Chromosome 6q deletion
in precursor T-cell lymphoblastic lymphoma and leukemia
of childhood and adolescence

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by
Burkhardt, Birgit
of
Stuttgart

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From the Center of Pediatrics
Department of Pediatric Hematology and Oncology
Head: Professor Dr. Alfred Reiter
of the Faculty of Medicine of the Justus Liebig University Giessen

First Supervisor and Committee Member: Prof. Dr. Alfred Reiter
Second Supervisor and Committee Member: Prof. Dr. Martin Stanulla M. Sc.
Committee Members: Prof. Dr. Dr. Hans Michael Piper
Prof. Dr. Rainer Renkawitz

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To Dorothea Neubert and Holger Burkhardt

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Parts of the data obtained in the current study were already published or submitted for publication:

B. Burkhardt, J. Bruch, M. Zimmermann, K. Strauch, R. Parwaresch, WD. Ludwig, L. Harder, B. Schlegelberger, F. Mueller, J. Harbott, A. Reiter.
Loss of heterozygosity on chromosome 6q14-24 is associated with poor outcome in children and adolescents with T-cell lymphoblastic lymphoma.
Leukemia, 2006;20:1422-1429.

A second manuscript with results of the project was recently submitted:
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Pediatric precursor T lymphoblastic leukemia and precursor T lymphoblastic lymphoma: Differences in common deleted regions of chromosome 6q and prognostic impact.
(Leukemia), submitted November 2006

Oral presentations:

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B. Burkhardt, J. Bruch, J. Salzburg, M. Zimmermann, A. Reiter
Clinical and biological significance of Loss of heterozygosity at chromosome 6q in children and adolescents with T-cell lymphoblastic lymphoma

Annual Meeting of the American Society of Hematology in Orlando, Florida at Dec. 9-12, 2006:
B. Burkhardt, A. Moericke, W. Klapper, F. Mueller, M. Schrappe, and A. Reiter.
Pediatric T-cell lymphoblastic leukemia and T-cell lymphoblastic lymphoma: Differences in the common deleted region and the prognostic impact of chromosome 6q deletions

1 Summary (English/German)

English:

Precursor T lymphoblastic lymphoma (T-LBL) is the second most common subtype of Non-Hodgkin Lymphoma (NHL) in children and adolescents. Favorable survival rates have been achieved with current combination chemotherapy regimens; however failure of frontline treatment is still fatal for the majority of patients. Currently there are no strong prognostic criteria known that would allow the minority of patients at risk of failure to be identified early enough to expose them to a more intense or new therapy.

Cytogenetic data from four index patients from clinical trial NHL-BFM 95 exhibited a common deleted region at chromosomal band 6q15-q16. Interestingly, all four patients suffered from a relapse. In the literature, chromosome 6q deletions have been reported for various hematological malignancies, but the prognostic impact is still inconclusive.

In the present study the frequency of chromosome 6q deletions in T-LBL, the common deleted region and the prognostic impact was analyzed. Secondly, identical analyses were performed in pediatric precursor T leukemia (T-ALL) patients as T-LBL and T-ALL are considered to be biologically closely related. Both groups were treated uniformly according to an ALL-BFM-type treatment strategy.

6q deletions were examined by loss-of-heterozygosity analysis (LOH) of 25 microsatellite markers on chromosome 6q14-q24. A total of 1,671 markers were successfully analyzed from 108 T-LBL patients. LOH was detectable in 21 patients. Markers D6S1682 and D6S468 flanked a chromosomal region which was affected by deletion in 13 cases. The cumulative incidence of relapse was 9±3% for LOH negative versus 63±12% for LOH positive patients *(P* < 0.001). In comparison, a total of 3,109 markers were successfully analyzed from 127 T-ALL patients. LOH was detected in 16 patients, with proximal interstitial deletions in 15 cases. Markers D6S1627 and D6S1644 flanked the 4.3-Mb common deleted region. LOH at 6q was not associated with outcome.

Thus, we conclude that LOH on chromosome 6q14-q24 was associated with a high risk of relapse in children with T-LBL and that the pattern of 6q deletions and the prognostic impact differed between pediatric T-LBL and T-ALL. These results might indicate differences in the biology of the cells in pediatric T-LBL and T-ALL.

German:

Bei Kindern und Jugendlichen sind lymphoblastische T-Zell Lymphome (T-LBL) der zweit häufigste Subtyp der Non-Hodgkin Lymphome. Mit modernen Chemotherapie-Regimen werden bei diesen Patienten hohe Überlebenswahrscheinlichkeiten erzielt. Im Falle eines Rezidivs sind die Überlebenswahrscheinlichkeiten jedoch sehr gering. Bisher konnten keine Risikofaktoren etabliert werden, die eine frühzeitige Identifizierung der Hoch-Risiko-Patienten erlauben würden, um diese frühzeitig mit intensiverer Therapie behandeln zu können.

Bei der Auswertung zytogenetischer Befunde von Patienten der Studie NHL-BFM 95 konnten vier Index-Patienten identifiziert werden, die alle vier eine Deletion im Chromosom 6q aufwiesen. Die gemeinsame Verlustregion der vier Fälle umfasst die chromosomalen Banden 6q15-q16. Interessanterweise erlitten alle vier Patienten ein Rezidiv. Die Literaturanalyse ergab, dass 6q Deletionen bereits bei einer Vielzahl hämatologischer Erkrankungen berichtet wurden, die prognostische Bedeutung bisher aber ungeklärt ist.

In der vorliegenden Studie wurden die Häufigkeit, die gemeinsame Verlustregion und die prognostische Bedeutung von 6q Deletionen bei Kindern und Jugendlichen mit T-LBL untersucht. In einem zweiten Schritt wurden die gleichen Analysen bei Kindern und Jugendlichen mit akuter lymphoblastischer T-Zell Leukämie (T-ALL) durchgeführt, da T-LBL und T-ALL als biologisch eng verwandte Erkrankungen gelten. Beide Patientengruppen waren einheitlich nach der ALL-BFM Strategie behandelt worden. Die Evaluation von Chromosom 6q Deletionen wurde mittels Loss-of-heterozygosity Analysen (LOH) durchgeführt, wobei ein Set von 25 Mikrosatellitenmarkern in den chromosomalen Banden 6q14-q24 untersucht wurde.

Bei 108 T-LBL-Patienten wurden in Summe 1.671 Marker erfolgreich untersucht. LOH von Mikrosetellitenmarkern wurde bei 21 Patienten nachgewiesen. Die Marker D6S1682 und D6S468 flankieren eine Region, die bei 13 der 21 Patienten von der Deletion betroffen war. Die kumulative Inzidenz für ein Rezidiv war 9±3% bei LOH-negativen Patienten versus 63±12% bei LOH-positiven Patienten *(P* < 0.001).

Im Vergleich dazu wurden bei insgesamt 127 T-ALL Patienten in Summe 3.109 Marker erfolgreich analysiert. Bei 16 Patienten wurde ein LOH von Mikrosetellitenmarkern nachgewiesen. 15 der 16 Fälle wiesen eine gemeinsame Verlustregion von 4,3 Mb im proximalen Anteil von Chromosom 6q flankiert von den Markern D6S1627 und D6S1644 auf. LOH im Chromosom 6q war bei T-ALL Patienten nicht mit dem Rezidiv-Risiko assoziiert.

Zusammenfassend konnte bei T-LBL Patienten die Assoziation von 6q Deletion mit einer hohen Rezidiv-Wahrscheinlichkeit gezeigt werden. Im Vergleich zwischen T-ALL und T-LBL konnten sowohl bezüglich der prognostischen Bedeutung als auch bei den Mustern der 6q Deletionen Unterschiede gezeigt werden, die möglicherweise auf biologische Unterschiede zwischen T-LBL und T-ALL hindeuten.

2 Introduction

In children and adolescents precursor T lymphoblastic neoplasms are classified into two diseases: acute lymphoblastic T-cell leukemia (T-ALL) and lymphoblastic T-cell lymphoma (T-LBL).

Several study groups including the BFM-group (Berlin-Frankfurt-Münster) distinguish between leukemia and lymphoma based on the primary site of involvement. Patients with less than 25% lymphoblasts in the bone marrow and no peripheral blasts are diagnosed with lymphoblastic lymphoma; in case of 25% or more blasts in the bone marrow patients are diagnosed with leukemia.[1;2]

Both, T-ALL and T-LBL are neoplasms of lymphoblasts committed to the T-cell lineage. The postulated cell of origin is the precursor T lymphoblast which differentiation is arrested at discrete stages of maturation.[3-5] Thus, the malignant clones in patients with T-ALL or T-LBL are thought to originate from normal lymphoid progenitor cells arrested at early stages of T-cell maturation.

2.1 T-cell maturation

Lymphocytes are characterized by their potential to express highly diverse antigen receptors. This diversity is achieved during the process of lymphocyte maturation from bone marrow derived progenitors to mature lymphocytes in the peripheral lymphoid tissue. The process during lymphocyte maturation that generates this diversity is unique for all cell-types in the human organism. The so called somatic recombination is based on enzymatic deletion of deoxyribonucleic acid (DNA) sections which allows that initially separated germline DNA sequences are brought together. Different combinations of gene segments result in the diversity of the lymphocyte repertoire.

The steps of T-cell development include 1) lineage commitment and proliferation, 2) expression of antigen receptor genes, and 3) selection of T-cells expressing useful antigen receptors.

1) Lineage commitment: Pluripotent hematological stem cells in the bone marrow give rise to all lineages of hematological cells. Early precursors of T lymphocytes leave the bone marrow and circulate to the thymus, where further maturation takes place. The process of early T-cell maturation is associated with progressive restriction of the developmental potential of the cells. The earliest recognizable thymic precursor lost the capacity for megakaryocytic or erythroid differentiation, but retain T-cell, natural killer cell (NK), and dendritic cell and possibly B-cell and myeloid potential.[6] Beside lineage commitment early T-cell maturation is characterized by interleukin-7 (IL-7) driven high mitotic activity of the progenitor cells.

2) Expression of antigen receptor genes: Next step of differentiation is the rearrangement of the T-cell receptor (TCR) gene loci. The recombination of the receptor gene sequences and the expression of the antigen receptor genes is the key event in the T-cell maturation process. The mature TCR is a heterodimer, either of an α chain and a covalently linked β chain (TCRαβ) or in the minority of T-cells a heterodimer composed of a γ chain and a δ chain (TCRγδ).

The genes encoding the TCRα chain are located at chromosome 14q11, the genes encoding the TCRβ chain are at chromosome 7q34 and for TCRγ chain at chromosome 7p15. The TCRδ chain locus is contained within the TCRα chain locus. Each germline TCR locus includes variable (V) segments, joining (J) segments and constant (C) gene segments. The TCRβ and the TCRδ also have diversity (D) segments. The segments are separated by intervening DNA. The numbers of gene segments per TCR locus are listed in Table 1 and the germline organization of a TCR locus is shown exemplarily for the TCRβ chain locus in Figure I.

Table 1. Number of gene segments of TCR loci

	TCRα	TCRβ	TCRγ	TCRδ
Localized at chromosome	14q11	7q34	7p15	14q11
Variable (V) segments	45	50	5	2
Diversity (D) segments	0	2	0	3
Joining (J) segments	55	12	5	4
Constant (C) segments	1	2	2	1

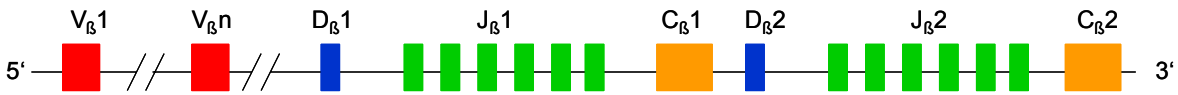


Figure I. Germline organization of human TCRβ chain locus. V: variable segments, J: joining segments, C: constant segments, D: diversity segments (mod. from Abbas and Lichtman, 2003[7])

Prior to transcription and translation of TCR loci, the somatic recombination of the loci is necessary. In the process of somatic recombination the functional genes encoding for the variable region of the TCR are formed. The segments of the germline loci that are initially separated from another are brought together by enzymatic introduction of double-stranded breaks into the germline DNA, deletion of intervening sequences and re-ligation of DNA as shown schematically in Figure II. The somatic recombination is mediated by the so-called V(D)J recombinases, which recognize specific recombination signal sequences (RSS) flanking the

rearranging segment. The segments are combined randomly and the different combinations produce different antigen receptors.[8]

