[11]/46,XX[9]	41	6	58	15	56	5
9	35	F	46,XX[4]	46,XY, inv(7)(p15q34)[4]	75	2	93	1	0	2
10	15	F	46,XY,-7, +r,inc[14]/46,XY[7]	46,XY,r(7).inc[14]/46,XY[7]	0	34	0	49	ND	ND
11	25	F	46,XX[9]	46,XX,t(7;7)(p15;q34)[3]/46,XX[6]	71	2	41	7	74	1
12	9	M	Data not available	No available mitoses: inv(7) or t(7;7)(p15;q34)	66	3	66	3	0	15
13	43	F	46,XX[9]	46,XX,inv(7)(p15q34)[9]	86	11	79	53	43	0
14	13	M	46,XY[15]	No available mitoses: inv(7) or t(7;7)(p15;q34)	1	11	38	4	7	0

Abbreviations: IF, immunophenotype; ND: not done.
Patients 11–14 were referred as patients TL43 to TL46 in Soulier *et al.*[4]
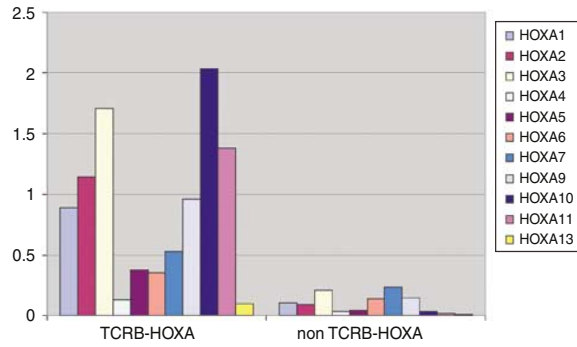[a]In percentage of blasts.

Figure 3 Geometric mean of expression levels of different *HOXA* cluster genes in *TCRβ-HOXA* rearranged cases versus non-rearranged cases.

cases. As expected, *TCRβ-HOXA* rearranged cases showed an upregulation of especially 5′ *HOXA* cluster genes compared to non-*TCRβ-HOXA* cases (Figure 3). However, upregulation of individual *HOXA* cluster genes was statistically significant only for genes *HOXA3 (P=0.007)*, *HOXA9 (P=0.0002)*, *HOXA10* ($P=0.001$) and *HOXA11* ($P=1.05 \times 10^{-5}$). Interestingly, case no. 10 carrying the *TCRβ-HOXA* fusion on a ring chromosome 7 showed an expression pattern of *HOXA* cluster genes more in favor of the 3′ located *HOXA* cluster genes (*HOXA1, HOXA2, HOXA3*) than 5′ located genes *HOXA9, HOXA10, HOXA11* and *HOXA13*. This might suggest a different breakpoint localization compared to breakpoints clustered in the *HOXA9* gene in three other *TCRβ-HOXA* rearranged cases (see below).

Clustered breakpoints in TCRβ-HOXA *rearranged T-ALL cases*

Long-distance PCR using *HOXA9* forward and *TCRβ-Dβ1* reverse primers as suggested by Soulier *et al.*[4] was successful in three out of 10 patients. This revealed amplicons with various lengths for each patient: patient 2, 2–3 kb; patient 8, 6–7 kb; and patient 9, 4–5 kb (not shown). Sequencing analysis showed that all three patients had breakpoints within a 2.9 kb region of intron 1A (total length: 3.8 kb) of the *HOXA*9 gene and within a 1.1 kb region of the *TCRβ* gene. Breakpoints within the *TCRβ* gene were located at the JB segments: JB2.7 (case no. 2), JB2.5 (case no. 8) and JB2.1 (case no. 9) (Figure 4). RSS sequences were found at the *TCRβ*, JB segments at 50–100 bp upstream from the respective breakpoints, suggesting aberrant VDJ recombination as possible mechanism leading to this translocation. The fact that this analysis was unsuccessful in the remaining seven patients, including the *TCRβ-HOXA* triplication, might suggest the existence of other breakpoints located 3′ of *HOXA9* gene or alternative *TCRβ* breakpoints.

Discussion

In this study, we report the findings of a retrospective screening of 424 T-ALL patients in search for specific clinical and biological characteristics of T-ALLs carrying a *TCRβ-HOXA* rearrangement, which we and others[4] recently described as a new cytogenetic entity.[3] This large-scale study revealed a slightly lower incidence of the abnormality than previously assumed, that is, 3.3% (14/424) compared to our first report (5%).[3] The median age at diagnosis was situated in the third decade (25.9 years; range 9–49 years) and affects both men and women (M/F: 8/6). Clinical findings were not significantly
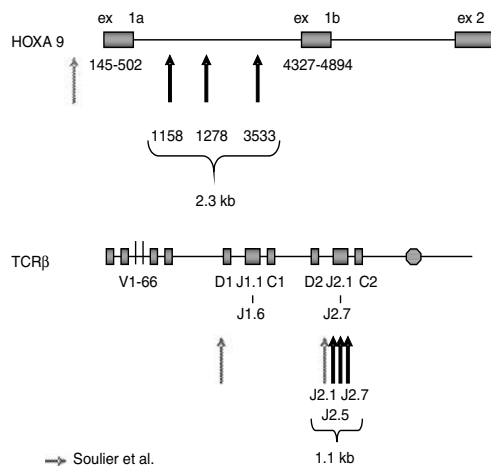
Figure 4 Genomic breakpoint position of three *TCRβ-HOXA* rearranged T-ALL (black arrows). Breakpoint positions of *TCRβ-HOXA* patients from Soulier *et al.* are indicated by white arrows.

Figure 5 Genomic structure of the *HOXA10* locus with alternative mRNA transcripts *HOXA10a* (full length) and *HOXA10b* (short transcript).

different from T-ALL in general.[27] The number of patients collected so far ($n = 14$) is rather small to make conclusions regarding survival. Nonetheless, four out of 14 patients deceased 24 ($n = 3$) and 48 ($n = 1$) months after diagnosis, which is comparable to the overall survival in T-ALL.

The reported typical immunological profile of *TCRβ-HOXA* rearranged cases (CD2−, CD4+, CD8−) was confirmed in all but three cases. T-ALLs used to be classified according to the European Group for the Immunological Characterization of Leukemias (EGIL) classification in T1 and T2 (immature T-ALL) and T3 and T4 (mature T-ALL), largely depending on the expression of CD1a.[18] With this classification, the *TCRβ-HOXA+* T-ALLs could be assigned to the group of mature T-ALLs (T3–T4). Recently, a *TCR*-based classification of T-ALLs was described, which demonstrated that T-ALLs largely reproduce normal thymic development and allowed separation of cases into *TCRαβ+*, *TCRγδ+* T-ALLs and immature/uncommitted, *TCR* and cytoplasmic *TCRβ*-negative cases.[28] Furthermore, the authors demonstrated that specific oncogenetic subclasses of T-ALL were associated with a specific, age-independent stage of maturation arrest. In line with this classification, it seems that the oncogenic pathways leading to the *TCRβ-HOXA* rearrangements are mostly situated at the immature stage of thymic development based on the lack of expression of surface *TCR*.

Interestingly, the 14 *TCRβ-HOXA* rearranged cases failed to show additional molecular/cytogenetic features frequently found in T-ALL such as *HOX11*, *HOX11L2*, *SIL-TAL1* deregulation or *CALM-AF10* rearrangements and *NUP214-ABL1* amplification. Recently, the first T-ALL patient carrying a *TCRδ-HOXA* rearrangement was described and most interestingly, this case carried a *CALM-AF10* aberration,[29] raising questions regarding mechanistic or oncogenic synergy between *CALM-AF10* and *HOXA* rearrangements. The lack of additional molecular/ cytogenetic features in the *TCRβ-HOXA* rearranged cases is in contrast to other cytogenetic subgroups like *NUP214-ABL1* amplified T-ALL, which is associated with *HOX11*, *HOX11L2* upregulation, but further suggests that the *TCRβ-HOXA* rearranged T-ALLs have a unique oncogenic pathway not shared with other known oncogenic events. However, deletions of 9p21 harboring the tumor suppressor genes *CDKN2A* (encoding p14 and p16) were present in nine out of 14 cases, suggesting a
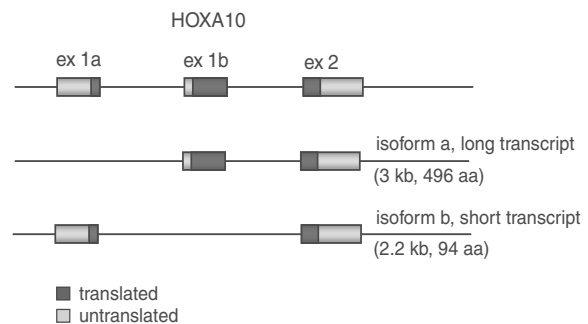
multistep pathogenesis with deletion of a tumor suppressor gene acting in concert with activation of *HOXA* proto-oncogenes. Similarly, activating *NOTCH1* mutations were present in as much as 67% (8/12) of *TCRβ-HOXA*-positive T-ALLs, which is in line with previous findings in T-ALL.[30] These findings further suggest that *HOXA* oncogenes might cooperate with *NOTCH1* in T-ALL pathogenesis.

Expression profiling of 10 *TCRβ-HOXA*-positive cases confirmed our previous findings, that is, a significant upregulation of *HOXA* cluster genes *HOXA3* ($P = 0.007$), *HOXA9* ($P = 0.0002$), *HOXA10* ($P = 0.001$) and *HOXA11* ($P = 1.05 \times 10^{-5}$), whereas another study found different levels of upregulation of all *HOXA* genes in this subgroup.[4] This discordance might be due to a different approach towards normalizing gene expression data and lack of statistical analysis. Interestingly, Soulier *et al.*[4] pointed to the presence of a specific short alternative *HOXA10b* transcript, which was exclusively present in the *TCRβ-HOXA* rearranged cases. Real-time quantitative RT-PCR for expression of *HOXA10b* revealed a specific expression in all *TCRβ-HOXA*-positive cases ($n = 14$), whereas *MLL+* and *CALM-AF10+* cases lacked expression of this alternative transcript. This finding further suggests that the *TCRβ-HOXA* subgroup of T-ALL has at least a specific oncogenic pathway not shared with other cytogenetic subgroups.

A hallmark of homeobox gene expression seems to be a high frequency of alternative splicing events leading to transcripts that would encode partial homeobox proteins lacking either the homeodomain or transcriptional regulatory domains, or containing alternative putative regulatory regions.[31,32] Alternatively, spliced homeobox-containing cDNAs from the *HOXA10* gene were cloned first from two myeloid leukemia cell lines[32] and shared the homeodomain and 3′ flanking regions but had unique 5′ flanking regions (Figure 5). Expression of the full-length transcript was detected predominantly in cell lines with a myeloid phenotype, whereas the short transcript was the major transcript in a B-cell line (CESS) and a T-cell line (MOLT). The splicing of *HOXA10* in normal bone marrow and primary samples of myeloid leukemias seemed to be different from that observed in leukemic cell lines,[33] with the full-length *HOXA10* transcript being the predominant transcript in this group, whereas immortalized cell lines contained the additional short transcript. However, in this study, no *HOXA10* expression was detected in cell lines or primary samples with lymphoid or erythroid features, whereas we found *HOXA10* expression in 25.2% of primary T-ALLs and in one T-ALL cell line (RPMI 8402). These conflicting results could be due to the small number of T-ALL samples analyzed ($n = 6$) and the lower

sensitivity of the methods used (RNase protection assay) in this study. The functional role of the N-terminal region of homeobox proteins is largely unknown. However, Zappavigna et al.[34] reported the first evidence that sequences in the N-terminal region of a HOX protein influence transcriptional activity.