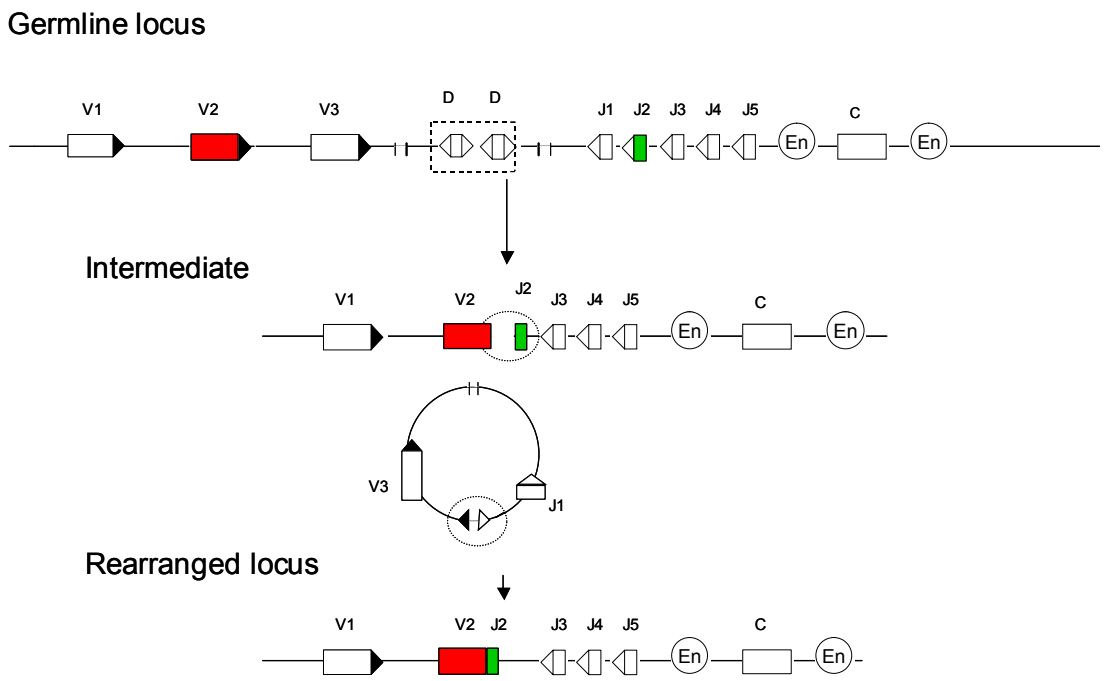
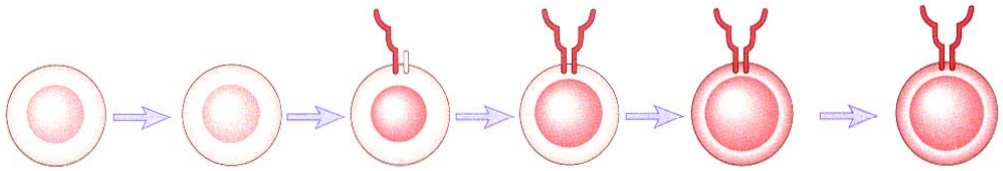


Figure II. V(D)J recombination. The V(D)J recombinases recognize the recombination signal sequences and bring noncontiguous exons together by deletion of intervening DNA in most cases. The DNA in the rearranged T-cell is therefore fundamentally different from the DNA in cells with germline DNA and the process is irreversible. V: variable segments, D: diversity segments, J: joining segments, C: constant segments, En: Enhancer elements, solid and open triangles: recognition sequences for the recombinase enzyme complex (mod. from Kirsch et al., 1997[9])

The somatic recombination of TCR loci starts with the TCRδ chain, followed by TCRγ and TCRβ. Successful TCRβ rearrangement in the presence of pre-Tα chain, which is an invariant transmembrane protein that associates with the TCRβ chain, allows the expression of a pre-TCR on the cell surface. The cells at this state of maturation are double positive for CD4 and CD8, express cytoplasmic CD3 (cyCD3) and undergo massive proliferation. This process is known as β-selection. Beta-selection is followed by the rearrangement of TCRα gene locus and the replacement of the pre-TCR expression by TCRαβ expression on the cell surface.

3) Selection of T-cells expressing useful antigen receptors: In the third phase of T-cell maturation the immature lymphocytes are selected based on the expressed antigen receptor with preservation of T-cells with useful receptors and elimination of potentially harmful self-

antigen reactive T-cells. Positive selection of T-cells ensures the development of T-cells whose receptors bind with low affinity to the self major histocompatibility complex (MHC) which identifies the particular organism. In parallel these selected T-cells are able to recognize foreign peptides displayed by the same MHC molecules on the antigen-presenting cells in the periphery. The following functional maturation of positively selected T-cells includes the expression of several intracellular and surface molecules which are involved in the lymphocyte activation and effector functions. The steps of T-cell maturation and the TCR loci recombination are summarized in Figure III.



Stage of maturation	stem cell	pro-T	pre-T	double positive	single positive	naive mature T-cell
TCR DNA	germline DNA	germline DNA	recombined β chain	recombined β, α chain	recombined β, α chain	recombined β, α chain
TCR expression	none	none	pre-T receptor (β chain/pre-T α)	membrane TCRαβ	membrane TCRαβ	membrane TCRαβ
Surface markers	CD44+, CD25-	CD44+, CD25+	CD44+, CD25+	CD4+, CD8+	CD4+ CD8- or CD4- CD8+	CD4+ CD8- or CD4- CD8+
Anatomic site	BM	thymus				periphery

Figure III. Stages of T-cell maturation (mod. from Abbas and Lichtman, 2003[7]). TCR: T-cell receptor, BM: bone marrow

The maturation of the T-cells including the rearrangement of the TCR loci is required for the generation of the immune response of the organism. However, the somatic recombination of TCR loci is catalyzed by a complex of enzymes including RAG1, RAG2 and DNA breakage and joining enzymes. The activity of these DNA-destabilizing enzymes in combination with the modification of the chromatin makes the DNA highly amenable to DNA breakage and rejoining events. This lymphocyte specific genetic instability is an important factor in consideration of the process of malignant transformation of the cells. The malignant transformation can occur at any stage of T-cell maturation. The subsequent clonal and uncontrolled proliferation of T-cells arrested at immature stages of development results in the clinical manifestation and diagnosis of precursor T-cell lymphoblastic lymphoma or leukemia.

2.2 Lymphoblastic lymphoma

Definition of lymphoblastic lymphoma

The designation of precursor T-cell lymphoblastic lymphoma (T-LBL) is used to describe precursor T-cell neoplasia with predominantly lymph node-based or thymic disease most commonly affecting the anterior mediastinum. The entity of lymphoblastic lymphoma has been recognized by most classifications of lymphoid neoplasms, including the Kiel classification[10] and Working Formulation systems[11]. The WHO (World Health Organization) classification has unified precursor T-cell lymphoblastic leukemia (T-ALL) and T-LBL as precursor T-cell lymphoblastic leukemia/lymphoma.[4]

Epidemiology of lymphoblastic lymphoma

Lymphoblastic lymphoma (LBL) is a rare disease that accounts for approximately 2% of all Non-Hodgkin lymphoma.[12] LBL can be subdivided in LBL of T-lineage (T-LBL) and precursor B-cell lineage (pB-LBL) comprising 80% and 10% of cases. In about 10% of cases, the immunophenotype can not be defiend.[4;12;13;13-23] The median age of pediatric T-LBL at diagnosis is 8.8 years and the male to female ratio is 2.5:1.[18]

There is no clear evidence for a change in the incidence of lymphoblastic lymphoma in the recent years.[24;25]

Diagnosis of lymphoblastic lymphoma

T-LBL is distinguished from T-ALL based on amount of lymphoblasts in the bone marrow at the time of initial diagnosis. Patients with less than 25% lymphoblasts in the bone marrow and no blasts in the peripheral blood are diagnosed with lymphoblastic lymphoma.

The histopathological and immunophenotypic features of lymphoblastic lymphoma are distinctive, so that diagnosis if properly approached, should be rarely a problem. The diagnosis can be established in two ways: In case of malignant effusions and/or significant bone marrow infiltration the diagnosis can be established by means of cytomorphology on cytospin preparations and immunophenotyping of cell suspensions, or in case of missing fluid specimen, diagnosis can be established by tissue examination coupled with immunophenotypic analysis after tumor biopsy.

Differential diagnoses are the pB-LBL and the Burkitt Lymphoma. Both can be distinguished by immunophenotypic analysis of the cells. Further differential diagnostic considerations include other small round-cell tumors. CD99, which is characteristically present on Ewing's Sarcoma and peripheral neuroectodermal tumor, is also expressed in lymphoblastic malignancies.[26]

Distinction from thymoma is complicated by the fact that both tumors have a thymic T-cell phenotype, but the morphology of the cells is generally different, and the characteristic epithelial distribution of thymoma is not present in lymphoma. In addition, cases of thymoma are rare in childhood and adolescence.

Morphology of lymphoblastic lymphoma

Morphologically, LBL show diffuse pattern of tissue involvement by a uniform population of small to medium-sized blastic cells. The lymph node shows complete effacement, with involvement of the capsule. The size of the lymphoblasts ranges from small cells with very condensed nuclear chromatin and no evident nucleoli to larger blasts with finely dispersed chromatin and relatively prominent nucleoli. The nuclear cytoplasmic ratio is high. Mitotic cells are invariably present and there are often apoptotic bodies in T-LBL. The classically described morphologic findings of T-LBL correspond to L1 (or L2) acute lymphoblastic leukemia in the French-American-British Classification of acute lymphoblastic leukemia.[27]

Immunophenotype of lymphoblastic lymphoma

The immunophenotype of the T-LBL cells corresponds with defined stages of intrathymic T-cell differentiation.[28;29] Precursor-T-cell lymphoblastic lymphoma most commonly express CD7, CD5, and CD2, whereas CD3 is present cytoplasmatically (cyCD3) or on the surface (sCD3).[30] LBL are usually TdT positive, a marker that distinguishes them from all other types of lymphoma.[15;31-35] In addition to TdT, CD34, and CD99 (MIC2)[26] are also expressed by the majority of T-LBL and proved useful in distinguishing this malignancy from other NHL.

CD4 and CD8 can be expressed in any combination: alone, neither, or both together. The expression of antigens corresponds to different stages of T-cell maturation with pro/pre-T-cells expressing cyCD3, CD7, CD2, CD5 or CD8, intermediate T-cell with positivity of CD1a, CD4, CD8 and CD3 and mature T-cells expressing membrane-bound CD3, and either CD4 or CD8, while CD1a is negative. In the NHL-BFM series most of the T-LBL were diagnosed with intermediate immunophenotype.[36]

T-cell lymphoblasts may demonstrate clonal rearrangement of the T-cell receptor genes, but this does not have to be lineage-specific.[37;38] Only CD3 is considered as a lineage specific marker.[4] Examination of histological section of T-cell lymphoblastic lymphoma/leukemia could identify several cases with expression of CD79a, which is considered to be a marker for B-lineage.[39;40] Also CD10 expression is variable in T-LBL.[41] Aberrant expression of myeloid-associated antigen, a feature of some T-ALL, can occur in T-LBL.[41-43] Interestingly, a small number of T-LBL may undergo a phenotypic switch to acute myeloid leukemia (AML).[44;45]

Although some of these lineage switches may occur secondary to treatment, examples of T-cell lymphoblastic malignancies that contain a very small blast population with committed myeloid maturation features have been reported.[20]

Genetic alterations and pathogenesis of lymphoblastic lymphoma

Due to the scarcity of adequate tumor tissue, reported cytogenetic or molecular genetic studies of T-LBL are rare and include small numbers of patients.[46-50] Additionally, many reports describing molecular and cytogenetic findings of T-LBL include cases of ALL and vice-versa. Thus, an accurate summary of cytogenetic abnormalities in T-LBL devoid of the influence of ALL is still lacking. Cytogenetic data of a small series of six children with T-LBL showed that clonal aberrations are common and most frequently involve the T-cell receptor gene regions. The aberrations were similar to aberrations in T-ALL[51], which is in line with earlier reports.[47;50;52]

It was reported, that the t(9;17) translocation might appear more commonly in T-lymphoblastic lymphoma than in T-cell acute lymphoblastic leukemia. Patients with this translocation often present with a mediastinal mass and have an aggressive disease course.[50;53]

The t(8;13)(p11;q11-14) has been described in rare cases of T-LBL that present with myeloid hyperplasia and eosinophilia.[54;55]

The t(10;11)(p13-14;q14-q21) was described in T-ALL, AML and T-LBL.[56-58]

The lack of cytogenetic and molecular genetic data on T-LBL hampers the understanding of the pathogenesis and molecular basis of T-LBL. The principal mechanisms are thought to be analogue to the pathogenic mechanisms in T-ALL.[59] However, currently this hypothesis is not proven with experimental data. In the WHO classification it is stated, that the etiology of T-LBL and T-ALL is largely unknown.[4]

Clinical features of lymphoblastic lymphoma

Common presentation sites of T-LBL are the mediastinum and pleural effusions causing respiratory impairment. Pericardial effusion with resulting cardiac tamponade may also occur in individual cases. In addition, symptoms and signs of superior vena cava obstruction may be present. Further manifestation sites are lymph nodes, liver, spleen, skin, bone, testis and soft tissue. T-LBL cells have the potential for dissemination to the central nervous system (CNS), but this is rare in T-LBL with a frequency of about 3% in the NHL-BFM group.[18] Bone marrow (BM) infiltration occurs in almost 20% of the cases, however, the amount of infiltration lymphoblasts in the bone marrow must be less than 25% per definition.

Prognostic factors in lymphoblastic lymphoma

Reliable and accurate methods for predicting prognosis are required to achieve adequate treatment with the least invasive regimens. Identification of clinical and/or biological prognostic factors is essential for individual risk adapted treatment of the patients. Specific chromosomal aberrations in hematological malignancies have been well known for years and in some entities even serve as diagnostic criteria or criteria for risk stratification.[60;61] However, in T-LBL the available genetic data are to few to prove prognostic impact of a specific genetic alteration and until now no genetic alteration or immunophenotypic marker with stable prognostic impact has been identified.

Concerning clinical parameters, few small retrospective series of adult patients with lymphoblastic lymphoma identified adverse clinical prognostic factors such as advanced age, Ann Arbor stage of disease, bone marrow or CNS involvement, lactate dehydrogenase (LDH) level, B symptoms, time to attainment of complete response or International Prognostic Index Group.[19;37;47;62-65] Only few parameters have been consistent across different series, most have varied in the different studies.

In pediatric LBL Riopel and colleagues showed adverse prognostic effect of advanced Ann Arbor stage of disease, bone marrow involvement, CNS involvement, elevated LDH level and B symptoms.[26] However, in the series of the BFM-group including 290 uniformly treated pediatric T-LBL patients, the only parameters with prognostic impact were B symptoms and critically ill condition at diagnosis.[66]

In conclusion, there are no consistent data for T-LBL on prognostic parameters. Hence no clear rationale exists for the stratification of T-LBL patients into different risk groups with differences in the treatment intensity. The only stratification criterion in use for the ongoing clinical trial of the NHL-BFM group is the stage of disease with reduced treatment intensity for patients with stage I or II disease. However, in T-LBL less than 5% of patients are diagnosed with limited stage I or II disease. Therefore, in practical terms more than 95% of patients receive the identical chemotherapy without any individual risk adaptation of treatment intensity.

Treatment and outcome of lymphoblastic lymphoma

Combination chemotherapy with or without local radiotherapy resulted in event-free survival rates of 64-90% for children suffering from lymphoblastic lymphoma.[36;67-74] In early studies the outcome of patients with lymphoblastic lymphoma was fatal for the majority of the patients. The introduction of intensive chemotherapy and radiation therapy protocols produced marked improvements in outcome. Protocols such as the LSA_2-L_2 regimen combined intensive chemotherapy with central nervous system irradiation.[75] For children with LBL various strategies

have been implemented to further improve the treatment outcome achieved with LSA_2-L_2.[36;67;69;70;76] The French Society of Pediatric Oncology incorporated courses of high-dose methotrexate into an LSA_2-L_2 backbone with excellent results.[67] In the NHL-BFM-group 5-year event-free survival of 85-90% has been achieved with a regimen whose consolidation phase includes four courses of high-dose methotrexate (5g/m² given every 2 weeks).[36;77] This improvement in outcome may be the result of higher intracellular levels of methotrexate polyglutamates achieved with the higher dose of methotrexate.[78] In addition to an improved outcome, the BFM-strategy contains reduced cumulative doses of cytostatic agents with a high risk for late effects compared with the LSA_2-L_2 backbone. Other refinements in therapy that are thought to contribute to improved treatment outcome include the incorporation of a re-induction phase, as well as the incorporation of L-asparaginase.[36;70;76]

Relapse of lymphoblastic lymphoma

Relapses of T-LBL occur early (within the first year after diagnosis) and involve the local site of initial manifestation in most of the cases. With currently available rescue-treatment strategies a significant number of patients fail to achieve a stable second remission. Therefore the outcome after failure of frontline treatment is very poor with 5-years survival rates below 20%. Almost all patients who were successfully rescued received high-dose chemotherapy followed by allogenic stem-cell transplantation.[79-82]

2.3 Acute lymphoblastic leukemia

Epidemiology of lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is the most common type of cancer in children.[25;83] The majority of pediatric ALL account for precursor B-cell immunophenotype (pB-ALL), while about 15% of cases express T-lineage associated antigens.[4;28;84;85] The median age at diagnosis is 8.1 years and the male to female ratio in childhood and adolescent T-ALL is 3:1.[84]

Diagnosis of lymphoblastic leukemia

Diagnosis of ALL is established by cytomorphological examination and flow cytometry analysis of bone marrow or peripheral blood. Differential diagnostic considerations for T-ALL include ALL of B-lineage, T-LBL, minimally differentiated AML and reactive bone marrow. T-ALL, pB-ALL and AML can be distinguished from each other by immunophenotypic analysis of the malignant cells. The differentiation of T-ALL and T-LBL is based on the blast count in the bone marrow. The diagnosis of T-ALL requires 25% or more lymphoblast infiltration in the bone marrow.

Morphology of lymphoblastic leukemia

The morphologic features of the cells are indistinguishable from the morphology of T-LBL cells with small to medium sized blast cells with scant cytoplasm, condensed to dispersed chromatin and high nuclear to cytoplasmic ratio.

Immunophenotype of lymphoblastic leukemia

T-ALL and T-LBL cells are similar in basic immunophenotyping. The lymphoblasts express TdT and variable CD1a, CD2, CD3, CD4, CD5, CD7 and CD8[86-88]. CD10 might be positive and CD79a positivity has been reported in some cases.[39;40;89] Co-expression of myeloid associated antigens CD13, CD33 and CD117 were detectable in considerable numbers of patients.[41;90]

Genetic alterations and pathogenesis of lymphoblastic leukemia

How many different mutations are required for leukemic transformation still remains to be elucidated. As leukemic cells are thought to derive from normal lymphoblasts arrested at defined stages of early maturation, every cell function or pathway that ensures homeostasis of the T-cell maturation system is a potential target for disruption. Some nonrandom cytogenetic alterations were described for T-ALL cells e.g. deletions of chromosome 9p or 6q, trisomy 8, 14q11 breakpoints, 11q23 breakpoints, 14q32 translocations, and 7q32-q36 breakpoints.[91-95] Translocations involving the T-cell receptor (TCR) loci either TCRαδ at chromosome 14q11 or TCRβ locus at chromosome 7q34 have been detected in 15% to one third of the patients.[96;97] The translocations lead to a deregulated expression of the partner gene by juxtaposition with the regulatory region of one of the T-cell receptor loci. Translocation partner genes include several genes with oncogenic properties such as MYC(at chromosome 8q24), TAL-1(1p32), RBTN/LMO1(11p15), RBTN/LMO2(11p13), HOX11(10q24) and LCK(1p34).[93;98] The TCR breakpoints in many cases resemble TCR recombination signals, implying that the genetic alteration occurred during TCR rearrangement.[99-103]

NOTCH1 was discovered as partner gene of the TCR locus in the translocation t(7;9) which was described in rare cases of T-ALL.[104] Subsequently the essential role of NOTCH1 for T-cell maturation was shown.[105;106] Interestingly, recent functional screenings of T-ALL samples revealed, that gain-of-function mutations of NOTCH1 can frequently be detected in T-ALL.[107] Currently, lots of efforts are undertaken to further elucidate the role of NOTCH1 activation in the pathogenesis of T-ALL. NOTCH1 is suggested to act as a master transcriptional regulator that contributes to T-cell transformation by regulating cell growth.[108] Weng and colleagues could identified c-Myc as direct target of NOTCH1 in T-ALL cells[109] and Chiang and colleagues

identified a regulatory sequence of NOTCH1 whose function influences the leukemogenic activity of NOTCH1[110].

Alteration of TAL-1 on chromosome 1p32 is considered to be one of the most common nonrandom genetic aberrations in T-ALL. In about 10-25% of cases TAL-1 locus is deregulated by a small interstitial deletion resulting in the SIL-TAL-1 fusion gene with aberrant expression of TAL-1.[111-115] Translocations involving the TAL-1 locus were estimated to occur in 1-3% of T-ALL.[92;116] TAL-1 (or stem cell leukemia gene SCL) encodes a basic helix-loop-helix transcription factor which is expressed in erythroid, myeloid, megakaryocytic, and hematopoetic stem cells[117;118] and is required for normal hematopoesis.[119;120] However, this gene is not expressed in normal T-lineage cells.[121] Recent data indicate, that in T-lineage progenitor cells transcriptional effects downstream of the aberrant expression of TAL-1 are amplified in a complex transcriptional network that results in the disruption of critical mechanisms that control cell homeostasis during T-cell development.[108]

The deletion of chromosome 9p occurs more frequently in T-ALL than in other immunologic subsets. It is observed in about 30% of cases by cytogenetic analysis and a higher percentage by molecular testing with even 70% or higher deletion-rate in T-ALL.[122-126] This deletion results in the loss of CDKN2A and CDKN2B. These gene loci code for p14 and p15 which have been implicated for a role in the biology of T-ALL.[127-132] Also inactivation of the transcripts at transcriptional and post-transcriptional level have been reported.[132;133] The transcripts of the locus are involved in the upstream regulation of retinoblastoma (Rb) protein thus blocking the cell cycle at the G1 phase[134] and in the regulation of p53 protein, which ensures cell cycle arrest and attempt to repair genotoxic damage before replicating DNA.[135;136]

The ATM gene product (Ataxia teleangiectasia mutated gene) is another protein which plays a central role in mediating cellular signaling in response to DNA damage. Hereditary mutations of the ATM gene cause ataxia teleangiectasia, a degenerative neurological disorder, which is characterized by immunodeficiency and increased risk of developing hematological malignancies, especially of T-cell origin.[137;138] In addition, acquired ATM alterations were described in children with lymphoid malignancies implicating ATM in leukemogenesis.[139;140] A recent report identified five polymorphisms of the coding part of ATM in children with T-ALL, which were associated with higher white blood cell count and unfavorable outcome.[141]

The review of the wide range of genetic alterations in T-ALL led to the understanding, that these chromosomal rearrangements are a hallmark of the biological diversity of T-ALL cells and are likely the result from defects in underlying cellular control mechanisms. Defective cell cycle surveillance mechanisms are likely to be the major factors leading to both, deregulated proliferation and chromosomal abnormalities that are associated with leukemic cells.[29;142;143]

Clinical features of lymphoblastic leukemia

By definition all T-ALL patients have at least 25% lymphoblast count in the bone marrow and/or detectable lymphoblasts in the peripheral blood at initial diagnosis. T-lineage ALL is often associated with high leukocyte count in the peripheral blood and a mediastinal mass.[144-147] Other sites are peripheral lymph nodes, skin, liver, spleen, Waldeyer's ring, CNS and gonads.

Prognostic factors in lymphoblastic leukemia

A number of risk factors were described in different clinical trials. Differences in risk stratification and treatment as well as the improvement of outcome in recent trials complicate the identification of stable and valid prognostic factors.

Concerning clinical characteristics, a recent review stated that the prognostic impact of age and leukocyte count have little clinical significance in T-ALL.[148] In an analysis of the ALL-BFM trials the relapse rate was slightly lower in T-ALL patients under 10 years of age.[84] However, the association between different factors such as T-lineage immunophenotype, elevated age and hyperleukocytosis hamper the assessment of the direct and independent impact of age on the prognosis. Male gender has been reported to be associated with poor prognosis.[149] However the adverse effect of male gender has been abolished in clinical trials in which the overall 5-year event-free survival rate is about 80% or more.[150;151]

Regarding biological features of the leukemic cells, the prognostic impact of immunophenotypic characteristics of ALL were analyzed in several studies. However, the lack of standardized criteria for the classification of subgroups, the different treatment strategies applied and the association of immunophenotypic parameters with certain cytogenetic aberrations complicated the assessment of the independent prognostic impact of immunophenotypes. In the past, ALL with T-cell immunophenotype was generally considered to be at high risk[148], mainly due to the association of T-lineage immunophenotype with numerous unfavorable clinical features. Therefore children with T-ALL were reported to have a worse prognosis than non-T-ALL.[85;152;153] Investigations of the prognostic impact of the maturation stage of T-ALL lymphoblasts indicate a favorable outcome of CD1a-positive intermediate phenotype and inferior outcome of patients with pro-T-cell immunophenotype.[29;154-158] Some studies could show associations between the expression of particular immunophenotypic markers and the outcome of patients e.g. a worse outcome for CD10 negative T-ALL.[85;146;159] A larger Children's Cancer Group series reported a statistically significant positive correlation between the CD2 expression level and event free survival.[160] And it was reported that CD3 positivity combined with an abnormal karyotype was a significant adverse risk factor[161], in contrast another study found no prognostic significance of CD3 expression[85]. In the same way the co-expression of myeloid-associated antigens remains a

controversial prognostic factor with similar outcome for patients with and without myeloid-antigen co-expression in some studies[29;90;154], but inferior results in others[162;163].

Cytogenetic abnormalities have been described in pediatric ALL and were used for risk-stratification in clinical trials e.g. in cases with hyperdiploidy or specific translocations, such as t(4;11) and t(9;22). However, the majority of these specific aberrations occur in pB-ALL rather than in T-ALL.[164] In T-ALL aberrant karyotypes particularly involving the T-cell receptor genes were described, but none of these cytogenetic abnormalities have been shown to have prognostic significance.[92;93;95] The data on the prognostic impact of molecular genetic detection of homozygous deletion of the CDKN2A locus in childhood T-ALL are inconsistent but there might be an unfavorable impact.[128]

Recently Breit et al. showed that activating NOTCH1 mutations in pediatric T-ALL patients predict a more rapid early treatment response and favorable long term outcome in the context of the treatment strategy ALL-BFM 2000.[165] In contrast, Zhu et al. showed statistically significant inferior relapse-free survival for adult patients with T-ALL positive for NOTCH1 mutation compared with NOTCH1 mutation negative T-ALL patients. In the pediatric age-group NOTCH1 mutation positive patients tended to an inferior outcome, but this trend was no longer significant.[166]

Gene expression profile studies showed that most cases of T-ALL can be grouped on the basis of involvement of one or more specific oncogenes such as HOX11, HOX11L2, TAL-1 plus either LMO1 or LMO2, LYL1 plus LMO2, and MLL-ENL.[167] Over-expression of these specific oncogenes has been reported to confer favorable or unfavorable prognosis[168-172], however the numbers were small and the data partly inconsistent. Therefore no solid prognostic genetic marker has been identified and reached general acceptance so far.