Interestingly, we found one patient showing overexpression of both *HOXA10* and *HOXA10b*, which revealed a triplication of *HOXA* and *TCRβ* flanking clones with deletion of the distal clones on a ring chromosome 7. In this case, *HOXA10* overexpression was not only due to the juxtaposition of these two genes, but also due to gene dosage. Ring chromosome formation may occur by two mechanisms: (1) double-strand breaks in each arm of a chromosome with subsequent fusion of the proximal broken ends; and (2) fusion of dysfunctional telomeres from the same chromosome.[35] FISH with telomeric probes showed absence of both 7pter and 7qter in the ring chromosome, suggesting that the first mechanism was responsible for ring formation in this patient. Acquired ring chromosomes have been found in many types of human neoplasia, especially in mesenchymal tumors but infrequent in acute leukemia.[35,36] In this particular patient, the ring chromosome showed little or no size variation, and lacked telomeric sequences but carried multiple[3–6] copies of the *TCRβ-HOXA* juxtaposition. So far, proto-oncogene amplification by ring chromosome formation was mostly described in solid tumors such as dermatofibrosarcoma protuberans, which is characterized by a reciprocal t(17;22)(q22;q13) or more commonly by supernumerary ring chromosomes containing amplified sequences from chromosomes 17, 22 and 8.[37,38] In leukemia, the mechanism of proto-oncogene activation by amplification on ring chromosomes was never reported in lymphoid leukemia but only demonstrated in rare myeloid leukemia cases. These amplifications on ring chromosomes have been described in the following myeloid leukemia cases: three cases with *MLL* amplification,[39] one with *ETV6* amplification[40] and two cases with *AML1* amplification on ring chromosomes.[41] Several amplification mechanisms have been proposed, that is, looping out of extra chromosomal sequences[42] without evidence of chromosomal rearrangements, breakage–fusion–bridge cycles that can be triggered by fragile site induction[43] and a translocation–deletion–amplification model.[44,45] Most of these mechanisms rely on unequal segregation of chromosome sequences during mitosis.

Based on karyotypic analysis, the *TCRβ-HOXA* rearrangement could be readily detected in two patients with exclusively abnormal metaphases, whereas two patients showed a del(7)(p15). This suggests that the rearrangement is not fully cryptic, but detection depends on the percentage of good-quality abnormal clonal metaphases within the karyotype and the banding technique (R-banding). Additional karyotypic aberrations were found in five separate cases and included add(6) 6(qter), del(6)(q14), add(5)(q31), del(9)(p21), del(9)(p12p24), +11 and +21.

Genomic breakpoints in three out of 10 patients showed a clustered pattern in the *TCRβ* and *HOXA* gene locus and were located between the JB2.1 and JB2.7 segments at the *TCRβ* locus (1.1 kb) and within the intron 1A of the *HOXA*9 gene (2.6 kb). The fact that this could not be demonstrated in the remaining seven patients, including the *TCRβ-HOXA* triplication, might suggest the existence of other breakpoints in the *HOXA* and/or *TCRβ* regions. RSS sequences were found at the *TCRβ*, JB segments at 50–100 bp upstream from the respective breakpoints, suggesting aberrant VDJ recombination as possible mechanism leading to this translocation. This is in line with previous reports of genomic breakpoints within *TCR* genes[46,47]

suggesting aberrant VDJ recombination as the most important mechanism leading to these translocations. However, recently, a t(7;14)(p15;q11) involving *HOXA* genes on chromosome 7p and *TCRδ* genes at 14q11 failed to show RSS-like sequences on the derivative chromosome 7, suggesting that other mechanisms might be involved in this *TCR* rearrangement.[29]

In conclusion, the present study covers the largest group of *TCRβ-HOXA* rearranged T-ALLs identified so far and summarizes clinical, immunophenotypical and molecular genetic characteristics of this subgroup. Most interestingly, this series includes the first case of oncogene triplication by ring chromosome formation in T-ALL, that is, triplication of the *TCRβ-HOXA* fusion, probably as a secondary genetic event subsequent to chromosomal rearrangement.

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CHAPTER II:

Paper 4:

> **_HOXA_ cluster deregulation by a _TCRδ-HOXA_ chromosomal translocation in a _CALM-AF10+_
> T-ALL.**
> Leukemia, 2006, Jun 20(6):1184-1187. (SCI= 6.6)

HOXA cluster deregulation in T-ALL associated with both a *TCRD-HOXA* and a *CALM-AF10* chromosomal translocation

Recognized T-cell acute lymphoblastic leukemias (T-ALLs) oncogenic pathways include transcriptional deregulation of oncogenes by juxtapositioning to the T-cell receptor β (*TCRB*) or α/δ (*TCRA/D*) enhancer loci, resulting in overexpression of genes such as *LMO2*, *LMO1*, *LYL1*, *TAL1/SCL*, and homeodomain genes such as *TLX1/HOX11*, *TLX3/HOX11L2* or *HOXA*. A V(D)J error-type mechanism has been proposed to explain such recurrent chromosomal events but other processes are likely to intervene, particularly those mediating the chromosomal break at the locus bearing the proto-oncogene.[1] Evidence in favor of V(D)J-mediated errors include the presence of non-templated nucleotides (n-diversity) at the chromosomal junction and of recognition signal sequences (RSS) or RSS-like sequences at the vicinity of the breakpoints on the derivative chromosomes, although putative RSS-like sequences are usually incomplete.

The *HOXA* gene cluster on chromosome 7p15 has recently been described as a new recurrent *TCRB* partner in T-ALL.[2,3] In cases with *TCRB-HOXA* translocation, a variable but consistent combination of individual *HOXA* genes on both sides of the breakpoint appears to be upregulated. The juxtapositioning of *TCRB* enhancer elements and the *HOXA* locus disruption in itself might deregulate the whole cluster expression. In other cases, *trans*-regulating factors can deregulate the whole *HOXA* cluster. The MLL protein directly regulates *HOX* family genes[4] and chimeric MLL oncoproteins are recognized to mediate *HOX* deregulation in AML and B- and T-lineage ALL.[5] T-cell acute lymphoblastic leukemia with *CALM-AF10* fusions also demonstrate *HOXA* cluster upregulation,[2,6] but the mechanisms mediating gene deregulation in these cases remain to be explored.

We here describe cytogenetic and molecular findings in the first case of T-ALL with both *CALM-AF10* and a t(7;14)(p15;q11), juxtaposing the *HOXA* cluster to the *TCRD* Jδ1 gene segment.

A 29-year-old male patient with hepatosplenomegaly but no mediastinal involvement was diagnosed with T-ALL at Purpan Hospital (Toulouse, France). White blood cell count was 42×10^9/l. Immunophenotyping and real time quantitative-polymerase chain reaction (RQ-PCR) revealed a TCR$\gamma\delta$+, CD4/8 double positive, CD1a- phenotype expressing high levels of *RAG-1* and pre-T-cell receptor alpha (*PTCRA*) transcripts, previously reported as UPN 3749.[7] Karyotype analysis showed 46,XY, t(7;14)(p15;q11),t(10;11)(p13–14;q14–21),add(18)(q23) [11]/46,XY[9]. RT-PCR analysis revealed the presence of a *CALM-AF10* fusion as predicted by the t(10;11)(p14;q21).[7]

Southern blot analysis of DNA extracted from a bone marrow sample at diagnosis revealed two rearranged *Bgl*II-digested Jδ1 fragments (data not shown). Multiplex *TCRD* PCR showed one clonal Vδ1–Jδ1 rearrangement but the second rearranged allele was not identified, and as such did not correspond to a classical *TCRD* rearrangement.

*Pvu*II digestion and Jδ1 amplification by ligation-mediated-polymerase chain reaction (LM-PCR) generated a product of approximately 350 bp (Figure 1a), which on sequencing was shown to contain a Jδ1–*HOXA* junction, with the intact start of Jδ1 separated by nine n-diversity nucleotides from an intronic fragment of chromosome 7, located between the *HOXA6* and *HOXA7* genes. A specifically designed PCR confirmed the breakpoint sequence (Figure 1b).

To search for the derivative breakpoints, the ligated *Pvu*II-digested DNA was amplified with the Der(14)S-specific primer, corresponding to the translocated telomeric part of chromosome 7. Two fragments of 600 and 700 bp were obtained (Figure 1c). Sequencing revealed that the 600 bp fragment corresponded to the genomic sequence of chromosome 7, whereas the 700 bp fragment showed chromosome 7 rearranged with the δREC1 segment on chromosome 14. A specifically designed primer pair confirmed the junction (Figure 1b) and revealed that the leukemic clone had already undergone Dδ2–Dδ3 rearrangement.
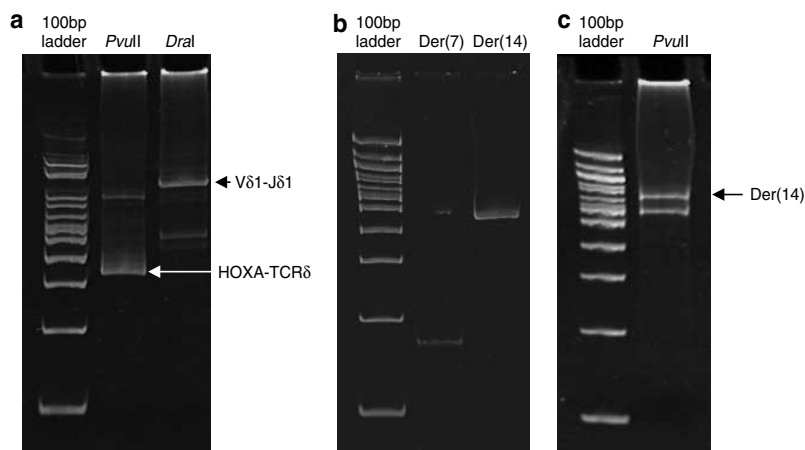

Figure 1 (**a**) Run of *Dra*I and *Pvu*II-digested LM-PCR product using the Jδ1-specific primer. The t(7;14) junction was amplified from the *Pvu*II-digested fragment appearing around 350 bp. The other *Dra*I-digested product of interest measuring around 1 kb represents the Vδ1–Jδ1 rearrangement of the second allele. The two products for the *Dra*I and *Pvu*II digestions appearing around 450 and 900 bp represent the expected germline products of 458 and 827 bp, respectively, amplified from the background population. (**b**) Direct PCR designed to amplify the der(7) and (14) junctions showing products of expected length. (**c**) Run of *Pvu*II-digested LM-PCR product using the DerS specific primer. The 600 bp product corresponds to the genomic chromosome 7 sequence. The 700 bp product corresponds to the der(14).

Figure 2 The nucleotide sequence of both derivatives implicated in the t(7;14) translocation. Underscored are recognition signal sequences (RSS) or RSS-like sequences in the vincinity of the breakpoints. Lowercase letters denote non-templated nucleotides at the junction.

Figure 3 Sequence of events leading to the final translocation. Exons are represented by boxes. Triangles represent recognition signal sequences and show their orientation. E = enhancer.