The ALL-BFM group implemented the vivo response to prednisone as an important prognostic parameter. In trial ALL-BFM 83 a 7-day prednisone prephase was introduced for the reduction of morbidity derived from tumor lysis. The reduction of lymphoblasts in peripheral blood was evaluated. The in vivo response to the 7-day prednisone prephase was shown to be of strong prognostic significance and the a leukemic blast cell count in peripheral blood of 1,000 per µl or more at day 8 defined a subgroup of ALL patients with a very high risk of relapse.[173] Therefore the early prednisone response was introduced as additional risk stratification criterion in trial ALL-BFM 86 and remained the most important prognostic factor for the subsequent ALL-BFM trials.[2;152;174]

In the ongoing clinical trial ALL-BFM 2000 patients are stratified into three risk groups based on the in vivo prednisone response, the presence or absence of the translocations t(9;22) or t(4;11), and the cytomorphological response in the bone marrow at day 33. Patients are further

stratified according to minimal residual disease (MRD) kinetics. MRD kinetic is assessed at two different time points, at day 33 and week 12 of treatment. Allele-specific polymerase chain reaction (PCR) protocols are used for quantitative detection of leukemic clone-specific T-cell receptor gene rearrangements.[175]

Treatment and outcome of lymphoblastic leukemia

Previous studies showed poorer outcomes for patients with T-lineage ALL compared to B-lineage.[145;153;161;176;177] The criteria used for risk classification were different, complicating the comparisons between groups. Nevertheless T-ALL patients were assigned to more intensive treatment elements compared to patients with pB-ALL.[173;178-181] Recent studies have reported improved outcomes in pediatric T-ALL[150;151;182-185], which suggest that for T-ALL patients when treated by intensive therapeutic regimens the risk might be almost similar to that of B-lineage ALL.

In the BFM-strategy based protocols, the outcome of T-ALL patients improved clearly in trial ALL-BFM 86 compared with previous trial ALL-BFM 83; most likely due to the introduction of high-dose methotrexate in trial ALL-BFM 86.[153]

Relapse of lymphoblastic leukemia

The outcome of patients who suffered a relapse of T-ALL is dismal because only a small number of patients (about 20%) can be saved with intensive chemotherapy followed by bone marrow transplantation (BMT).[186;187]

Distinction between T-cell lymphoblastic lymphoma and leukemia

Both diseases share common characteristics as immunophenotypic features, the morphology of the lymphoblasts but also clinical characteristics as the median age at diagnosis and the favorable outcome after ALL-type chemotherapy. Besides the similarities there are obvious differences between the two diseases. In lymphoma patients an anterior mediastinal mass represents the primary site of disease while in leukemia patients bone marrow involvement is the predominant site of disease. Interestingly the typical sites of relapse differ also, with predominantly local relapse in lymphoma patients[36;80] and systemic relapse in T-ALL[186]. These differences in the distribution of the disease might indicate differences in the homing of lymphoblasts between lymphoma cells and leukemia cells. This raises the question whether T-LBL and T-ALL represent one disease with two different presentations or whether the cells of the two diseases carry different biologic potentials.

Because of the overlapping morphologic and immunophenotypic features, the distinction between acute lymphoblastic leukemia and lymphoblastic lymphoma is considered by some to be largely arbitrary.[188-190] The International Lymphoma Study Group and the WHO have designated these malignancies precursor T lymphoblastic leukemia/lymphoma in the updated REAL and WHO classification.[4;191;192]

However, subtle immunophenotypic, molecular, and cytogenetic differences suggest that acute lymphoblastic leukemia and lymphoblastic lymphoma might be biologically different in certain aspects.[14;15;20;28;193-195]

Immunophenotype

T-ALL and T-LBL show overlapping immunophenotypic features[4], but some studies stated that lymphoblastic lymphomas in general have antigen expression profiles consistent with a more mature stage of intrathymic T-cell development than does T-cell acute lymphoblastic leukemia.[14;15;20;22;193-196] However, attempts at separating lymphoblastic lymphoma from acute lymphoblastic leukemia by immunophenotypic features have been largely unsuccessful. Interestingly, it was reported on a small series, that T-ALL express more frequent the TCRγδ than it can be detected in T-LBL.[197] However, larger comparative series analyzed with current immunophenotypic techniques and classified according to agreed classification systems are lacking.

Genetics

Compared with T-ALL, there is relatively little literature on cytogenetic studies or molecular analyses in T-LBL.[59] The elucidation of genetic alteration in T-LBL is hampered by the scarcity

of specimen for detailed genetic characterization. It is stated in the literature and current text books that the typical chromosomal aberrations reported in T-ALL can also be found in T-LBL.[4;20] However, larger series of cytogenetic and molecular genetic data in T-LBL are missing.

Concerning molecular genetic analyses, Raetz and colleagues compared gene expression profiles of nine T-LBL and ten T-ALL samples. Though the number of samples was limited, the authors reported significant and clear distinctions in the expression profiles between T-LBL and T-ALL using both unsupervised and supervised methods of data analysis.[198]

Baleydier and colleagues performed paraffin tissue microarray immunophenotyping, T-cell receptor rearrangement genotyping by Southern blot and PCR and RQ-PCR quantification of pTa, RAG1, HOXA5/A9, HOX11/HOX11L2, LMO1/2, LYL1, TAL-1, SIL-TAL, CALM-AF10 and NUP214-ABL (fusion-) transcripts in a retrospective series of 44 T-LBL (12 patients < 16 years). The authors concluded that pediatric T-LBL show different oncogenic profiles compared to those of T-ALL arrested at the same stage of maturation.[199]

In addition, the incidence of NOTCH1 mutations were examined comparatively for T-ALL and T-LBL samples and analyzed with respect to possible differences regarding clinical features or stage of maturation arrest of the T-lymphoblasts. 46 T-ALL samples and 44 T-LBL samples obtained from adult and pediatric patients were analyzed by direct sequencing for mutations in exons 6, 27 and 34. The authors reported NOTCH1 mutations in 48% of T-ALL samples and 41% of T-LBL samples. The type of mutations differed insofar, as combined mutations of the HD (heterodimerisation) domain and the PEST domain were seen in 6/22 T-ALL and 6/18 T-LBL, HD-mutations-only were seen in 16/22 T-ALL and 7/18 T-LBL, while PEST-mutations-only were seen in none of the T-ALL samples but 5/18 T-LBL samples.[200]

Clinical characteristics

The best known difference between T-ALL and T-LBL is the primary site of involvement. T-ALL patients present with bone marrow involvement and lymphoblasts in the peripheral blood often accompanied by thrombocytopenia. In T-LBL patients the primary manifestation of the lymphoma is the anterior mediastinum with pleural and/or pericardial effusions.

Splenomegaly and adenopathy was reported to be more frequent in T-ALL than in T-LBL.[188] The frequency of CNS involvement in T-ALL is more than twice of the frequency of CNS involvement in T-LBL.[18;84] However, in general the clinical characteristics show a larger overlap between pediatric T-LBL and T-ALL, e.g. a high portion of T-ALL patients are diagnosed with mediastinal mass.

Treatment

In the past the prognosis for T-ALL patients was inferior compared with the outcome of T-LBL patients despite treatment with comparable intensive chemotherapy protocols.[23;36;153] However, these differences were equalized due to the improvement in outcome for T-ALL patients.

2.4 Deletions of chromosome 6q

The NHL-BFM study center in Giessen and the Oncogenetic Laboratory in the department of Pediatric Hematology and Oncology of the Justus Liebig University Giessen serve as national reference laboratories for cytomorphology, molecular genetics and cytogenetics in childhood and adolescent NHL. These laboratories receive tumor, blood and bone marrow samples from patients in Germany for central reference analysis. In addition, the Institute of Human Genetics, University Hospital Schleswig-Holstein, Campus Kiel, head Prof. Dr. R. Siebert (former Prof. Dr. W. Grote) served as national reference laboratory for cytogenetics in the trial NHL-BFM 95.

During the last years a considerable number of samples for cytogenetic analysis could be examined to allow a retrospective analysis of the available data. This analysis of the cytogenetic data of T-LBL patients revealed four patients with a deletion of chromosome 6q. The full karyotype of these four patients is depicted in Table 2. Interestingly, all four patients exhibited a common deleted region at chromosomal band 6q15-q16 as illustrated in Figure IV (common deleted region highlighted with light grey color) and all four patients had suffered a relapse.

Table 2. Karyotypes of the four index patients with cytogenetic detectable deletion of chromosome 6q. All four patients suffered a relapse.

index patient I	46,XX,del(6)(q1?2q1?6),t(9;17)(q34;q22)
index patient II	46,XY,del(6)(q12q24)
index patient III	46,XY,t(1;5)(q32;q35), del(6)(q15q25)
index patient IV	at relapse: 45~47,XY,del(6)(q1?4q2?1),t(9;14)(q34;q11),+?20,+mar

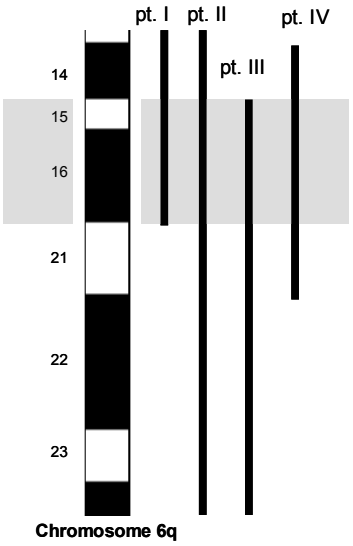


Figure IV. Deleted region of chromosome 6q in the four index patients (pt.) detected by cytogenetic analyses. The black bars indicate the deleted region in each of the four cases.

A review of the literature revealed that deletions of chromosome 6q have been reported in a variety of hematological malignancies such as lymphoid malignancies[201-212], B-cell Non Hodgkin Lymphoma[213-218], follicular lymphoma[219;220], chronic lymphocytic leukemia (CLL)[221-223], Waldenstrom macroglobulinemia[224;225], or (pediatric) acute lymphoblastic leukemia[93-95;226-241].

Chromosome 6q deletions have also been described in a variety of solid tumors such as cystic carcinoma[242], pancreatic cancer[243], hepatocellular carcinoma[244], melanoma[245], prostate cancer[246;247], breast cancer[248], gastric carcinoma[249], and lung cancer[250].

These data led to the suggestion, that a tumor suppressor gene is localized on chromosome 6q. A number of studies on hematological malignancies narrowed down different minimal regions of deletion[202;205;206;213;216;221;237;251], and two studies identified candidate tumor suppressor genes localized in the minimal deleted region of the study samples, but their tumor suppressor role has not yet been confirmed. [201;226]

Concerning the prognostic value of 6q deletions in hematological malignancies, some studies claim deletions of chromosome 6q to be of no prognostic impact[93;228;240;252], while other studies associate them with inferior treatment response and an increased risk for relapse.[207;215;216;220;222;225;236;241;253;254]

Focused on T-LBL, there were no data about 6q deletions available in the literature. In pediatric acute lymphoblastic leukemia the frequency of 6q deletions was reported between 8% to 18% in

cytogenetic analyses[92;93;95;227;228] and 15-32% with molecular genetic analyses[204;240;255]. Differentiated according to immunophenotype, 6q deletions were more frequent in T-ALL compared with precursor B-cell ALL.[233;234;236;252]

Eight published studies of ALL samples and in some studies ALL and NHL samples which were based on fluorescence in situ hybridization or loss of heterozygosity analyses identified a common deleted region in the respective sample series.[204-206;226;229;231;240;254] The relative positions of the common deleted regions are summarized in Figure V.