The translocation was balanced, with loss of only a single nucleotide from chromosome 7 (Figure 2). The δREC-chromosome 7 translocation most probably happened during attempted Dδ3–Jδ1 joining (Figure 3). The addition of n-nucleotides at the junctions is compatible with a V(D)J error-type translocation mechanism. However, the heptamer-like sequence in the vicinity of the der(7) breakpoint diverges from the consensus heptamer sequence.

This case is of particular interest as it represents the first description of a *TCRD-HOXA* translocation, as all nine previously reported cases of T-ALL with *HOXA* cluster translocations involved the *TCRB* locus. The *HOXA* breakpoint between *HOXA6* and *HOXA7* in the present case is telomeric to the four *TCRB-HOXA* cases, which clustered in a 2.6-kb region between *HOXA10* and *HOXA9*,[2] and to the three additional *TCRB-HOXA* cases where the breakpoint was clustered in intron 1A of the *HOXA9* gene locus (Cauwelier et al., unpublished

data). The relative incidence of *TCRB* and *TCRD-HOXA* translocations needs to be tested by Fluorescence *in situ* hybridisation (FISH), as *TCRB-HOXA* translocations are difficult to detect on classical caryotyping. The localization of the *TCRD* and *HOXA* loci makes cryptic t(7;14)(p15;q11) unlikely if mitosis is representative of the leukemic clone.

As for other TCR translocations, to what extent TCR-*HOXA* rearrangements represent V(D)J errors is not yet clear. Soulier *et al.* described two heptamer-like sequences beside the *HOXA* breakpoints on the derivative chromosome 7. In our case, junctional diversity at the der(7) junction and at the δRec–Dδ2–Dδ3 junction on the der(14) is in keeping with RAG-induced events. However, the heptamer-like sequence 5' to the breakpoint on chromosome 7 is not concensus (underscored in Figure 2). It can therefore be postulated that the strand break on chromosome 14 and the illegitimate ligation to chromosome 7 are RAG-induced, whereas the mechanism initiating the break

on chromosome 7 remains to be defined. Although illegitimate V(D)J recombination has been proposed to be involved in most Ig/TCR–proto-oncogene recurrent lymphoid translocations, functional models do not always support this assumption and suggest that for certain recurrent translocations the breaks at the non-immune locus are initiated by other, as yet unknown, mechanisms.[1] The present case is likely to belong to this category.

As expected, FISH analysis confirmed the colocalization of *TCRD* and *HOXA* probes in six of eight mitosis and in 80% of interphase nuclei. The *CALM-AF10* translocation was confirmed by FISH in more than 80% of interphase nuclei, thus demonstrating the existence of the two translocations in the vast majority of cells and excluding the possibility of two distinct clones (Figure 4a and b), in accordance with the karyotype showing both translocations in 11 mitosis. Given that *CALM-AF10+* T-ALL overexpress HOXA genes,[2,6] albeit by an unknown mechanism, it was surprising to find another *HOXA* genetic abnormality in the same leukemic clone.

We therefore analyzed *HOXA* transcript levels in order to assess the relative impact of *CALM-AF10* and *TCRD-HOXA* on
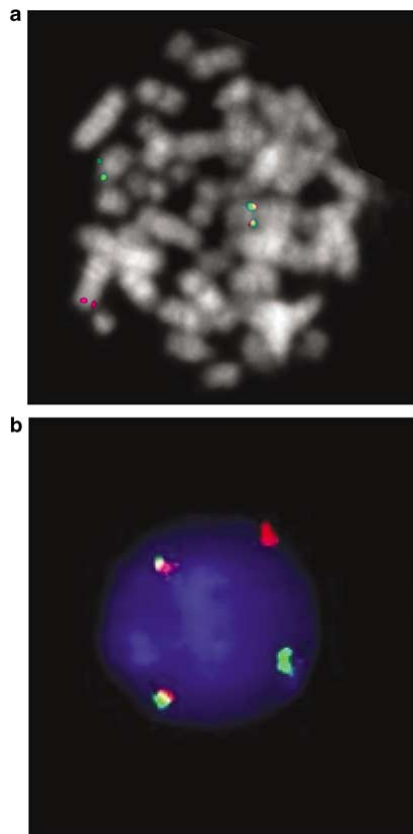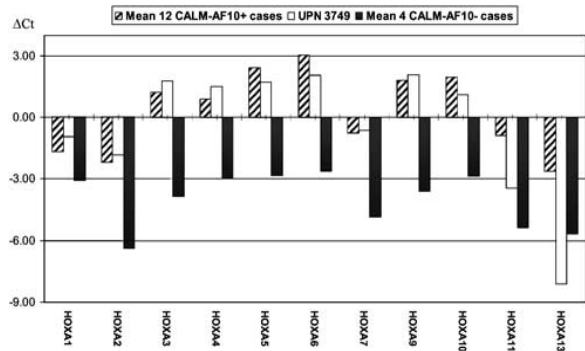
Figure 5 RQ-PCR quantification of the *HOXA* transcripts. Absolute ΔC_t (cycle threshold) value $= TBP\ C_t - HOXA\ C_t$.

HOXA overexpression. We compared transcript expression of the whole *HOXA* cluster in this case to a series of 12 other *CALM-AF10+* T-ALL with no evident karyotypic abnormalities involving chromosome band 7p15 (Figure 5). As expected, *HOXA* cluster genes were overexpressed although the presence of the *HOXA-TCRD* translocation did not give rise to a clearly distinct pattern of *HOXA* gene expression in comparison to *CALM-AF10+* T-ALL without apparent *TCRD-HOXA* rearrangement, in particular with respect to the *HOXA6* and *HOXA7* genes adjacent to the breakpoints. This would suggest that rupture of the *HOXA* locus by *TCRD* is sufficient to lead to generalized *HOXA* deregulation on both sides of the breakpoint, not restricted to the genes adjacent to the breakpoint, as previously described for *HOXA-TCRB*.[2] *HOXA* expression may therefore be deregulated by elements other than the *TCRD* (or *TCRB*) enhancers. Alternatively, as this case also demonstrates a *CALM-AF10* fusion, the *HOXA* profile may be determined in this case by *CALM-AF10* rather than *HOXA-TCRD,* thus explaining the absence of distinct *HOXA* profiles when compared with 'isolated *CALM-AF10*' T-ALLs. This is compatible with a predominant *trans*-mediated influence of *CALM-AF10* on *HOXA* deregulation, which a *cis*-mediated effect of the *TCRD-HOXA6/7* juxtaposition does not over-ride.

This case expressed both a surface TCRγδ receptor and *PTCRA*, which is normally restricted to the TCRαβ lineage. We have previously shown that TCRγδ+ PTCRA+ T-ALLs often demonstrate deregulation of orphan homeobox-like genes (*HOX11/TLX1* or *HOX11L2/TLX3*)[8] and that these cases are characterized by high RAG-1 levels, which suggest active, but unproductive V(D)J activity with respect to a complete TCR. Such a scenario is conducive to V(D)J errors, as described here for the δRec-Jδ-HOXA rearrangement identified in the majority of the leukemic clone.

In conclusion, we report that *HOXA* deregulation by TCR juxtapositioning is not restricted to *TCRB*, with impact for screening practice. The coexistence of *HOXA-TCRD* and *CALM-AF10* in this case does not modify the *HOXA* deregulation profile observed in *CALM-AF10* T-ALLs. This would suggest a dominant *trans*-mediated mechanism of *HOXA* deregulation by *CALM-AF10*, which merits to be explored.

Figure 4 (**a**) Fluorescence *in situ* hybridization (FISH) result using *TCRA/D* distal (green) and *HOXA* proximal (orange) FISH probes showing a fusion signal in six of eight mitosis. (**b**) FISH result showing two *CALM-AF10* fusion signals.

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CHAPTER III: GENERAL DISCUSSION

General discussion

The purpose of the present study was to screen a large number of T-ALLs to search for cryptic, chromosomal aberrations targeting T cell receptor loci (TCR) and eventually detect putative new T cell oncogenes. During previous work on childhood ALL from our group, a cryptic chromosomal rearrangement affecting the *TCRB* locus (7q34) in a childhood T-ALL patient was detected [1]. This observation was the trigger to study a large series of childhood and adult T-ALL patients to search for additional cryptic rearrangements and possibly new target genes of especially the *TCRB* locus.

In a first study, 94 T-ALLs were analyzed using **FISH** with *TCRB* flanking FISH clones [2]. This analysis uncovered the presence of a new recurrent chromosomal aberration affecting the *TCRB* gene locus (7q34) and the *HOXA* gene locus (7p15) in an inversion, inv(7)(p15q34), in 5 of 94 patients. To further delineate the respective breakpoints, *TCRB* and *HOXA* covering FISH clones were carefully selected and applied to all 5 inv(7) patients, which refined the breakpoints within the *HOXA* gene cluster between the *HOXA5* and *HOXA10* gene (12kb). To further address the clinical, immunophenotypical and molecular genetic findings of this chromosomal aberration, we studied 330 additional T-ALLs. This revealed **TCRB-HOXA rearrangements** in 5 more patients and together with 4 patients identified in the study from Soulier [3], brings the total to 14 in 424 patients screened (3.3%) [4]. Nine cases were **inv(7)(p15q34)**, whereas two patients showed a **t(7;7)(p15;q34)** and one case with **TCRB-HOXA triplication** due to ring chromosome formation. This interesting observation is discussed in detail below. For the two patients left, no metaphases were available which hampers a distinction between an inv(7) and a t(7;7). Cloning of the breakpoints was successful in three cases and unraveled clustered breakpoints within the *HOXA* gene cluster (intron1a of *HOXA9*; 2.3 kb region) as well as in the *TCRB* gene locus (J*B*2.1 to J*B*2.7; 1.1kb region). This was slightly different to the breakpoints found in 4 patients carrying the *TCRB-HOXA* rearrangement in the study of Soulier et al. [3]. They characterized the breakpoints within the *HOXA* cluster 3' to the *HOXA10* gene and within intron 1b of *HOXA9* and within the *DB1* gene of the *TCRB* locus respectively. Nevertheless, the region encompassing the *HOXA10* and *HOXA9* genes remains the major breakpoint cluster region.

This initial study also demonstrated the unexpected high frequency of *TCRB* rearrangements in as much as 20% of T-ALLs (19/94) screened. This finding was confirmed in a second study where we analyzed 32 additional patients (total n=126) and 19 T-ALL cell lines for chromosomal aberrations affecting *TCRB* locus and the other TCR loci (*TCRaδ* and *TCRγ*) [5]. *TCRB* chromosomal rearrangements were found in 19% (24/126) whereas only 3.1% (4/126) of these aberrations were detected upon conventional karyotyping. In five other patients, partial deletions or additional material of chromosome 7 were found, suggesting a chromosome 7 alteration in 3.9 % (5/126). However, 11.1% (14/126) of these *TCRB* alterations remained undetected upon previous banding analysis. In other words, within the group of *TCRB* rearranged cases (n=24), most rearrangements remained undetected (58.3%; 14/24) when excluding failures. This high percentage of cryptic *TCRB* rearrangements could be partly explained by the distal localization of the *TCRB* locus (7q34)

together with a distal chromosomal position of the partner genes involved namely inv(7)(p15q34), t(7;7)(p15;q34), t(7;11)(q34;q24) and t(7;10)(q34;q24). Given these remarkable results on rearrangements affecting the *TCRB* locus, we also tested the involvement of the two other TCR loci (*TCRaδ*: 14q11 and *TCRγ*: 7p15) in this series of patients and T-ALL cell lines using flanking FISH probes for these respective loci. This resulted in the first assessment of the incidence of chromosomal TCR rearrangements in T-ALL [5]. Similarly to the *TCRB* locus, breakpoints in the *TCRaδ* locus were detected in 17.4% using FISH whereas standard cytogenetics only detected 9.4% *TCRaδ* rearrangements. Given the proximal chromosomal position of this locus (in contrast to the *TCRB* locus) these rearrangements should be readily detectable on G-banding analysis. Some of these rearrangements remained undetected due to the involvement of chromosome 14 in more complex karyotypes, whereas two cases showed a marker chromosome possibly harboring the derivative chromosome 14. In line with previous reports, no *TCRγ* rearrangements were observed in this series. Apparently, these *TCRγ* rearrangements are only detected in T-cell tumors in patients with ataxia telangiectasia (ATM) where these rearrangements are frequently found [6]. A possible explanation for the high incidence of *TCRB* and *TCRaδ* rearrangements and the lack of alterations affecting the *TCRγ* locus could be the length of the genes (*TCRaδ*: 1,000 kb; *TCRB*: 620 kb; TCRγ: 160 kb) with greater susceptibility to double strand breaks and subsequent translocations along with increasing length of the respective gene.