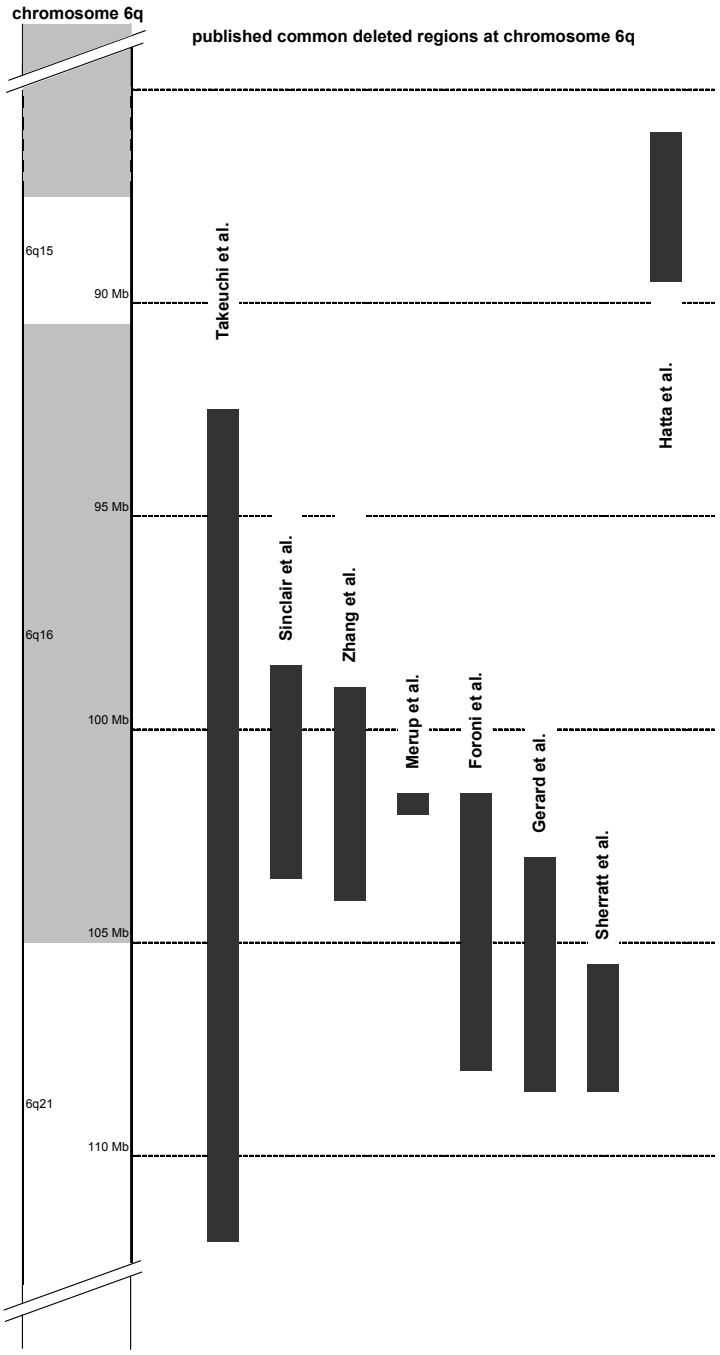


Figure V. Regions of minimal deletion in ALL (and NHL) identified in published studies mapping 6q deletion with fluorescence in situ hybridization or loss of heterozygosity

The prognostic impact of 6q deletions in T-ALL has not been determined yet. It was reported that the early response to treatment was inferior in ALL patients with 6q deletions compared to patients without 6q deletions.[252] Four studies indicated an association of 6q deletions with poor outcome. In two cytogenetic analyses of adult ALL patients deletions of 6q were associated with reduced event free survival and higher relapse rates.[236;241] Focused on T-ALL, a karyotype analyses of 354 pediatric T-ALL cases reported 6q deletions in 38 patients (11%). Twenty-seven of these 38 patients suffered a relapse.[92] In addition, Foroni et al. reported inferior outcome in T-ALL patients with del(6)(q16q21) compared to patients without this aberration.[254] However, in some studies there was no prognostic value of 6q deletions in ALL patients.[240;252;255]

In conclusion neither the frequency nor the prognostic impact of chromosome 6q deletions was analyzed in pediatric T-LBL so far. Four index patients of the NHL-BFM studies might indicate a possible association of 6q deletions with increased risk of relapse in T-LBL.

T-ALL is considered to be a biologically closely related disease or according to some, simply the different clinical manifestation of the same disease. The available data on the impact of 6q deletions in T-ALL are inconsistent. Therefore, the impact of chromosome 6q deletions and the relationship of pediatric T-LBL and T-ALL remain to be elucidated.

2.5 Study objectives

In T-LBL patients, the clinical impact of chromosome 6 deletions is still unclear, due to the lack of genetic data. In T-ALL the impact of 6q deletions is complicated due to the inconsistent reports in the literature. The published studies on 6q deletions in hematological malignancies imply, that 6q deletions might have different effects in different biological entities. In addition, the prognostic value might depend on the treatment applied. Therefore, this retrospective study was focused on a systematic comparison of chromosome 6q deletions in pediatric T-LBL compared with pediatric T-ALL. To allow detailed outcome analyses the current retrospective study was restricted to patients uniformly treated according to ALL-BFM type treatment strategies.[77;153;256]

The first phase of the project was focused on T-LBL samples. The following study objectives were defined:

- Analysis of the frequency of chromosome 6q deletions in T-LBL
- Identification of the common deleted region in T-LBL
- Evaluation of the prognostic impact of 6q deletions in T-LBL

In the second phase of the project the study objectives were extended and transferred on T-ALL, a biologically closely related disease. The following study objectives were defined:

- Analysis of the frequency of chromosome 6q deletions in T-ALL
- Identification of the common deleted region in T-ALL
- Evaluation of the prognostic impact of 6q deletions in T-ALL
- Comparison of T-LBL and T-ALL concerning the frequency of chromosome 6q deletions, the common deleted regions of 6q deletions and the prognostic impact
- Discussion whether the molecular genetic pattern of 6q deletions add data on the question concerning biologic similarities and differences between T-LBL and T-ALL

Based on the limitations concerning the availability of tumor samples, especially in the T-LBL cases, it was intended to perform fluorescence in situ hybridization (FISH) to examine the above mentioned study objectives. The FISH technique allows the investigation of interphase nuclei and does not necessarily require metaphase nuclei as cytogenetic analysis does. Therefore, the spectrum of evaluable sample types is larger in FISH technique and includes also tumor touch imprints and cytospin preparations of malignant effusions.

3 Material and methods

3.1 Materials

3.1.1 Reagents for Fluorescence in situ hybridization (FISH)

Table 3. Reagents for FISH reaction

Lysogeny Broth (LB) Medium containing Chloramphenicol*
Agarose, PeqGOLD Universal Agarose, Peqlab, Erlangen, Germany
I2-Propanol, Sigma, Steinheim, Germany
H_2O
10x NT-buffer*
0.1 M ß-Mercaptoethanol*
dNTP, Roche, Mannheim, Germany*
Spectrum Green-dUTP, Vysis, Downers Grove, USA
Spectrum Orange-dUTP, Vysis, Downers Grove, USA
Cy3-2'-deoxy-uridine-5'-triphosphate, Amersham Biosciences, Buckinghamshire, UK
Biotin-16-2'-deoxy-uridine-5'-triphosphate, Roche, Mannheim, Germany
Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate, Roche, Mannheim, Germany
DNA Polymerase I, 5 U/µl, Roche, Mannheim, Germany
Deoxyribonuclease I, Invitrogen, Karlsruhe, Germany
DNA ladder III and VI, Roche, Mannheim, Germany
1% Ethidium-bromide, Roth, Karlsruhe, Germany
0.5 M EDTA, Invitrogen, Karlsruhe, Germany
Human cot DNA (1 mg/ml), Invitrogen, Karlsruhe, Germany
Salmon sperm DNA (10 mg/ml), Sigma, Steinheim, Germany
3 M sodium acetate*
30%, 50%, 70%, 90%, 100% Ethanol, Riedel de Haen, Sigma, Seelze, Germany*
CEP Hybridization mix, Vysis, Downers Grove, USA
Fixative (methanol:acetic acid)*
0.1x SSC*
2x SSC*
0.01 M HCl*
Pepsin solution*
1x PBS*
Formamide solution*
Rubber cement Fixogum, Marabu, Tamm, Germany

Continue Table 3	
	1x SSC*
	2x SSC+Tween 0.1%*
	4x SSC+Tween 0.05%*
	Avidin-FITC, Roche, Mannheim, Germany
	Antidigoxigenin-Rhodamin, Roche, Mannheim, Germany
	1% BSA, Promega, Madison, USA
	Vectashield, Mounting Medium with DAPI, Burlingame, USA
	5% BSA, Promega, Madison, USA

* Solutions are described in detail in appendix 8.2 Solutions, buffers and media.

3.1.2 Reagents for DNA preparation

Table 4. Reagents for DNA preparation	
100% Ethanol	100% Ethanol, Riedel de Haen, Sigma, Seelze, Germany*
Chloroform	Trichlormethan Chloroform $\geq$ 99%, Roth, Karlsruhe, Germany
Sodium citrate	Sodiumcitrate-Dihydrate, Merck, Darmstadt, Germany
Sodium hydroxide	Sodium hydroxide, Roth, Karlsruhe, Germany

3.1.3 Reagents for PCR

Table 5. Reagents for PCR reaction	
dNTP	ROTI®-Mix PCR3 (10nM), Roth, Karlsruhe, Germany
Polymerase	AmpliTaq Gold, Applied Biosystems, Weiterstadt, Germany
$MgCl_2$	$MgCl_2$ Solution 50 mM, Invitrogen, Karlsruhe, Germany
Buffer	GeneAmp 10x PCR Buffer (contains 15 mM $MgCl_2$), Applied Biosystems, Weiterstadt, Germany
H_20	LiChrosolv, Merck, Darmstadt, Germany

3.1.4 Reagents for fragment length analysis

Table 6. Reagents for fragment length analysis	
Polymer	Performance Opti. Polymer6 3100 (POP-6), Applied Biosystems, Weiterstadt, Germany
Buffer	Buffer (10x) with EDTA, Applied Biosystems, Part. No. 402824
HI-DI	Hi-Di™ Formamide, pH 7.1, Applied Biosystems
Size standard	Genescan™ 400 HD [ROX™] Standard, Applied Biosystems single-stranded labeled fragments: 50, 60, 90, 100, 120, 150, 160, 180, 190, 200, 220, 240, 260, 280, 290, 300, 320, 340, 360, 380, 400

3.1.5 Patients' samples

In T-LBL cases, the samples were collected in the reference laboratories of the study NHL-BFM between April 1995 and May 2004. The study center and reference laboratory for cytomorphology and genetics at the Justus Liebig University Giessen, head Prof. Dr. A. Reiter provided bone marrow and blood smears, tumor touch imprints, cytospin preparation of malignant effusions, frozen tumor cells and frozen cells from malignant effusions. The reference laboratory for pathology at the Lymph Node Registry at the University in Kiel, head Prof. Dr. H. Wacker (former Prof. Dr. R. Parwaresch) provided DNA extracted from paraffin embedded tumor biopsies. The reference laboratory for immunophenotyping at the Robert-Rössle-Klinik at the HELIOS Klinikum Berlin-Buch, Charité Medical School, Berlin, head Prof. Dr. W.-D. Ludwig and the Institute of Human Genetics, University Hospital Schleswig-Holstein, Campus Kiel, head Prof. Dr. R. Siebert (former Prof. Dr. W. Grote) provided tumor cells of malignant effusions or tumor cells frozen in DMSO.

In T-ALL the samples were collected between August 1999 and July 2002. The DNA of the initial tumor samples and blast-free follow-up samples were provided by the ALL-BFM study center and reference laboratory for cytomorphology and MRD monitoring, head Prof. Dr. M. Schrappe.

3.2 Molecular genetic methods

3.2.1 Fluorescence in situ hybridization (FISH)

In situ hybridization is a technique for the analysis of structural and numerical chromosome aberrations, as it allows the visualization of defined sequences of nucleic acids at the cellular and subcellular level. The method is based on site-specific annealing (hybridization) of single-stranded DNA (probe) to denatured complementary sequences on cytological preparations (target).

The protocol used in the current study was adapted from the early work of Gall and Pardune, who described the technique of in situ hybridization.[257] This early technique of isotopic in situ hybridization was later modified using non-isotopic, in particular fluorescence in situ hybridization (FISH) protocols. FISH is a technique used for the detection of target DNA by hybridization of the samples with a complementary probe which is coupled with fluorochromes. The probes are synthesized with incorporated fluorescence molecules or reporter molecules which can be recognized with fluorochromes-labeled reporter binding molecules. Figure VI illustrates schematically the technique of FISH analysis. A major advantage of the FISH-technique is, that it does not require metaphase nuclei, but also works with interphase nuclei. This is of great significance in cases where metaphase chromosome spreads cannot be prepared.