This study also demonstrated the involvement of possible **new putative T-cell oncogenes** at different chromosomal positions: chromosome 11q24, 20p12, 20q12 and 6q22. Another interesting observation was the presence of rearrangements of both *TCRaδ* and *TCRB* loci in six patients and one cell line. Five of these patients were children, possibly reflecting the higher susceptibility for errors in VDJ recombination as a consequence of greater antigen exposure during childhood. In four patients, involvement of different T-cell oncogenes by both *TCRB* and *TCRaδ* genes was confirmed using FISH with the appropriate flanking BAC clones. Interestingly, two patients carried a translocation between the two TCR loci. Rearrangements between two TCR loci, *TCRγ* and *TCRaδ*, have been reported in a high frequency in ATM patients. This aberration has also been found in T-lymphocytes from healthy individuals at very low frequencies, suggesting that rearrangements affecting both TCR loci are not merely chromosomal aberrations associated with tumorigenesis but could represent the capacity of the recombinase system to enlarge immune diversity [7, 8].

Besides the inversion, inv(7)(p15q34) and translocation, t(7;7)(p15;q34) cases, we also found a patient carrying a **triplication of the *TCRB-HOXA*** fusion on a ring chromosome 7 [5]. This is the first report of *HOXA10* and *HOXA10b* overexpression in a *TCRB-HOXA* rearranged case not only due to juxtaposition of these two genes, but also to gene dosage. Ring chromosome formation may occur by double strand breaks in each arm of a chromosome with subsequent fusion of the proximal broken ends or by fusion of dysfunctional telomeres from the same chromosome. Given the absence of both telomeres 7q and 7p in our patient we assume the first mechanism was responsible for ring formation in our patient. Acquired ring chromosomes have been found in many types of human neoplasia, especially in mesenchymal tumors but infrequent in acute leukemia [9, 10]. In leukemia, the

mechanism of proto-oncogene activation by amplification on ring chromosomes was only demonstrated rarely in myeloid cases but not in lymphoid leukemia so far.

Gene expression analysis showed consistent increased expression of especially *HOXA10* and *HOXA11* in all *TCRB-HOXA* positive cases compared to T-ALL cases lacking this rearrangement [4]. Given the breakpoint position within or 3' to the *HOXA9* gene and the sustained high expression of the 5' located *HOXA10* and *HOXA11* genes, we propose two possible mechanisms leading to this aberrant *HOXA* gene expression. *First*, the disruption of the *HOXA* gene cluster could interfere with the normal program of sequential up and downregulation following the 3' to 5' localization of the *HOXA* genes with 3' genes being expressed in the more primitive cells and 5' genes in more differentiated cells. Evidence for involvement of *HOXA* genes in developing thymocytes came from a study from Taghon et al. [11] where high *HOXA10* expression was found in the earliest T cell precursors in human thymus and showed subsequent downregulation in CD4 and CD8 single positive mature T cells. Physical disruption of the *HOXA* gene cluster could therefore block downregulation of *HOXA10* and *HOXA11* required to allow cells to complete their differentiation and maturation. The *second* mechanism that could explain high *HOXA10* and *HOXA11* expression in these patients, is that enhancers embedded within the *TCRB* locus lead to sustained high expression levels of *HOXA10* and *HOXA11*. Given the 10-fold higher expression levels of *HOXA10* and *HOXA11* observed in the *TCRB-HOXA* positive patients compared to normal developing thymocytes, we favor the second hypothesis.

As mentioned in the introduction, *HOX* genes play a significant role not only in regulating body formation but also in organization and regulation of hematopoiesis and leukemogenesis. First evidence for involvement of *HOX* genes in human leukemia came from the identification of translocations involving the nucleoporin gene (*NUP98*) and *HOXA9* in rare cases of AML and subsequently a number of *NUP98* fusions with other *HOX* genes have been described [12-14]. Until recently, a role for *HOXA* cluster genes in T-ALL was only suggested by Ferrando et al. [15] who demonstrated increased expression levels of a subset of *HOX* genes in *MLL* rearranged T- and B-ALL. In these cases however, the transcriptional activation of *HOXA* cluster genes was a direct effect of *MLL* since both *HOXA7* and *HOXA9* were proven to be direct targets. First evidence for a direct role of *HOXA* genes in T-ALL was demonstrated in our initial study [1] and, together with previous studies that showed impairment of final T cell differentiation upon enforced *HOXA10* expression in cord blood cells, strongly suggest that the *HOXA* gene cluster is of crucial importance in normal T cell development. Shortly after we published the new recurrent cytogenetic abnormality involving the *HOXA* and *TCRB* gene locus with elevated *HOXA* gene expression, an other cytogenetic T-ALL subgroup was described showing elevated *HOXA* gene expression namely the *CALM-AF10* positive T-ALL with the t(10;11)(p12;q14-q21) [16]. Gene expression data of a large group of T-ALLs further confirmed the existence of a *HOXA* expressing group of T-ALLs consisting of *MLL, CALM-AF10* and *TCRB-HOXA* rearranged cases [3]. All these findings support the importance of *HOXA* genes not only in myeloid but also in lymphoid leukemogenesis.

Given the significant transcriptional upregulation of especially *HOXA10* and *HOXA11* in patients carrying the *TCRB-HOXA* rearrangement, we further analyzed 170 T-ALL patients (including

14 *TCRB-HOXA* positive patients) for the expression of *HOXA10* in order to validate the association of this chromosomal rearrangement and ectopic *HOXA10* expression [5]. This revealed overexpression of *HOXA10* in 25.2% (43/170) of cases, whereas only 8.2% (n=14) carried the *TCRB-HOXA* rearrangement. In 4.2% of *HOXA10* overexpressing cases, either *CALM-AF10* or *MLL* rearrangements were present. For the remaining 12.8% of patients with ectopic *HOXA10* expression, the exact mechanism of *HOXA* deregulation remains unclear. One possible explanation is that some of the cases included as *HOXA10* positive, represent normal *HOXA10* expression reflecting their stage of differentiation arrest. Therefore, *HOXA10* expression is not a useful screening marker for *TCRB-HOXA* rearrangements. Interestingly, gene expression data from the group of Soulier [3], pointed to the expression of an alternative short *HOXA10* transcript, *HOXA10b* in the subgroup of *TCRB-HOXA* positive cases. We therefore analyzed all *HOXA10* expressing T-ALLs for expression of this short transcript which was detected in all 14 *TCRB-HOXA* positive cases and could not be detected in *MLL* and *CALM-AF10* positive T-ALLs [5]. As a consequence, expression of this short *HOXA10b* transcript could serve as a screening marker for the *TCRB-HOXA* aberration. Alternative splicing seems to be a hallmark of homeobox gene expression, leading to transcripts that encode partial homeobox proteins, lacking either the homeodomain, transcriptional regulatory domains, or containing alternative regulatory regions. Alternatively spliced homeobox-containing cDNAs from the *HOXA10* gene were first cloned from two myeloid leukemia cell lines [17]. These two cDNAs share the homeodomain and 3' flanking regions but have unique 5' flanking regions. The full length transcript could be detected in normal bone marrow and primary myeloid leukemic samples, while immortalized cell lines contained the additional short *HOXA10b* transcript, suggesting a role for *HOXA10b* in immortalization. Differences in biological functions have been suggested for many alternatively spliced homeobox genes but Zappavinga et al.[18] for the first time provided evidence that sequences in the N-terminal region of a *HOX* protein influence transcriptional activity.

Based on karyotypic analysis using R banding techniques, the *TCRB-HOXA* rearrangement could only be detected in two out of 14 patients carrying an inv(7)(p15q34). The aberration was suggested in two other patients by the presence of a del(7)(p15). Additional random clonal aberrations were found in 5 patients such as: add(6)(qter), del(6)(q14), add(5)(q31), del(9)(p21), +11 and +21, which might suggest the need for other genetic alterations to generate an overt leukemia. Other molecular-cytogenetic features frequently found in T-ALL such as *HOX11, HOX11L2, SIL-TAL1* deregulation*, CALM-AF10* rearrangement or *NUP214-ABL1* amplification were absent in this series. The lack of these additional molecular-cytogenetic features in this cytogenetic subgroup is in contrast to other T-ALL subgroups like *NUP214-ABL1* amplified T-ALL which is associated with *HOX11* or *HOX11L2* upregulation. This suggests that the *TCRB-HOXA* rearranged T-ALLs have a different oncogenic pathway not shared with other known oncogenic events. Deletions of 9p21 (*CDKN2A* and *CDKN2B*) and activating *NOTCH1* mutations however, were found in a high proportion (64% and 67% respectively) of *TCRB-HOXA* rearranged cases suggesting a multistep pathogenesis with deletion of tumor suppressor genes (9p21) or activated *NOTCH1* signaling cooperating with *HOXA* oncogenes.

HOXA deregulation by TCR juxtapositioning is not restricted to the *TCRB* locus as we detected the first case of ***TCRδ-HOXA*** rearrangement in a *CALM-AF10* positive T-ALL [19]. The

breakpoint within the *HOXA* cluster was situated between the *HOXA6* and *HOXA7* genes, which is telomeric to the clustered breakpoints in the *TCRß-HOXA* cases. In these cases, the breakpoints were clustered in a 2.6 kb region between *HOXA9* and *HOXA10* [3] and within intron 1a of *HOXA9* [4]. Similarly to the *TCRß-HOXA* positive cases, *HOXA* cluster genes were overexpressed under the influence of enhancers within the *TCRδ* locus. Given that *CALM-AF10* positive T-ALLs overexpress *HOXA* genes, it was surprising to find another genetic abnormality disrupting the *HOXA* gene cluster in the same leukemic clone. In order to assess the relative impact of *CALM-AF10* and *TCRδ-HOXA* aberrations on *HOXA* overexpression, *HOXA* transcript levels of *HOXA1* to *HOXA13* of *CALM-AF10+* cases were compared to expression levels in this particular patient. Both showed a similar upregulated expression of *HOXA3* to *HOXA6*, *HOXA10* and *HOXA11*. This would suggest that rupture of the *HOXA* locus by *TCRδ* is sufficient to lead to generalized *HOXA* deregulation on both sides of the breakpoint and not restricted to the genes adjacent to the breakpoint as we described for the *HOXA-TCRß* rearrangement. *HOXA* expression may be regulated by elements other than the TCR enhancers (cis-mediated) like the trans-mediated influence of the *CALM-AF10* fusion which overrides the cis-mediated effects.