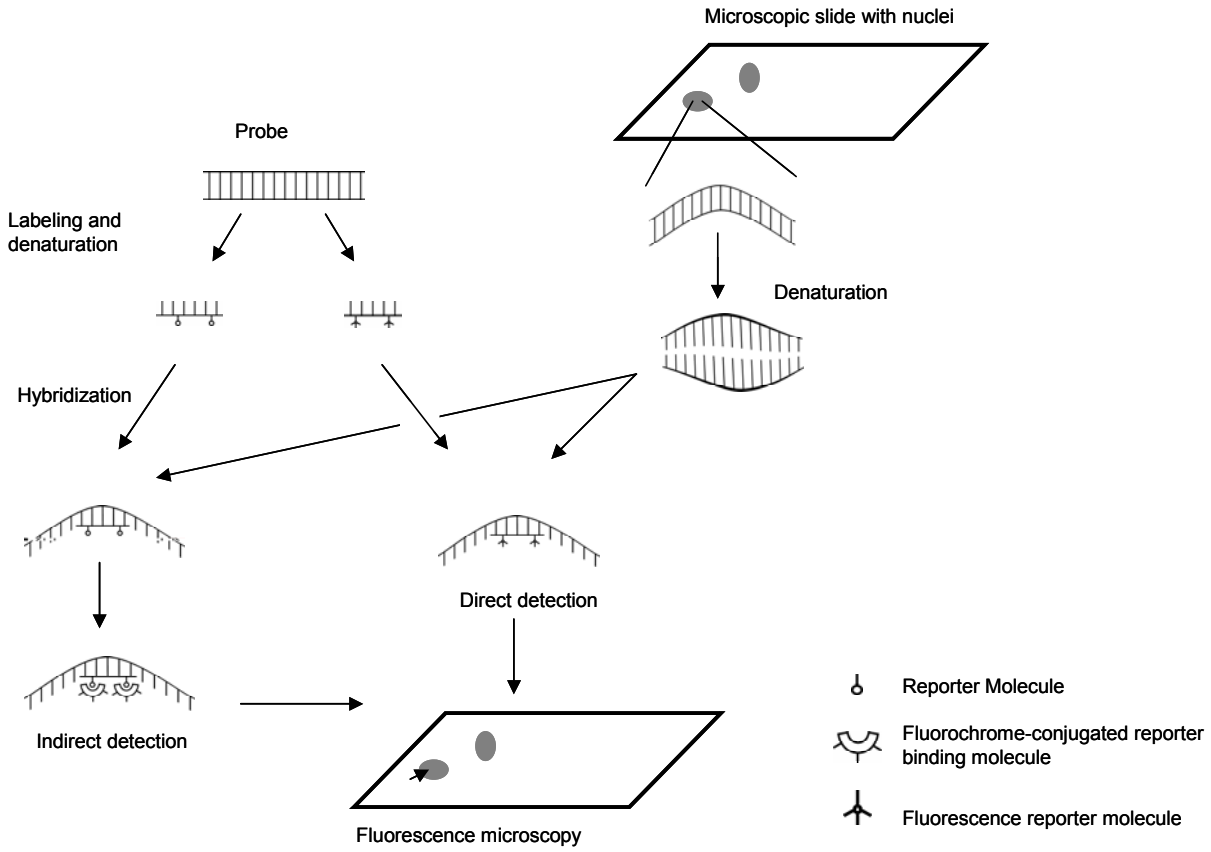


Figure VI. Schematic illustration of fluorescence in situ hybridization (FISH)

3.2.1.1 Selection of bacterial clones, culture of bacteria and DNA preparation

Commercial FISH probes are available for different chromosomal loci in the human genome. These commercial probes are already labeled with fluorescent dyes and can be used for hybridization without further modifications. However, the number and localization of the commercially available FISH probes for human chromosome 6q were not sufficient for the study objective to narrow down the common deleted region in the proximal part of chromosome 6q.

Therefore bacterial artificial chromosome (BAC) clones were used in the current study. These clones of *Escherichia coli* bacteria contain a plasmid with specific human DNA sequences. The size of these plasmids varies, depending on the particular human DNA locus sequence. Typical BAC clones used for FISH analyses contain plasmids of 100 to 200 kb. After expansion of the bacteria, the DNA can be prepared, labeled and used as specific FISH probes. The available BAC clones for genetic markers in the human genome are depicted in current genome data bases (Internet references I, II, III). In the current study the selection of the BAC clones for the preparation of FISH probes was based on the analysis of the reported common deleted regions of 6q in the literature and the common deleted region of the four relapsed T-LBL index patients. The genetic markers D6S1601, D6S283 and D6S246 were affected by the relevant reports of common deleted regions in the literature and the markers were within or close to the common deleted region in the four index patients. The chromosomal localization of the marker is schematically depicted in Figure VII.

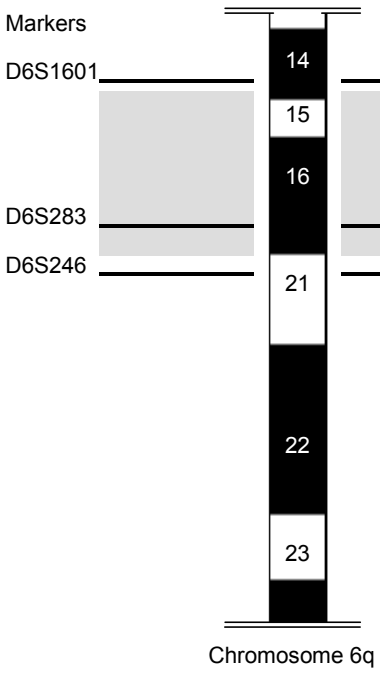


Figure VII. Schema of the localization of the genetic markers D6S1601, D6S283 and D6S246 on human chromosome 6q. Light grey infill: common deleted region of the four relapsed index patients of the NHL-BFM series

Via data base research BAC clones with plasmid of complementary DNA sequences were identified (I, II, III). BAC clones were obtained from BACPAC Resources Online (IV). Table 7 summarizes the name of the clones and the chromosomal localization of the clones that were tested in the current project.

Table 7. BAC clones tested in the study

Clone	Chromosomal locus	Marker
RP3-435K13	6q14.3	D6S1601
RP1-33L1	6q14.3	D6S1601
RP11-602G22	6q14.3	D6S1601
RP11-133H4	6q16.3	D6S283
RP11-259I14	6q16.3	D6S283
RP11-487F5	6q16.3	D6S283
RP11-423D17	6q16.3	D6S283
RP11-951B10	6q16.3	D6S283
RP 1-111B22	6q21	D6S246
RP3-429G5	6q21	D6S246
RP11-815N24	6q21	D6S246
RP11-15H7	6q24.1	

The aliquots of the BAC clone stocks were cultured in a preparatory culture in 5 ml Lysogeny Broth (LB) medium with Chloramphenicol at 37°C overnight on a vibraxer. Aliquots of the preparatory culture were again cultured in 50 ml LB medium with Chloramphenicol at 37°C overnight. DNA was prepared using JETSTAR Midi Prep, GENOMED, Loehne, Germany. The DNA preparation with this kit was based on a modified alkaline/sodium dodecyl sulfate (SDS) procedure. After neutralization the lysates were applied onto a column and the plasmid DNA was bound to the anion exchange resin. After washing the bound plasmid DNA was eluted from the column. According to the manufacturer the kit was preferentially designed to extract and purify high copy plasmid DNA from *E.coli* cultures. The components of the kit and the protocol for DNA extraction are summarized in Table 8.

Table 8. DNA preparation with JETSTAR Midi Prep, GENOMED, Loehne, Germany

Components
Solution E1 (Cell Re-suspending)
Solution E2 (Cell Lysis)
Solution E3 (Neutralization)
Solution E5 (Column Washing)
Solution E6 (DNA Elution)

Continue Table 8

Protocol

1. Equilibration of the columns with 10 ml solution E4 under gravity flow.
2. For harvesting of the cells, the *E. coli* cells were pelleted by centrifugation at 6 000 rpm at 4°C for 15 min and the medium was removed.
3. Re-suspension of the cells with 4 ml solution E1.
4. Cell lysis with 4 ml solution E2 and incubation at room temperature for 5 min.
5. For neutralization 4 ml E3 solution were added and immediately mixed by inverting the tube 5 times, incubation for 10 min.
6. The equilibrated column was loaded with the lysate from step 5.
7. The column was washed with 10 ml E5 solution by gravity flow. This step was repeated once.
8. For elution 5 ml E6 solution were added and the eluate was collected
9. For the precipitation of the DNA 3.5 ml isopropanol were added. Centrifugation at 15 000 rpm at 30°C for 5 min. The plasmid DNA was washed with 70% ethanol and re-centrifuged at 15 000 rpm for 5 min. Air dry of the pellet. Pellet was re-dissolved in 10-15 µl H_2O.

3.2.1.2 Nick translation

For the detection procedure of the DNA probes, the plasmid DNA was labeled enzymatically according to a Nick translation protocol. Two techniques were used for labeling of the BAC probes: 1) probe labeling with either biotin or digoxigenin as reporter molecules and indirect detection of hybridized probe via avidin or antibodies or 2) direct labeling with nucleotides conjugated directly with fluorescent dyes. Table 9 summarizes the different labeling dyes tested in this study. Labeling with Cy3-dUTP for red signals and labeling with Biotin/Avidin-FITC for green signals revealed highest quality of the fluorescence signals.

Table 9. Labeling of DNA probes

<u>Indirect detection</u>	
Biotin	Biotin-16-dUTP/Avidin-FITC (Roche)
Digoxigenin	Digoxigenein-11-dUTP/Antidigoxigenin-Rhodamin (Roche)
<u>Direct detection</u>	
Cy3	Cy3-dUTP (Amersham)
Spectrum Green	Spectrum Green-dUTP (Vysis)
Spectrum Orange	Spectrum Orange-dUTP (Vysis)

The following protocol was used for the nick translation (Table 10). Because fluorescent dyes are subject to photobleaching (fading), the preparations were not permanent. During the whole procedure the dyes were kept away from light.

Table 10. Reaction and protocol of nick translation

Components	
H_20	x µl
10 x NT buffer	5 µl
ß-Mercaptoethanol	5 µl
dNTP	5 µl
Biotin-16-/Cy3-dUTP	1 µl
DNA (2 µg)	x µl
Polymerase I	2 µl
DNAse I (1:6 000)	1 µl
	50 µl

Protocol

1. ´ The reaction was incubated in a 15°C water bath for 2 h.
2. The reaction was bedded on ice, until the actual size of the probe molecules was determined.
3. An aliquot of 6 µl was taken, gel loading buffer and 1 µl 0.5 M EDTA were added and the reaction was loaded on a 1.5% agarose gel (100 V, 30 min).
4. DNA was visualized in the gel by ethidium bromide and photographs were taken during UV illumination.
5. Fragments should be smaller than 600 bp but larger than 200 bp.
6. For enzyme inactivation 1 µl 0.5 M EDTA was added and the reaction was incubated at 65°C for 10 min.
7. For precipitation: careful mixture of
   - 44 µl DNA probe (ca. 1 800 ng)
   - 90 µl (90 µg) human cot-1-DNA (1 mg/ml)
   - 2 µl (20 µg) salmon sperm-DNA (10 mg/ml)
   - 1/10 volume (= 14 µl) 3 M sodium acetate (pH 5,2)
   - 2.5-3 volumes abs. ethanol (400 µl; -20°C)

   Incubation at –20°C over night.
8. Centrifugation at 14 000 rpm and 4°C for 30 min.
9. The supernatant was removed and the pellet was washed with 2-3 volumes (400 µl) 70% ethanol (-20°C).
10. Centrifugation at 14 000 rpm and 4°C for 12 min.
11. The supernatant was removed and the pellet was air dried at room temperature for 20-30 min.
12. Elution of the pellet in 6 µl H_2O.
13. After optimal elution of the pellet, 14 µl hybridization mix (pH 7-7,5) was added, final concentration of the probe: 90 ng DNA/µl.

3.2.1.3 Hybridization of probes

To increase probe accessibility and to reduce fluorescence background staining, the microscopic slides with the target samples were pre-treated as described in Table 11.

Table 11. Pre-treatment of microscopic slides

1. Fixation of the microscopic slides in fixative for 20 min.
2. For rehydration slides were put through a series of 70%, 50% and 30% ethanol and 0.1x SSC at room temperature for 1 minute each.
3. To increase the permeability of the membranes, the slides were incubated in 2x SSC (85°C) for 20 min.
4. Slides were cooled down to 37°C.
5. Equilibration of slides in HCl solution (37°C) for 3 min.
6. Addition of 300 µl Pepsin solution and incubation at 37°C.

   The period of incubation was adjusted to the thickness of the cells on the microscopic slide and the duration of storage of the slide prior to FISH analysis. In general, incubation period was increased with increasing thickness of the cells and longer storage period. The incubation time according to thickness of the cells and storage period was as follows:

Type of sample and thickness	stored > 1 year	stored > 2 years	stored > 4 years
Tumor touch imprint thin	25 min	30 min	40 min
Tumor touch imprint middle	30 min	35 min	45 min
Tumor touch imprint thick	35 min	40 min	50 min
Cytospin preparation normal	25 min	30 min	40 min
Cytospin preparation thick	30 min	35 min	45 min

7. Washing in 1x PBS for 3 min.
8. Slides were put through a series of 70%, 90% and 100% ethanol at room temperature for 2 minutes each.
9. Air dry (10 min to 1 day).

Denaturation of the target samples on the microscopic slides was performed as described in Table 12.

Table 12. Denaturation of target samples

1. 150 µl 70%/2x SSC Formamide denaturation solution were applied on the slide and covered with a coverslip 24x60 mm.
2. The target samples were denatured thermally with incubation on a heater at 85°C for 25 min.
3. The coverslips were removed carefully and the slides were put through a series of 70% ethanol for 2 min and 90% and 100% ethanol for 1 minute each at room temperature for dehydration.
4. Air dry for 10 min.

The denaturation of the probes, the hybridization of probes and target samples and the subsequent washing procedure for probes labeled for direct detection with Cy3-dUTP are described in Table 13.