Our study on **clinical, cytogenetic and molecular characteristics** of the *TCRß-HOXA* rearranged cases [4] showed a median age at diagnosis of 25.9 years (range 9-49 y) which affects both men and women (M/F: 8/6) whereas *TCRß* negative T-ALL showed a lower age of onset (median 16.6 years)(P=0.07) and a pronounced male predominance (M/F: 12:1) (P=0.07). Clinical findings were not significantly different from other T-ALL. Immunophenotypically, T-ALL can be classified into T1,T2 (immature) and T3,T4 (mature) subtypes using the EGIL classification [20] which is largely depending on the expression of CD1a. The *TCRß-HOXA* rearranged T-ALLs could be assigned to the group of mature T-ALLs (T3-T4) based on the surface expression of CD1a (T3) and sCD3 (T4) but showed a characteristic immunophenotypic profile of CD2-, CD4+, CD8- and TCR- which is more in favor of a differentiation stop in an immature T cell. Recently, a TCR based classification of T-ALLs was described which demonstrated that T-ALLs largely reproduce normal thymic development and allow separation of cases into TCRαß+, TCRγδ+ T-ALLs and immature, sTCR (surface TCR) negative and cTCRß (cytoplasmic TCRß) negative cases [21]. Furthermore, the authors demonstrated that specific oncogenetic subclasses of T-ALL (*HOX11, HOX11L2, SIL-TAL1* and *CALM-AF10*) were associated with a specific, age-independent stage of maturation arrest with *TAL1* and *HOX11* being specific to the αß lineage, *HOX11L2* belonging to an intermediate αß/γδ population and *CALM-AF10* specific to the γδ lineage [22]. In line with this classification, it seems that the oncogenic pathways leading to the *TCRß-HOXA* rearrangements are almost exclusively situated at the immature stage of thymic development based on the lack or weak expression of sTCR.

In conclusion, our data for the first time point to a role for class I homeobox genes in T-cell oncogenesis and strongly support the previously proposed role for *HOXA* genes in thymocyte development.

Our results offer several **future** research **perspectives**. First of all, given the finding of a new recurrent cytogenetic aberration in T-ALL, it is indicated that all newly diagnosed T-ALLs should be checked for the presence of a *TCRB-HOXA* rearrangement. This should allow us to make eventual correlations or predictions towards treatment outcome and survival. Secondly, previous undetected partner chromosome regions such as 6q22, 11q24, 20p12 and 20q12 need to be analyzed in detail which could result in the detection of new genes involved in T-cell oncogenesis. Thirdly, an intriguing finding is the overexpression of *HOXA10* in as much as 12.8% of T-ALLs with unknown underlying mechanism. Further studies could reveal other mechanisms of transcriptional activation besides the known cis- and trans-mediated activation pathways.

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Samenvatting

T-cel acute lymfoblasten leukemie (T-ALL) is een aggressieve maligniteit uitgaande van de voorlopers van de T cel en komt zowel voor bij kinderen (10-15% van de kinderen met ALL) als bij volwassenen (25% van volwassenen met ALL). Het is een vorm van leukemie die in meerdere aspecten afwijkt van de veel vaker voorkomende B-cel ALL en een minder gunstige overleving kent niettegenstaande talrijke ontwikkelingen van nieuwe behandelingsstrategieën gedurende de laatste 50 jaar. Zoals andere vormen van leukemie ontstaat deze vorm van kanker door een opeenstapeling van genetische veranderingen (mutaties) in een hematopoietische voorlopercel. De meest frequente mutaties bij acute leukemie vormen de chromosomale herschikkingen waarin genen zijn betrokken die coderen voor transcriptiefactoren. Bij T of -B cel lymfoïde leukemieën komen deze transcriptiefactoren vaak onder invloed van regulatorische sequenties gelegen in genen die coderen voor de T-cel (TCR) of immunoglobuline receptoren (IG). Als gevolg van deze structurele herschikking ontstaat vervolgens een ongecontroleerde transcriptionele opregulatie van deze transcriptiefactoren.

In de voorbije 20 jaar zijn talrijke studies uitgevoerd die de diagnostische en prognostische waarde van het detecteren van chromosomale herschikkingen hebben aangetoond. Bovendien vormt het cytogenetisch onderzoek vaak de eerste stap in de richting van het identificeren van kandidaatgenen die mede verantwoordelijk zijn voor het ontstaan van een bepaald soort leukemie. Ondanks het belang van het cytogenetisch onderzoek dient rekening gehouden te worden met de beperkte resolutie (10Mb) waardoor een aantal chromosoomherschikkingen kunnen worden gemist. Fluorescentie in situ hybridisatie (FISH) kan een antwoord bieden op de beperkte resolutie van het klassieke chromosomenonderzoek en vormt dan ook een belangrijk onderdeel in de diagnostiek en onderzoek van hematologische maligniteiten.

In de huidige studie hebben wij ons gefocuseerd op het ontdekken van nieuwe oncogenen in een weinig frequente subklasse van leukemie, T-ALL. Een vorige M-FISH studie uitgevoerd bij kinderen met ALL en complexe chromosoomafwijkingen leidde tot de ontdekking van een cryptische chromosomale herschikking van de *TCRB* locus (7q34) en een nog onbekend oncogen gelegen op chromosoom 11q24 in een translocatie t(7;11)(q34;q24). Dit was de aanzet tot het screenen van een grote groep T-ALL patiënten (n=94) met het oog op het detecteren van additionele cryptische herschikkingen van deze *TCRB* locus met behulp van de fluorescentie in situ hybridisatie (FISH) techniek. Uit deze studie bleek dat herschikkingen van de *TCRB* locus veel vaker voorkomen (20%) dan aangenomen op basis van conventionele karyotypering (7%). Dit werd bevestigd in onze studie waarin slechts 3.1% van de *TCRB* chromosomale herschikkingen gedetecteerd werden met klassieke banderingstechnieken. De meest interessante bevinding was echter het ontdekken van een nieuw partner gen voor de *TCRB* locus in 5 van de 94 patiënten: de *HOXA* cluster genen op chromosoom 7p15. Deze 5 patiënten waren drager van een nieuwe recurrente herschikking, namelijk een inversie inv(7)(p15q34) met breukpunten gelegen in de *HOXA* cluster genen (7p15) enerzijds en de *TCRB* locus anderzijds (7q34). Dit was het eerste rapport waarin betrokkenheid van de *HOXA* cluster genen in chromosomale herschikkingen in T-ALL werd beschreven. Bovendien gaf deze herschikking aanleiding

tot overexpressie van *HOXA10* en *HOXA11* genen in het bijzonder. Samen met voorafgaande bevindingen van *HOXA* genexpressie in de maturatie van normale T cellen, kunnen we veronderstellen dat *HOXA* clustergenen een rol spelen in het onstaan van T-ALLs die drager zijn van deze nieuwe herschikking. Dit vormt een aspect van de T-cel oncogenese dat tot noch toe onbekend was gebleven.

In de tweede studie werden naast de *TCRβ* locus ook de andere TCR loci, *TCRαδ* (14q11) en *TCRγ* (7p15) onderzocht op chromosomale herschikkingen in 126 T-ALL patiënten en 19 T-ALL cellijnen. Deze studie bracht aan het licht dat ook de frequentie van de *TCRαδ* herschikkingen onderschat werd. Bovendien werden chromosomale herschikkingen van zowel de *TCRβ* als de *TCRαδ* locus samen aangetroffen, waarbij beide loci meestal verschillende T-cel oncogenen troffen alsook onderling herschikking vertoonde in 2 patiënten. Naast het voorkomen van talrijke niet eerder beschreven chromosomale herschikkingen in T-ALL, werden ook recurrente herschikkingen gedetecteerd met mogelijks nieuwe T-cel oncogenen op chromosomen 11q24, 20p12 en 6q22.

In een derde multi-centrische studie werden in totaal 424 T-ALLs retrospectief onderzocht op het voorkomen van de *TCRβ-HOXA* herschikking en werden klinische, immunofenotypische en moleculair-genetische data van deze patiënten nagegaan. Deze studie bracht 5 bijkomende *TCRβ-HOXA* positieve patiënten aan het licht welke het totaal op 14 patiënten brengt (3.3%) waarvan 9 inv(7)(p15q34), 2 t(7;7)(p15;q34) en 1 *TCRβ-HOXA* triplicatie tengevolge van ringvorming. Voor de twee resterende patiënten konden geen mitosen worden gevonden waardoor een onderscheid tussen een inv(7) en een t(7;7) werd bemoeilijkt. Voor 170 van de 424 patiënten werd genexpressie analyse uitgevoerd voor het *HOXA10* gen om de specifieke correlatie met de aanwezigheid van een *TCRβ-HOXA* herschikking na te gaan. Overexpressie van *HOXA10* werd echter vastgesteld in 25.2% van de T-ALLs waarvan slechts 8.2% drager waren van een *TCRβ-HOXA* herschikking (n=14). Expressie van het korte *HOXA10* transcript (*HOXA10b*) echter werd bijna exclusief aangetroffen in *TCRβ-HOXA* positieve patiënten (n=14), waardoor een rol in de *TCRβ-HOXA* gemedieerde oncogenese werd gesuggereerd. De chromosomale breukpunten werden gekloneerd in 3 van de 10 patiënten en toonde een clustering in zowel de *TCRβ* locus (1.1 kb) als in de *HOXA* gen cluster (3.8 kb). Dit was lichtjes verschillend van de breukpunten gevonden bij 4 patiënten van de Franse onderzoeksgroep, doch alle breukpunten blijven gegroepeerd in de regio rond de *HOXA9* en *HOXA10* genen. Andere moleculaire en/of cytogenetische afwijkingen die vaak voorkomen in T-ALL (*SIL-TAL1, CALM-AF10, HOX11, HOX11L2*) werden niet aangetroffen in de *TCRβ-HOXA* herschikte patiënten behalve deletie 9p21 en *NOTCH1* activerende mutaties welke aangetroffen werden in respectievelijk 64% en 67% van de *TCRβ-HOXA* herschikte stalen.

In een vierde korte studie beschrijven we het voorkomen van de eerste *HOXA* chromosomale herschikking met de *TCRδ* locus in een T-ALL patiënt die tevens drager is van een *CALM-AF10* translocatie.

Samengevat kunnen we stellen dat we dankzij de FISH techniek erin geslaagd zijn een aantal nieuwe chromosomale herschikkingen te detecteren binnen de T-ALL groep welke met de klassieke karyotypering over het hoofd zouden worden gezien.

Summary

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of T-cell precursors that develop both in children (10-15% of childhood ALL) and adults (25% of adults ALLs). This type of leukemia differs in many features from a B-lineage ALL and despite a marked improvement of treatment success rates for B- ALL over the past 50 years, less success has been achieved in the treatment of T-ALL. Similar to other types of leukemia, T-ALL is caused by a multistep mutagenesis involving various genetic alterations within a hematopoietic precursor stem cell. Chromosomal translocations affecting genes that encode transcription factors are the most common alterations in acute leukemia. In T or B lymphoid leukemias, these genes are frequently translocated downstream to the regulatory sequences of genes encoding chains of the T-cell receptor (TCR) or immunoglobulin (IG) molecules, resulting in inappropriate expression of the translocated transcription factors.