Table 13. Denaturation of probes, hybridization and post-hybridization washing for probes with Cy3-dUTP labeling for direct detection

1. The DNA probes were denatured thermally with incubation at 75°C for 5 min.
2. 5 µl DNA probe was applied on the slide, covered with a coverslip 18x18 mm and sealed with rubber cement.
3. The hybridization reaction was carried out at 37°C overnight in a moist chamber.
4. The rubber cement was removed carefully and microscopic slides were collected in 2x SSC (37°C) for 2 min.
5. Incubation in 2x SSC (37°C) for 3-5 min on a vibraxer for the removal of the coverslip.
6. Washing in 1x SSC (72°) for 8 min.
7. Washing in 2x SSC + Tween for 5 min.
8. Washing in 1x PBS at room temperature for 3 min.
9. Slides were put through a series of 70%, 90% and 100% ethanol at room temperature for 2 minutes each.
10. Air dry for 10 min.
11. 20 µl Vectashield were applied on the slide, a coverslip 24x50 mm was added and sealed with rubber cement (Fixogum).
12. Storage at 4°C.

For the probes labeled with biotin-16-dUTP for indirect detection, the protocol was modified as specified in Table 14.

Table 14. Denaturation of probes, hybridization and post-hybridization washing for probes with indirect detection

Steps 1-6 as described in Table 13.

7. Incubation of the slides in 4x SSC + Tween at room temperature for 5 min.
8. For blocking 60 µl 5% BSA were applied on the slide, coverage with a coverslip 24x60 mm.

9a. Incubation in a moist chamber at 37°C for 15 minutes.

9b. In parallel: preparation of the fluorochrome-conjugated reporter binding molecules:

Dilution of the Avidin-FITC 1 : 400 in 1% BSA
Centrifugation at 12 000 rpm for 3 min.

10. Careful removal of the coverslip; collection of the slides in 4x SSC + Tween.
11. For detection, 60 µl of the solution with Avidin-FITC were applied on the microscopic slide and covered with a coverslip.
12. Incubation in a moist chamber at 37°C for 20 min.

continue Table 14

13. Careful removal of the coverslip.
14. Washing in 4x SCC + Tween (43°C) on a vibraxer for 3 min, repeated once.
15. Washing in 1x PBS at room temperature for 3 min.
16. For dehydration slides were put through a series of 70%, 90% and 100% ethanol at room temperature for 2 min each.
17. Air dry for 10 min.
18. 20 µl Vectashield were applied on the slide and a coverslip was added.
19. Storage at 4°C.

Repetitive elements in genomic DNA are known to cause unspecific fluorescent signals due to unspecific hybridization of the repetitive sequences in the labeled DNA probe and the target DNA samples. To reduce unspecific hybridization of repetitive elements in the labeled probe with complementary sequences in the samples, human cot DNA and salmon sperm DNA were used. The reagents were added to the plasmid DNA probe in step 8 of the nick translation (Table 10). In principal, the labeled probe fragments are denatured together with an excess of unlabeled competitor DNA. The repetitive sequences in the excess of competitor DNA hybridize rapidly with the single-stranded repetitive elements of the target sample, while the less frequent specific sequences remain single-stranded. These sequences hybridize with the specific sequences of the labeled plasmid DNA.

3.2.1.4 Fluorescence microscopy

The signals of the locus specific DNA probes were visualized using an Axiophot fluorescence microscope with Pan-Neofluor objectives and a 100 Watt mercury lamp (Zeiss, Göttingen, Germany). The microscope was equipped with a high performance charge coupled device (CCD) camera (COHU, San Diego, USA).

Analyses of interphase nuclei included the examination of 200 nuclei per slide. Two fluorescence signals of a probe per nucleus indicated germline status for the particular locus. Detection of only one fluorescence signal per nucleus indicated the deletion of the second loci. The analyses were performed on 400x magnification. The cut-off for significant findings in a sample was defined by at least 5% aberrant findings in 200 analyzed nuclei.

The nick translation, preparation of samples, denaturation of probe and target DNA, hybridization, washing and fluorescence microscopy were performed in the Oncogenetic Laboratory, head Prof. Dr. J. Harbott in the Department of Pediatric Hematology and Oncology, Justus-Liebig University, Giessen.

3.2.2 Loss of Heterozygosity analysis (LOH)

Loss of heterozygosity (LOH) in a cell represents the loss of a single parent's contribution to part of the cell's genome. In a heterozygote, loss of heterozygosity can arise by two methods. In the first, a region of a chromosome is deleted, resulting in only one copy remaining. In the second, genetic recombination leaves the cell with two copies of the chromosomal region, but both come from the same parent. The first method is thought to be the by far more frequent event that results in LOH. Under the assumption, that the finding of LOH of a chromosomal locus corresponds to deletion of one copy of the respective locus, LOH analyses represent one technique to analyze deletions in the genome.

LOH can be identified in tumor cells by noting the presence of heterozygosity at a genetic locus in an organism's germline DNA. Then comparing the tumor DNA can reveal the absence of heterozygosity at that locus. LOH analysis is often done using polymorphic markers, such as microsatellites or single nucleotide polymorphisms, for which the two parents contributed different alleles. After polymerase chain reaction (PCR) amplification the polymorphic alleles can be detected e.g. using fluorescence detection.

In the current study LOH analysis was based on microsatellite marker analysis. These microsatellite markers are spread over the whole genome and characteristically contain repeats of di-, tri,- or tetra-nucleotide sequences (short tandem repeats STR). In certain markers, the number of these repeats is highly polymorphic, as exemplarily shown in the upper part of Figure VIII for the tri-nucleotide repeat with one to ten copies of the TAA-tri-nucleotide sequence in marker D6S2407. This results in the variable fragment length of microsatellite markers which can be detected by fragment length analysis (FLA).

In heterozygotes the analysis of germline DNA provides two alleles which differ in the size of the two fragments (middle part of Figure VIII). Comparative analysis of the respective marker in the tumor DNA of the same individual can reveal either retained heterozygosity or loss of heterozygosity, when only one fragment is detectable and the second fragment is lost (lower part of Figure VIII). The principal of LOH analysis based on fragment lengths polymorphism of microsatellite marker is illustrated in Figure VIII by one characteristic finding of LOH in a study patient. For the LOH analysis in this patient DNA was prepared from samples with germline DNA and separately from tumor samples of the same patient. The two template DNAs were then amplified separately by PCR using marker specific primer pairs (underlined sequences in Figure VIII). The PCR products were the subjected to electrophoresis on a genetic analyzer (ABI PRISM 3100, Applied Biosystems, Weiterstadt, Germany).

Marker D6S2407

TTC CAA GGA TCT GGC ATT TGT ATT TTT AAG AAT CTA AAA CTT
AAA GTA **TAA TAA TAA TAA TAA TAA TAA TAA TAA TAA** TCT
CAA AAA AAA ATC TAA AGC AGA ATT AAA TAT TTT CAT TGA TTT
GTG TTT TCT CTG ACT TGG CAT ATA GGT TGG CAC AGT TAA TTA

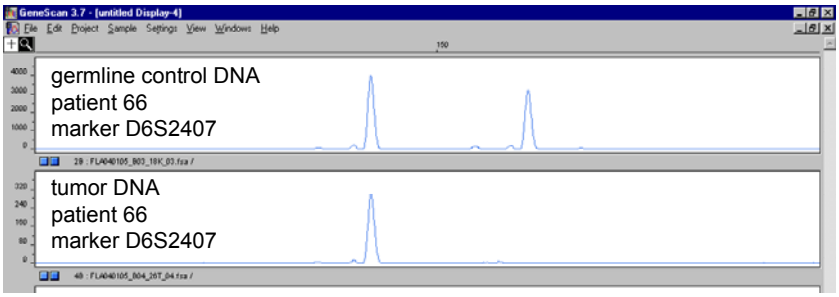


Figure VIII. Example of LOH detected in a study patient. The upper part shows the DNA sequence of marker D6S2407 in the human genome. The microsatellite marker contains a tri-nucleotide repeat sequence TAA, which can be present in the DNA in one to ten copies. The middle part of the figure shows the results of fragment length analysis of germline DNA in patient No. 66. The two peaks represent the two different sized alleles of the microsatellite marker indicating heterozygosity in the germline DNA of the patient with fragment length of 148 bp and 154 bp. The lower part of the figure shows the results of the fragment length analysis of the same marker in the corresponding tumor DNA of the identical patient. The allele with 154 bp is no longer detectable, representing a loss of heterozygosity. Underlined sequences: primer sequences.

3.2.2.1 Preparation of DNA in T-LBL samples

Prior to LOH analyses, DNA was prepared from the available samples. In T-LBL high-molecular-weight germline DNA was extracted from peripheral blood (PB) and bone marrow (BM) smears, or from frozen peripheral blood and bone marrow cells. No significant infiltration by lymphoma cells in the PB and BM smears was demonstrated by morphological analyses and additionally, in about half of the cases, no infiltration by lymphoma cells in the PB and BM was demonstrated by flow cytometry analysis. Morphological review was performed by Prof. Dr. A. Reiter, Hannover/Gießen. The central flow cytometry analysis was performed by Prof. Dr. W.-D. Ludwig, Berlin.

Initial tumor DNA from the time of diagnosis was prepared from tumor touch imprints, cytospin preparations of malignant effusions, frozen tumor cells from tumor biopsies or malignant effusions or from paraffin embedded tumor biopsies. Patients whose material was available only from the time of relapse and not from the initial diagnosis were not included in this analysis. As reported by others, representative sections from T-LBL samples showed minimal percentage of background stromal cells.[198]

Depending on the type of specimen, different DNA purification kits were used. DNA preparation from smears, cytospins or tumor touch imprints were performed with peqGOLD Forensic DNA Kit (PeqLab) or ChargeSwitch® Forensic DNA Purification Kit (Invitrogen). DNA preparation from frozen cells was performed using either E.Z.N.A. blood DNA kit (PeqLab) or peqGOLD TriFast kit (PeqLab). The DNA preparation is described in detail in the following paragraphs.

PeqGOLD Forensic DNA Kit, PeqLab, Erlangen, Germany

This DNA preparation kit was designed to recover small amounts of DNA quantitatively from different specimens, including blood stains. The components of the kit and the protocol used for DNA preparation are described in Table 15.

Table 15. DNA preparation with PeqGOLD Forensic DNA Kit

Components

Carrier suspension

Lysis Buffer D

Bone Lysis Enhancer

Elution Buffer D

Wash Buffer

Protocol

1. Samples were scratched from the slides using a sterile scalpel.
2. 1 ml Lysis Buffer D was added and incubated for at least one hour at room temperature.
3. 30 sec full speed centrifugation and transfer of the supernatant into a new reaction tube.
4. The carrier suspension was vortexed thoroughly and 15 µl were added to the reaction tube and then vortexed.
5. Incubation for 5 minutes at room temperature.
6. Centrifugation for 1 sec at 10 000 rpm to pellet down the carrier. The supernatant was discarded.
7. 1 ml wash buffer was added and the briefly vortexed to completely re-suspend the pellet. Centrifugation for 1 sec at 10 000 rpm. The supernatant was discarded.
8. 2 repeats of step 7, the residual fluid was spined down and the wash buffer was removed.
9. The pellet was dried by replacing the open tube on a heater at 60°C.
10. The pellet was re-suspended in 50 µl H_2O and incubated for 5 min at 60°C.
11. Centrifugation for 2 min at full speed. The DNA-containing supernatant was transferred into a fresh tube. This step was repeated once. Elution volume 100 µl.

ChargeSwitch® Forensic DNA Purification Kit, Invitrogen, Karslruhe, Germany

According to the manufacturer, the ChargeSwitch® kit was specifically designed to allow efficient purification of genomic DNA from forensic samples. The kit uses magnetic bead-based technology to isolate genomic DNA. The surface charge of the magnetic beads depends on the pH of the surrounding buffer with a positive charge in low pH conditions that binds negatively charged DNA backbone. Contaminants and proteins are not bound and washed away in an aqueous wash buffer. For the elution of DNA, the charge on surface of the bed is neutralized by raising the pH to 8.5.

Table 16. DNA preparation with ChargeSwitch® Forensic DNA Purification Kit

Components

ChargeSwitch® Lysis Buffer (L13)

ChargeSwitch® Magnetic Beads

Proteinase K 20 mg/ml in 50 mM Tris-HCl, pH 8.5, 5 mM $CaCl_2$, 50% glycerol

ChargeSwitch® Purification Buffer (N5)

ChargeSwitch® Wash Buffer (W12)

ChargeSwitch® Elution Buffer (E5) 10 mM Tris-HCl, pH 8.5

MagnaRack®, Invitrogen

Protocol

1. Samples were scratched from the slides using a sterile scalpel and transferred into a microcentrifuge tube.
2. 1 ml Lysis buffer and 10 µl Proteinase K were mixed.
3. Lysis Mix was added to the sample tube.
4. Incubation at 55°C for 30-60 min.
5. The supernatant was transferred to a fresh tube.
6. 200 µl purification buffer was added to the tube.
7. After vortexing, 20 µl magnetic beads were added to the sample.
8. Incubation at room temperature for 1-5 min; then the tube was placed in the MagnaRack® for 1 min.
9. Supernatant was removed and discarded; the tube was removed from the rack.
10. 500 µl wash buffer was added and mixed gently and the tube was placed in the MagnaRack® for 1 minute.
11. Supernatant was removed and discarded; the tube was removed from the rack.
12. The wash step was repeated once.
13. For eluting the DNA 100 µl E5 elution buffer were added to the tube and gently mixed until the beads were completely re-suspended.
14. Incubation at room temperature for 1-5 min, gently mixed again.
15. The tube was placed in the MagnaRack® for 1 min until beads form a tight pellet.
16. The elute containing the purified DNA was transferred to a new tube. Elution volume 100 µl.