During last 20 years, many studies have shown that chromosomal abnormalities found in leukemia patients were useful diagnostic and prognostic indicators. Moreover, cytogenetic analysis is often the first step towards the identification of leukemia-causing genes which are associated with the emergence of a certain type of leukemia. Despite the major contribution of cytogenetic analysis in unraveling genetic defects in malignant cells, this method suffers from limited resolution (10Mb) which can lead to overlooking of some chromosomal rearrangements. The limitations of classical karyotyping have been overcome by the introduction of fluorescence in situ hybridization (FISH) and offers important possibilities for diagnostic and prognostic purposes in hematological malignancies.

In the present study, we focused on the identification of putative new oncogenes in a minor subgroup of leukemias, T-cell acute lymphoblastic leukemia (T-ALL). A previous M-FISH study performed on childhood ALL carrying complex chromosomal aberrations, resulted in the identification of a cryptic chromosomal translocation, t(7;11)(q34;q24), affecting the *TCRB* locus (7q34) and an unknown oncogene on chromosome 11q24. This was the trigger to screen a large group of T-ALL patients (n=94) using fluorescence in situ hybridization (FISH) in search for additional cryptic rearrangements targeting the *TCRB* locus. This study unraveled that chromosomal rearrangements affecting the *TCRB* locus were much more frequent (20%) than assumed based on conventional karyotyping (7%). This observation was confirmed in our series of patients where only 3.1% of *TCRB* chromosomal aberrations were detected by classical banding techniques. Most interestingly, this screening study lead to the identification of a new *TCRB* partner gene in 5 of 94 patients: the *HOXA* cluster genes on chromosome 7p15. These 5 patients carried a new, recurrent rearrangement i.e. an inversion inv(7)(p15q34) with breakpoints within the *HOXA* cluster genes (7p15) and the *TCRB* locus (7q34) respectively. This was the first report of *HOXA* cluster genes being involved in chromosomal rearrangements in T-ALL. This new recurrent rearrangement lead to overexpression of *HOXA10* and *HOXA11* genes in particular. Together with previous findings of *HOXA* gene expression in developing T lymphocytes, we can assume that *HOXA* cluster genes are involved

in T-cell oncogenesis. This was the first report providing evidence for involvement of *HOXA* genes in T-cell oncogenesis.

In a second study, we analysed involvement of two other TCR loci, *TCRaδ* (14q11) and *TCRγ* (7p15) besides the *TCRB* locus (7q34) in a group of 126 T-ALL patients and 19 T-ALL cell lines. This analysis showed that the frequency of *TCRaδ* rearrangements was also underestimated so far. Moreover, chromosomal rearrangements of both the *TCRaδ* and the *TCRB* loci were found in this series of patients and mostly targeted different T-cell oncogenes whereas two patients carried rearrangements between the two TCR loci. Besides the finding of a series of previously unreported chromosomal rearrangements in T-ALL, this study also unraveled recurrent aberrations with putative new T-cell oncogenes on chromosomes 11q24, 20p12 and 6q22.

In a third multi-center study, a total of 424 T-ALL cases were analysed retrospectively to search for additional *TCRB-HOXA* positive cases and subsequently to address specific clinical, immunophenotypical and biological characteristics of T-ALLs carrying this aberration. This revealed *TCRB-HOXA* rearrangements in 5 additional patients, which brings the total to 14 cases in 424 patients (3.3%) and included 9 inv(7)(p15q34), 2 t(7;7)(p15;q34) and 1 *TCRB-HOXA* triplication due to ring chromosome formation. For the two patients left, no metaphases were available which hampers a distinction between an inv(7) and a t(7;7). For 170 of these 424 patients, gene expression analysis for *HOXA10* expression was performed to check the relationship between *HOXA10* overexpression and the presence of the *TCRB-HOXA* rearrangement. *HOXA10* overexpression was detected in 25.2% of cases including all cases with a *TCRB-HOXA* aberration (n=14; 8.2%). Expression of the short *HOXA10* transcript *(HOXA10b)* however was almost exclusively found in the *TCRB-HOXA* positive patients (n=14), suggesting a specific role for the *HOXA10b* short transcript in *TCRB-HOXA* mediated oncogenesis. Breakpoints of 3 out of 10 cases were cloned and showed a clustering in both the *TCRB* locus (1.1 kb) and the *HOXA* gene locus (3.8 kb). This was slightly different to the breakpoints found in 4 patients carrying the *TCRB-HOXA* rearrangement in the study of Soulier et al. where the breakpoints were found 3' to the *HOXA10* gene and within intron 1b of *HOXA9* respectively. Nevertheless, the region encompassing the *HOXA10* and *HOXA9* genes remains the major breakpoint cluster region.

Other molecular and/or cytogenetic aberrations frequently found in subtypes of T-ALL (*SIL-TAL1, CALM-AF10, HOX11, HOX11L2*) were not detected in the *TCRB-HOXA* rearranged cases except for deletion of 9p21 and *NOTCH1* activating mutations which were present in 64% and 67% respectively.

Finally, in a fourth small study, we describe the first case of a *HOXA* chromosomal rearrangement with the *TCRδ* locus in a T-ALL patient already carrying a *CALM-AF10* translocation.

In conclusion, we clearly illustrated the power of FISH in detecting new chromosomal rearrangements within the group of T-ALLs which could easily be overlooked by conventional karyotyping.

Résumé

La leucémie aigue lymphoblastiques de la lignée T (LAL-T) représente une forme de cancer agressif au départ de précurseurs lymphoïdes T survenant tant chez l'enfant (10 à 15% des LAL de l'enfant) que chez l'adulte (25% des LAL de l'adulte). Ce type de leucémie diffère des LAL de la lignée B en plusieurs aspects et notamment en termes de survie malgré le développement important de nouvelles stratégies thérapeutiques lors des 50 dernières années. Comme pour les autres formes de leucémie, son apparition est la conséquence d'une accumulation de plusieurs altérations génétiques d'un progéniteur lymphoïde de la lignée T. Des translocations chromosomiques impliquant des gènes qui codent pour des facteurs de transcription sont les altérations génétiques le plus fréquemment retrouvées dans les leucémies aigues. Le plus souvent ces gènes codant pour des facteurs de transcriptions sont par conséquence soumis à l'influence des séquences régulatrices des gènes codant pour les récepteurs T (TCR) ou pour les récepteurs à immunoglobulines aboutissant à leur expression de façon inappropriée.

Lors des 20 dernières années, de nombreuses études ont été effectuées démontrant la valeur diagnostique et pronostique de ces réarrangements chromosomiques. De plus, l'analyse cytogénétique est souvent à la base de l'identification de gènes candidats responsables d'un certain type de leucémie. Cependant, contenu de sa résolution limitée de 10 Mb, l'analyse cytogénétique classique peut passer à côté de certains réarrangements chromosomiques. L'hybridation de fluorescence in situ (FISH) surpasse ce problème et constitue ainsi un outil diagnostique et de recherche important dans les hémopathies malignes.

Cette étude a pour but l'identification de nouveaux oncogènes impliqués dans une forme plus rare de leucémie étant la LAL-T. Une étude M-FISH précédente réalisée sur des échantillons d'enfants atteints d'une LAL avec des aberrations chromosomiques complexes, a permis la découverte d'une translocation chromosomique t(7;11)(q34;q24). Ceci ayant mené à l'identification d'un réarrangement chromosomique cryptique du locus *TCRB* (7q34) et d'un nouvel oncogène situé sur le bras long du chromosome 11 (11q24). Cette découverte a constitué le point de départ d'un dépistage à large échelle (94 patients atteints d'une LAL-T) ayant pour but de détecter des réarrangements cryptiques additionnels du locus *TCRB* par FISH. Cette étude a montré que des réarrangements du locus *TCRB* étaient nettement plus fréquents (20%) que la fréquence rapportée par l'analyse cytogénétique chromosomale classique (7%). Ceci a été confirmé par notre étude où pas plus de 3.0% de réarrangements du locus *TCRB* ont été détectés par cytogénétique traditionnelle. La découverte la plus intéressante fût celle d'un nouveau gène partenaire du locus de *TCRB* dans 5 cas sur les 94 : les gènes appartenant au cluster *HOXA* sur le chromosome 7p15. Ces 5 patients étaient porteurs d'un nouveau réarrangement récurrent étant une inversion inv(7)(p15q34), avec des points de cassure situés dans les gènes appartenant au cluster *HOXA* (7p15) d'une part et au cluster *TCRB* (7q34) d'autre part. C'est la première fois qu'une implication des gènes appartenant au cluster HOXA a été décrite dans les réarrangements chromosomiques des LAL-T. De plus, ce réarrangement a mené à la surexpression des gènes *HOXA10* et *HOXA11* plus en particulier. En plus de la constatation dans des études précédentes du rôle des gènes *HOXA* dans le

développement des lymphocytes T, ces découvertes nous font supposer que les gènes appartenant au cluster *HOXA* jouent un rôle dans le processus oncogénique des LAL-T possédant ce nouveau réarrangement chromosomique.

Dans la deuxième partie du travail des réarrangements chromosomiques ont également été recherchés dans les autres loci TCR, *TCRaδ* (14q11) et *TCRγ* (7p15) dans un groupe de 126 patients atteints de LAL-T et 19 lignées cellulaires LAL-T. Cette étude a démontré que la fréquence des réarrangements de *TCRaδ* était également sous-estimée. De plus, une coexistence de réarrangements chromosomique de *TCRaδ* et de *TCRβ* impliquant le plus souvent des oncogènes cellulaires T différents, a été découverte dans cette série de patients et chez 2 d'entre eux il existait un réarrangement interne entre ces deux loci. Mis a part ces réarrangements chromosomiques non-décrits précédemment dans les LAL-T, d'autres réarrangements récurrents impliquant possiblement de nouveaux oncogènes ont été détectés sur les chromosomes 11q24, 20q12 et 6q22.

Une troisième étude multicentrique rétrospective a visé à étudier la survenue de nouveaux réarrangements *TCRβ-HOXA* dans une série de 424 patients avec LAL-T et son rapport avec des éléments cliniques, immunophénotypique et moléculaires. Cette étude a permis de mettre en évidence 5 autres patients avec réarrangements *TCRβ-HOXA* ce qui fait un total de 14 patients (3.3%) dont 9 avec une inversion inv(7)(p15q34), 2 avec une translocation t(7 ;7)(p15 ;q34) et 1 patient avec une triplication *TCRβ-HOXA* suite à la formation d'un chromosome en anneau. Quant aux 2 patients restants, la distinction entre une inv(7) et une t(7;7) n'a pu être faite en raison de la non-disponibilité de métaphases. Une analyse d'expression du gène *HOXA10* a été réalisée dans 170 cas sur 424 dans le but de rechercher une corrélation spécifique entre une surexpression de ce gène et la présence d'un réarrangement *TCRβ-HOXA*.Une surexpression a été retrouvée dans 25,2 % des cas incluant tous les cas porteurs d'un réarrangement *TCRβ-HOXA* (n=14 ; 8.2%). Le variant de transcrit court de *HOXA10 (HOXA10b)* a cependant été retrouvé presque exclusivement chez les patients présentant un réarrangement *TCRβ-HOXA* ce qui fait suggérer son rôle spécifique dans l'oncogenèse médiée par le réarrangement *TCRβ-HOXA*. Les points de cassure chromosomiques clonés dans 3 cas ont montré un regroupement des points aussi bien dans le locus *TCRβ* (1.1 kb) que dans le locus des gènes *HOXA* (3.8kb). Ceci est légèrement différent par rapport à l'étude de Soulier et al. où les points de cassure de 4 patients ont été retrouvés en position 3' par rapport au gène *HOXA10* et compris dans l'intron 1b du gène *HOXA9* respectivement. Néanmoins, la région englobant les gènes *HOXA10* et *HOXA9* reste la région majeure de regroupement des points de cassure. D'autres aberrations moléculaires et/ou cytogénétiques fréquemment retrouvées dans les LAL-T (*SIL-TAL1, CALM-AF10, HOX11, HOX11L2*), n'ont pas été retrouvées dans le groupe de patients présentant un réarrangement *TCRβ-HOXA*, mis à part une délétion 9p21 et des mutations activatrices *NOTCH1* dans respectivement 64% et 67% des cas.

Enfin, dans une quatrième petite étude, nous décrivons pour la première fois la survenue d'un réarrangement chromosomique *TCRδ-HOXA* chez un patient présentant une LAL-T étant également porteur d'une translocation *CALM-AF10*.

En conclusion, nous avons clairement démontré l'apport supérieur de l'analyse par FISH par rapport à l'analyse cytogénétique classique à la détection de nouveaux réarrangements chromosomiques au sein du groupe des LAL-T.

Dankwoord

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2000-2001: Postgraduate course in Hospital Management (KUL)

2002: Basic and advanced training course in Blood banking (Ortho Clinical Diagnostics)

Professional activities:

1997-2003: Assistant training in Clinical Biology

2003-2006: Ph.D. candidate Center for Medical Genetics, Ghent University

(Funded by the Interuniversity Attraction Poles, Phase V)

Awards:

Best oral presentation 6th International Symposium and Expert Workshop on Leukemia and Lymphoma; 17-19 March 2005, Amsterdam, The Netherlands.

B. Cauwelier, F. Speleman, N. Dastugue, J. Cools, B. Verhasselt, B. Poppe, N. Van Roy, J. Vandesompele, C. Graux, A. Uyttebroeck, M. Boogaerts, B. De Moerloose, Y. Benoit, D. Selleslag, J. Billiet, A. Robert, F. Huguet, P. Vandenberghe, A. De Paepe, P. Marynen, A. Hagemeijer.
A new recurrent inversion, inv(7)(p15q34), leads to transcriptional activation of *HOXA10* and *HOXA11* in a subset of T-cell acute lymphoblastic leukemias.

Publications:

(a1) Publications in international journals with peer review

M. Cokelaere, B. Cauwelier, K. Cokelaere, G. Flo, N. Houache, S. Lievens, M. Van Boven, E. Decuypere.
Hematological and pathological effects of 0.25 % purified simmondsin in growing rats.
Industrial Crops and Products. 2000 ; 165–171.

B. Cauwelier, F. Nollet, E. De Laere, M. Van Leeuwen, J. Billiet, A. Criel, A. Louwagie.
Simultaneous occurrence of myelodysplastic syndrome and monoclonal B lymphocytes.
Leuk Lymphoma. 2002 ; 43(1):191-193.

B. Cauwelier, B. Gordts, P. Descheemaecker, H. Van Landuyt.
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Eur J Clin Microbiol Infect Dis. 2004 ; 23(5):389-392.

F. Speleman*, B. Cauwelier*, N. Dastugue, J. Cools, B. Verhasselt, B. Poppe, N. Van Roy, J. Vandesompele, C. Graux, A. Uyttebroeck, M. Boogaerts, B. De Moerloose, Y. Benoit, D. Selleslag, J. Billiet, A. Robert, F. Huguet, P. Vandenberghe, A. De Paepe, P. Marynen, A. Hagemeijer. * Both authors contributed equally.
A new recurrent inversion, inv(7)(p15q34), leads to transcriptional activation of *HOXA10* and *HOXA11* in a subset of T-cell acute lymphoblastic leukemias.
Leukemia. 2005 ; 19(3):358-366.

B. Poppe, N. Dastugue, J. Vandesompele, B. Cauwelier, B. De Smet, N. Yigit, A. De Paepe, C. Recher, V. De Mas, A. Hagemeijer and F. Speleman.
EVI1 is consistently expressed as principal transcript in common and rare recurrent 3q26 rearrangements.
Genes Chromosomes Cancer. 2006 ; 45(4):349-356.

B. Poppe, B. Cauwelier, H. Van Limbergen, N. Yigit, J. Philippé, B. Verhasselt, A. De Paepe, Y. Benoit, F. Speleman.
Novel cryptic chromosomal rearrangements in childhood ALL detected by M-FISH.
Haematologica. 2005 ; 90(9):1179-1185.

B. Cauwelier, N. Dastugue, A. Hagemeijer, C. Herens, A. De Paepe, F.Speleman.
Cytogenetic study of 126 unselected T-ALL cases and 19 T-ALL cell lines reveals unexpected high incidence of cryptic rearrangements of the *TCRβ* locus with at least 2 new recurrent partner genes.
Leukemia. 2006 ; 20(7):1238-1244.

B. Cauwelier, H. Cavé, C. Gervais , M. Lessard , C. Barin, C. Perot , J. Van den Akker, F. Mugneret , C. Charrin, M.P. Pagès, M.J. Grégoire, P. Jonveaux , M. Lafage-Pochitaloff, M. J. Mozzicconacci, C. Terré, I. Luquet, P. Cornillet-Lefebvre, B. Laurence, G. Plessis, C. Lefebvre, D. Leroux, H. Antoine-Poirel, C. Graux, L. Mauvieux, P. Heimann, C. Chalas, E. Clappier, B. Verhasselt, Y. Benoit, B. De Moerloose, B. Poppe, N. Van Roy, K. De Keersmaecker, J. Cools, F. Sigaux, J. Soulier, A. Hagemeijer, A. De Paepe, N. Dastugue, R. Berger, F. Speleman.
Clinical, cytogenetic and molecular characteristics of 14 T-ALL patients carrying the *TCRB-HOXA* rearrangement: a study of the Groupe Francophone de Cytogénétique Hématologique (GFCH).
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A. De Weer, B. Poppe, B. Cauwelier, N. Van Roy, N. Dastugue, A. Hagemeijer, A. De Paepe, F. Speleman.

Screening for EVI1: ectopic expression absent in T-cell acute lymphoblastic leukemia patients and cell lines.

Cancer Genet Cytogenet. 2006 Nov;171(1):79-80.

I. Lahortiga, K. De Keersmaecker, P. Van Vlierberghe, C. Graux, B. Cauwelier, F. Lambert, N. Mentens, H. B. Beverloo, R. Pieters, F. Speleman, M. D. Odero, M. Bauters, G. Froyen, P. Marynen, P. Vandenberghe, I. Wlodarska, J. P.P. Meijerink, J. Cools.
Duplication of the *MYB* oncogene in T-cell acute lymphoblastic leukemia.
Submitted Nature Genetics 2006.

(a2) Publications in international journals

F. Nollet, B. Cauwelier, J. Billiet, D. Selleslag, A. Van Hoof, A. Louwagie, A. Criel .
Do B-CLL patients with Ig Vh3-21 genes compose a new subset of CLL?
Letter to the Editor. Blood. 2002 ; 100(3):1097-1098.

J. Bergeron, E. Clappier, B. Cauwelier, N. Dastugue, C. Millien, E. Delabesse, K. Beldjord, F. Speleman, J. Soulier, E. Macintyre, V. Asnafi.
HOXA cluster deregulation by a *TCRδ-HOXA* chromosomal translocation in a *CALM-AF10*+ T-ALL.
Letter to the Editor. Leukemia. 2006 ; 20(6):1184-1187.

B. Cauwelier, N. Dastugue, A. Hagemeijer, F. Speleman. inv(7)(p15q34),t(7;7)(p15;q34). Atlas Genet Cytogenet Oncol Haematol. October 2005 .

B. Cauwelier, F. Speleman. *HOXA11* (Homeobox A11). Atlas Genet Cytogenet Oncol Haematol. June 2006 .

(a3) Publications in national journals

B. Cauwelier, A. De Paepe A., F. Speleman : "Prehistorische" genen in acute lymfoblastenleukemie (ALL) bieden perspectieven voor moleculaire signaalweg-therapie. Artsenkrant 18/07/2005.

Oral presentations:

B. Cauwelier, F. Speleman, N. Dastugue, J. Cools, B. Verhasselt, B. Poppe, N. Van Roy, J. Vandesompele, C. Graux, A. Uyttebroeck, M. Boogaerts, B. De Moerloose, Y. Benoit, D. Selleslag, J. Billiet, A. Robert, F. Huguet, P. Vandenberghe, A. De Paepe, P. Marynen, A. Hagemeijer.
A new recurrent inversion, inv(7)(p15q34), leads to transcriptional activation of *HOXA10* and *HOXA11* in a subset of T-cell acute lymphoblastic leukemias.
6th International Symposium and Expert Workshop on Leukemia and Lymphoma, 17-19 March 2005, Amsterdam, Nederland.

B. Cauwelier, F. Speleman, N. Dastugue, J. Cools, B. Verhasselt, B. Poppe, N. Van Roy, J. Vandesompele, C. Graux, A. Uyttebroeck, M. Boogaerts, B. De Moerloose, Y. Benoit, D. Selleslag, J. Billiet, A. Robert, F. Huguet, P. Vandenberghe, A. De Paepe, P. Marynen, A. Hagemeijer.
A new recurrent inversion, inv(7)(p15q34), leads to transcriptional activation of *HOXA10* and *HOXA11* in a subset of T-cell acute lymphoblastic leukemias.
Société Française d'Hématologie, 31 April-1 March 2005, Paris, France.

B. Cauwelier. Nieuwe chromosomale defecten bij hematologische aandoeningen.
Interne Vorming CMG 10 May 2005, Ghent, Belgium.

N. Van Roy , B. Cauwelier, F. Speleman, N. Dastugue, J. Cools, B. Verhasselt, B. Poppe, J. Vandesompele, C. Graux, A. Uyttebroeck, M. Boogaerts, B. De Moerloose, Y. Benoit, D. Selleslag, J. Billiet, A. Robert, F. Huguet, P. Vandenberghe, A. De Paepe, P. Marynen, A. Hagemeijer.
A new recurrent inversion, inv(7)(p15q34), leads to transcriptional activation of *HOXA10* and *HOXA11* in a subset of T-cell acute lymphoblastic leukemias.
European Hematology Association, 2-5 June, 2005, Stockholm, Sweden.

B.Cauwelier, H. Cavé, N. Dastugue, P. Heimann, H. Antoine-Poirel, C. Lefebvre, D. Leroux, M. Grégoire, B. Verhasselt, Y. Benoit, B. De Moerloose, N. Van Roy, A. Hagemeijer, A. De Paepe, F. Speleman.
The *TCRB-HOXA* rearrangement in T-ALL leads to a specific increase of the alternative *HOXA10b* transcript.
EMBO/SEMM Workshop, 23-26 March 2006, Riva Del Garda, Italy.

B. Cauwelier, N. Dastugue, A. Hagemeijer, C. Herens, A. De Paepe, F. Speleman.
Screening des réarrangements des gènes du récepteur T dans les LAL-T: fréquence inattendue des remaniements du *TCRB* et identification de nouvelles régions partenaires.
Société Française d'Hématologie, 23-25 March 2006, Paris, France.

B. Cauwelier, H. Cavé, N. Dastugue, P. Heimann, H. Antoine-Poirel, C. Lefebvre, D. Leroux, M. Grégoire, B. Verhasselt, Y. Benoit, B. De Moerloose, N. Van Roy, A. Hagemeijer, A. De Paepe, F. Speleman.
The *TCRB-HOXA* rearrangement in T-ALL leads to a specific increase of the alternative *HOXA10b* transcript.
19th International Symposium on Technological Innovations in Laboratory Hematology, 25-28 April, Amsterdam, the Netherlands.

Poster presentations :

B. Cauwelier, F. Nollet, E. De Laere, M. Van Leeuwen, J. Billiet, A. Criel, A. Louwagie
Simultaneous occurrence of myelodysplastic syndrome and mature B cell lymphoma with different clonal origin recognized with FISH and Humara.
Belgian Hematological Society, February 2001, Gembloux, Belgium.

F. Nollet, B. Cauwelier, J. Billiet, D. Selleslag, A. Van Hoof, A. Louwagie, A. Criel
Analysis of clonal B cell CD38 and immunoglobulin variable region sequence status in relation to clinical outcome for B-CLL.
Belgian Hematological Society, February 2002, Brussel, Belgium.

F. Nollet, B. Cauwelier, J. Billiet, D. Selleslag, A. Van Hoof, A. Louwagie, A. Criel
Heavy chain somatic hypermutation analysis in Waldenström macroglobulinemia
and chronic lymphocytic leukemia.
International Conference on Malignant Lymphoma, 12-15 June 2002, Lugano, Switserland.

B. Cauwelier, B. Poppe, N. Yigit, A. De Paepe, Y. Benoit, N. Dastugue, F. Speleman
Detection of a cryptic *EVI1* gene rearrangement using a validated probe set for interphase FISH.
European Haematology Association, 10-13 June 2004, Geneva, Switserland.

B. Cauwelier, F. Speleman, N. Dastugue, J. Cools, B. Verhasselt, B. Poppe, N. Van Roy, J. Vandesompele, C. Graux, A. Uyttebroeck, M. Boogaerts, B. De Moerloose, Y. Benoit, D. Selleslag, J. Billiet, A. Robert, F. Huguet, P. Vandenberghe, A. De Paepe, P. Marynen, A. Hagemeijer.
A new recurrent inversion, inv(7)(p15q34), leads to transcriptional activation of *HOXA10* and *HOXA11* in a subset of T-cell acute lymphoblastic leukemias.
Belgian Hematological Society, January 2005, Genval, Belgium.

B. Cauwelier, F. Speleman, N. Dastugue, J. Cools, B. Verhasselt, B. Poppe, N. Van Roy, J. Vandesompele, C. Graux, A. Uyttebroeck, M. Boogaerts, B. De Moerloose, Y. Benoit, D. Selleslag, J. Billiet, A. Robert, F. Huguet, P. Vandenberghe, A. De Paepe, P. Marynen, A. Hagemeijer.
A new recurrent inversion, inv(7)(p15q34), leads to transcriptional activation of *HOXA10* and *HOXA11* in a subset of T-cell acute lymphoblastic leukemias.
Belgian Society for Human Genetics, 28 January 2005, Luik, Belgium.

B. Cauwelier, F. Speleman, N. Dastugue, J. Cools, B. Verhasselt, B. Poppe, N. Van Roy, J. Vandesompele, C. Graux, A. Uyttebroeck, M. Boogaerts, B. De Moerloose, Y. Benoit, D. Selleslag, J. Billiet, A. Robert, F. Huguet, P. Vandenberghe, A. De Paepe, P. Marynen, A. Hagemeijer.
A new recurrent inversion, inv(7)(p15q34), leads to transcriptional activation of *HOXA10* and *HOXA11* in a subset of T-cell acute lymphoblastic leukemias.
European Cytogenetics Conference, 4-7 June 2005, Madrid, Spain.

B. Cauwelier, F. Speleman, N. Dastugue, J. Cools, B. Verhasselt, B. Poppe, N. Van Roy, J. Vandesompele, C. Graux, A. Uyttebroeck, M. Boogaerts, B. De Moerloose, Y. Benoit, D. Selleslag, J. Billiet, A. Robert, F. Huguet, P. Vandenberghe, A. De Paepe, P. Marynen, A. Hagemeijer.
A new recurrent inversion, inv(7)(p15q34), leads to transcriptional activation of *HOXA10* and *HOXA11* in a subset of T-cell acute lymphoblastic leukemias.
37th Congress of the International Society of Pediactric Oncology, 21-24 September 2005, Vancouver, Canada.

B. Cauwelier, N. Dastugue, J. Cools, B. Verhasselt, B. Poppe, N. Van Roy, J. Vandesompele, C. Graux, A. Uyttebroeck, M. Boogaerts, B. De Moerloose, Y. Benoit, D. Selleslag, J. Billiet, A. Robert, F. Huguet, P. Vandenberghe, A. De Paepe, P. Marynen, A. Hagemeijer, F. Speleman.
Screening for *TCR* rearrangements in T-ALL lead to the identification of a subset of *TCRβ-HOXA* rearranged cases and at least two new recurrent partner genes.
Second Erasmus Workshop on Molecular Therapeutics in Acute Leukemia, 7-8 October 2005, Rotterdam, The Netherlands.

N. Van Roy, B. Cauwelier, F. Speleman, N. Dastugue, J. Cools, B. Verhasselt, B. Poppe, J. Vandesompele, M. Boogaerts, B. De Moerloose, Y. Benoit, D. Selleslag, J. Billiet, F. Huguet, P. Vandenberghe, A. De Paepe, P. Marynen, A. Hagemeijer.
Identification of genes involved in T-cell oncogenesis through FISH screening of *TCR* rearrangements in T-ALL.
ArrayCGH-MC, 19-21 October 2005, Bari, Italy.

B. Cauwelier, H. Cavé, N. Dastugue, P. Heimann, H. Antoine-Poirel, B. Verhasselt, Y. Benoit, B. De Moerloose, N. Van Roy, A. Hagemeijer, A. De Paepe, F. Speleman.
The *TCRB-HOXA* rearrangement in T-ALL leads to a specific increase of the alternative *HOXA10b* transcript.
Belgian Hematological Society, 27-28 January 2006, Genval, Belgium.

B. Cauwelier, H. Cavé, N. Dastugue, P. Heimann, H. Antoine-Poirel, B. Verhasselt, Y. Benoit, B. De Moerloose, N. Van Roy, A. Hagemeijer, A. De Paepe, F. Speleman.
The *TCRB-HOXA* rearrangement in T-ALL leads to a specific increase of the alternative *HOXA10b* transcript.
Belgian Society for Human Genetics, 19 February 2006, Antwerpen, Belgium.

A. De Weer, B. Poppe, B. Cauwelier, N. Van Roy, N. Dastugue, B. De Moerloose, Y. Benoit, L. Noens, A De Paepe, F. Speleman.
Recurrent t(3;17) translocations in AML with *EVI1* rearrangement: high resolution mapping of the 17q breakpoints.
Belgian Hematological Society, 27-28 January 2006, Genval, Belgium.

B. Cauwelier, H. Cavé, N. Dastugue, P. Heimann, H. Antoine-Poirel, B. Verhasselt, Y. Benoit, B. De Moerloose, N. Van Roy, A. Hagemeijer, A. De Paepe, F. Speleman.
The *TCRB-HOXA* rearrangement in T-ALL leads to a specific increase of the alternative *HOXA10b* transcript.
ESH/AACR Conference, 2-5 February 2006, Cascais, Portugal.

B. Cauwelier, H. Cavé, N. Dastugue, P. Heimann, H. Antoine-Poirel, B. Verhasselt, Y. Benoit, B. De Moerloose, N. Van Roy, A. Hagemeijer, A. De Paepe, F. Speleman.
The *TCRB-HOXA* rearrangement in T-ALL leads to a specific increase of the alternative *HOXA10b* transcript.
Topposter Wetenschapsdag UZ Gent, Het Pand, 30 March 2006, Ghent, Belgium.

J. Bergeron, E. Clappier, B. Cauwelier, C. Millien, F. Speleman, J. Soulier, E. Delabesse, K. Beldjord, N. Dastugue, E. Macintyre, V. Asnafi.
Caractérisation moléculaire d'une nouvelle translocation t(7;14) impliqueant le locus *TCRD* et les gènes *HOXA*.
Congrès annuel de la Société Française d'Hématologie, 23-26 Mars 2006, Paris, France.

Congresses, workshops and meetings:

Meeting of the EORTC Cytogenetic & Molecular Genetic Committee. UZ Gasthuisberg, 24-25 October 2003, Leuven, Belgium.

6th Post-ASH Meeting, Sheraton Brussels Airport, 19 December 2003, Zaventem, Belgium.

19th General Meeting of the Belgian Hematological Society, 30-31 January 2004, Brussels, Belgium.

4th Annual Meeting of the Belgian Society of Human Genetics, 19 March 2004, Ghent, Belgium.

9th Congress of the European Hematology Association, 10-13 June 2004, Geneva Palexpo, Geneva, Switzerland.

Najaarsvergadering Hematologie, Ijzertekort en ijzeroverbelading, 9 October 2004, AZ St.Jan AV, Bruges, Belgium.

7th Post-ASH Meeting, Sheraton Brussels Airport, 17 December 2004, Zaventem, Belgium.

4de Wetenschapsdag UZ Gent, 20 January 2005, Ghent, Belgium.

5th Annual Meeting of the Belgian Society for Human Genetics, 28-29 January 2005, Luik, Belgium.

20th General Meeting of the Belgian Hematological Society; January 2005, Genval, Belgium.

Groupe Francophone de Cytogénétique Hématologique, 3 February 2005, Paris, France.

6th International Symposium and Expert Workshop on Leukemia and Lymphoma, 17-19 March 2005, Amsterdam, the Netherlands.

Congrès annuel de la Société Française d'Hématologie (SFH), 31 April-1 May 2005, Maison de la Chimie, Paris, France.

Voorjaarsvergadering Hematologie, Chronische lymfatische leukemie en Ziekte van Waldenström, 28 May 2005, AZ St. Jan AV, Bruges, Belgium.

Second Erasmus Workshop on Molecular Therapeutics in Acute Leukemia, 7-8 October 2005, Rotterdam, the Netherlands.

Najaarsvergadering Hematologie, Myelodysplastische syndromen (MDS), 26 November 2005, AZ St.Jan AV, Bruges, Belgium.

21th General Meeting of the Belgian Hematological Society, 27-28 January 2006, Hotel Château du Lac, Genval, Belgium.

6th Annual Meeting of the Belgian Society for Human Genetics, 19 February 2006, Campus Drie Eiken, Antwerp, Belgium.

EMBO/SEMM Workshop on Homedomain proteins, hematopoietic development and leukemias, 23-26 March 2006, Astoria Park Hotel, Riva Del Garda, Italy.

Congrès annuel de la Société Française d'Hématologie (SFH), 23-25 March 2006, Maison de la Chimie, Paris, France.

5de Wetenschapsdag UZ Gent, 30 March 2006, Het Pand, Ghent, Belgium.

19th International Symposium on Technological Innovations in Laboratory Hematology, 25-28 April 2006, Grand Hotel Krasnapolsky, Amsterdam, the Netherlands.