E.Z.N.A. Blood DNA Kit, PeqLab, Erlangen, Germany

The E.Z.N.A. Blood kits use a reversible DNA-binding property of the HiBind® matrix for DNA purification. After lysis under denaturing conditions, samples were applied to the HiBind® spin columns, which bind DNA, while cellular debris, hemoglobin, and other proteins were washed away. DNA was finally eluted with water. The components of the kit and the protocol used for DNA preparation are specified in Table 17.

Table 17. DNA preparation with E.Z.N.A. Blood DNA Kit

Components

Buffer BL

DNA Wash Buffer Concentrate

HB Buffer

Elution Buffer

OB-Protease

RNase A

TE buffer 10 mM

HiBind® DNA Columns

Collection tubes 2 ml (other materials)

100% Ethanol (supplied by user)

Protocol

1. 250 µl sample were mixed with 25 µl protease and 250 µl BL Buffer.
2. 5 µl RNase A were added and sample was incubated at 70°C and once during incubation briefly vortexed.
3. 260 µl 100% Ethanol was added to the sample.
4. Half of the solution was transferred into a column which was assembled in a 2 ml collection tube; centrifugation at 8 000 rpm for 1 min.
5. Step 4 was repeated with the 2nd half of the solution.
6. The collection tube and the flow-through liquid were discharged and the column was placed into new 2 ml collection tube.
7. 500 µl HB Buffer were added on the column; centrifugation at 8 000 rpm for 30 sec. The flow-through was discarded.
8. The column was placed into a new collection tube and 600 µl wash buffer were added; centrifugation at 8 000 rpm for 1 min. The flow-through was discarded.
9. Step 8 was repeated once.
10. The column was placed into a new collection tube and centrifuged with full speed for 2 min.
11. The column was placed into a sterile 1.5 ml microfuge tube and 60 µl of pre-heated (70°C) H_2O were added. Incubation for 2 min.
12. Centrifugation with 8 000 rpm for 1 min to elute DNA from the column.
13. Step 11 and 12 were repeated once. Elution volume 100 µl.

peqGOLD TriFast, PeqLab, Erlangen, Germany

The TriFast kit was used for DNA preparation of tumor frozen cells. The advantage of this preparation is that it allows parallel preparation of DNA, RNA and proteins. The protocol used for DNA preparation is described in Table 18.

Table 18. DNA preparation with peqGOLD TriFast Kit

Components

TriFast

Supplied by user: Chloroform, Sodium citrate, Sodium hydroxide

1. Phase separation: 700 µl TriFast were added to the sample; incubation at room temperature for 5 min.
    a. After addition of 200 µl chloroform sample was shaken by hand for 15 sec. and then incubated at room temperature for 3-10 min.
    b. Centrifugation at 12 000 rpm and 4°C for 5 min which separated the mixture in 3 phases.
    c. 3 phases: lower chloroform phase (red) with DNA and proteins, interphase with DNA and proteins and a colorless upper phase with RNA.
1. DNA precipitation
    a. The aqueous phase was removed and 300 µl of 100% ethanol were added. Incubation at room temperature for 2-3 min.
    b. Centrifugation at 2 000 rpm at 4°C for 5 min.
2. DNA wash
    a. The supernatant was removed.
    b. The DNA pellet was washed with 1 ml 0.1 M sodium citrate/10% ethanol for 30 minutes. Centrifugation at 2 000 rpm at 4°C for 5 min.
    c. Step b was repeated once.
    d. The DNA pellet was suspended in 2 ml of 75% ethanol and incubated at room temperature for 15 min.
    e. Centrifugation at 2 000 rpm at 4°C.
3. DNA solubilization
    a. DNA pellet was dried at room temperature or on the heater at 37°C.
    b. Suspension in 8 mM NaOH by slowly passing the pellet through a wide bore pipette; amount of NaOH was adjusted to the pellet with final DNA concentration to 0.2-0.3 µg/µl.

Parts of the patients were included in an earlier study, in which the genotype of MTHFR and TNFα was examined.[258;259] For this analysis germline DNA preparation from PB or BM smears was performed by Dr. K. Seidemann, Medical School Hannover, Germany using Roche High Pure PCR Template Preparation kit.

Preparation of DNA from paraffin embedded tumor biopsies was performed by Dr. Wolfram Klapper, Institute of Pathology and Lymph node registry, University Hospital Schleswig-Holstein,

Campus Kiel, Germany. Paraffin embedded tumor specimens were cut with a microtome using a clean blade. Two to five sections of 5 µm thickness were transferred into a reaction tube for removal of paraffin by xylol treatment. After a proteinase K digestion at 42°C over night in Tris-Buffer, the DNA was purified in parts of the samples (see appendix 8.3), using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's manual.

After preparation, the DNA was stored at -20°C until usage. The available specimen, the DNA purification kit and the amount of prepared DNA for each case are specified in appendix 8.3 DNA preparation in T-LBL samples.

3.2.2.2 Preparation of DNA in T-ALL samples

In T-ALL mononuclear cells were isolated from BM or PB samples. Genomic DNA was prepared using the QIAamp DNA blood midi kit (Qiagen GmbH, Hilden, Germany). The isolation of the mononuclear cells and the DNA preparations using the QIAamp DNA blood midi kit were performed at the ALL-BFM Study center in Hannover/Kiel.

40 µl aliquots containing 50 ng DNA/µl were provided by the ALL-BFM study center. Tumor DNA was prepared from initial BM or PB samples with a blast percentage > 70%. Germ line DNA was prepared from follow-up samples of BM or PB without blast infiltration.

In a small number of cases no mononuclear cells were available for the preparation of DNA. In these cases either cells from malignant effusions or BM smears were used for DNA preparation. The DNA preparation was performed according to the protocols specified above. The available specimen, the DNA purification kit and the amount of prepared DNA for all cases are depicted in detail in the appendix 8.4 DNA preparation in T-ALL samples.

3.2.2.3 UV spectrometry

After all DNA preparations the quality and concentration of DNA was examined by UV spectrometry. DNA shows characteristic maximal absorption spectrum at wavelength of 260 nm. Therefore UV spectrometry at this wavelength allows the calculation of DNA concentration. For the calculation the following assumptions were made: An OD (optical density) of one unit corresponds to a concentration of ~50 µg/ml of doubled-stranded DNA.[260]

The typical contamination of DNA elutes are proteins which show characteristic absorption spectrum with maximum at 280 nm. The ratio between the readings at 260 nm and 280 nm provided an estimate of the purity of the DNA elutes. OD_{260} : OD_{280} of 1.8 to 2.0 were considered as pure DNA elutes.

The UV spectrophotometry was performed on a Ultraspec 1000, Pharmacia Biotech, Cambridge, UK, using 5 µl aliquots of elutes + 95 µl H_20 (1:20). All newly prepared DNA elutes

were checked for sufficient DNA quality and DNA concentration. If necessary, the elutes were diluted to DNA concentrations of ~ 5-10 ng/µl, depending on the type of sample and the kit used for DNA preparation.

3.2.2.4 Polymerase chain reaction

The polymerase chain reaction (PCR) provides a powerful technique for direct amplification of short segments of the genome.

The reaction contains the two primers, the template DNA, thermo stable DNA-polymerase (taq DNA-polymerase), all four 2`-deoxynucleoside 5`-triphosphates (dNTPs) dATP, dCTP, dGTP, dTTP, a buffer and magnesium chloride ions.

The PCR protocol starts with denaturation of the DNA preparation at 94°C. The double-stranded DNA is separated by heat into single-stranded DNA serving as a template for amplification. Amplification of the DNA is achieved by DNA polymerase producing a complementary DNA-strand. DNA polymerase needs a double-stranded sequence of DNA as a starting point. To provide this double-stranded DNA the single-stranded DNA is annealed with one forward and one reverse primer sequences of ~20 bases. Each primer is complementary to a site on the opposite strand determining the target microsatellite marker. After denaturation the temperature is lowered in an annealing step to 45°-53°C so both primers can ideally anneal to their complementary regions on the template DNA. The annealing temperature is modified according to the melting temperature of the specific primer pair. In a third step (extension) the temperature is raised to 72°, the temperature optimum for the taq DNA-polymerase. In the extension step new DNA-strands are synthesized complementary to the template DNA. The entire cycle is repeated 35 times resulting in copies of non-determined length (with only one primer at one end) but also copies with a length defined by the two primers. Throughout amplification the number of copies of non-determined length grows linearly whereas the number of copies of determined length grows exponentially. Therefore the predominant majority of products of determined length exist after 25-32 cycles.

The PCR was accomplished in programmable thermo-cyclers which guarantee quick and precise temperature changes.

3.2.2.5 Microsatellite markers

The microsatellite markers used in the present study were polymorphic short tandem repeat markers (STR). The samples were analyzed with a set of 25 microsatellite markers spanning a 64 Mb region on chromosome 6q14-q24.

Markers were chosen based on two criteria: The first selection criterion was that the markers had to cover chromosomal bands 6q15-q16, which represented the common deleted region in cytogenetic analysis of the four relapsed T-LBL index patients in the trial NHL-BFM 95, and the markers should cover the minimal deleted regions of reported deletions of proximal chromosome 6q in hematological malignancies.[204;205;226;229;231;240;251]

The second selection criterion was a preferred rate of >75% heterozygosity, as reported in the Genome Database (II).

The following microsatellite markers were analyzed: D6S1589, D6S2407, D6S251, D6S1609, D6S1627, D6S1004, D6S1644, D6S1043, D6S1274, D6S300, D6S1682, D6S1284, D6S1716, D6S1717, D6S468, D6S283, D6S1580, D6S1021, D6S447, D6S278, D6S261, D6S1657, D6S1639, D6S435, and D6S310. The localization of these markers is depicted in Figure IX schematically.

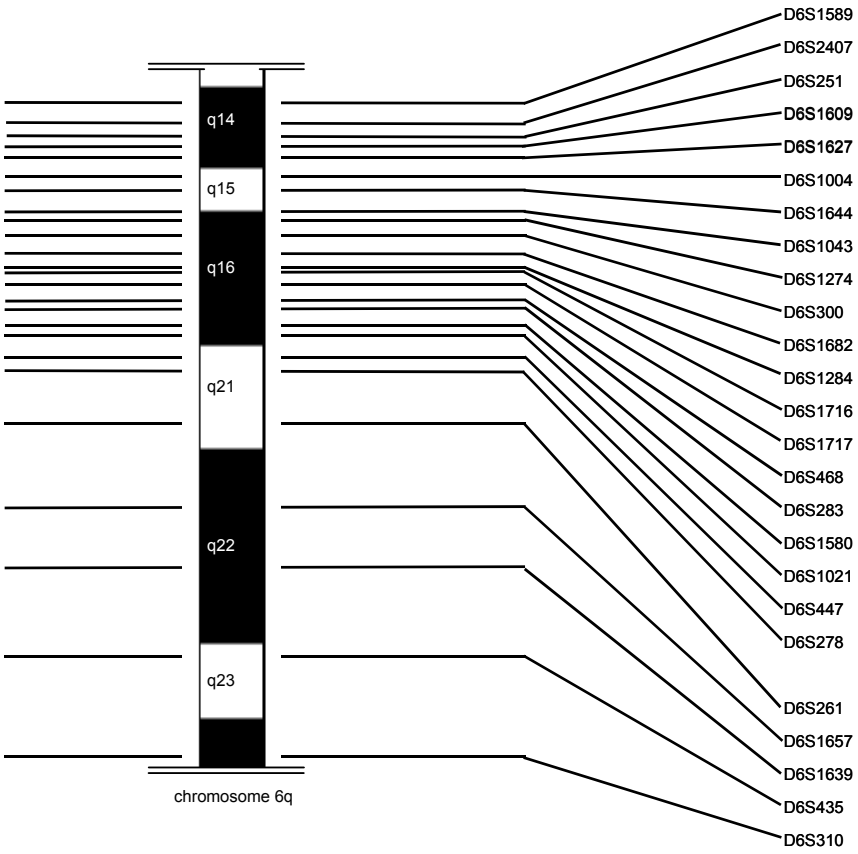


Figure IX. Schema of the localization of the 25 microsatellite markers used for LOH analysis in the present study

3.2.2.6 Primers for polymerase chain reaction

Primer sequences for amplifying the microsatellite marker sequences were retrieved from GDB and the ensembl data base (I, II). PCR primers were synthesized by MWG Biotech (Ebersberg, Germany) with the forward primer labeled at the 5' end with the fluorescent dye 6-FAM.

3.2.2.7 Polymerase chain reaction conditions

Paired normal and tumor DNA samples from each patient were amplified with AmpliTaq Gold enzyme (Applied Biosystems, Weiterstadt, Germany) in PCR reactions using 0.5-100 ng of genomic DNA as a template (as specified in appendix 8.3 and 8.4). PCR was performed in a 25 µl reaction volume containing 1 µl of 10 pmol/µl primer solution and 2.5 µl 10x standard PCR buffer. Thirty-five cycles were carried out in a thermal cycler under conditions specified in table 19-22.

Table 19. PCR reaction	
genomic DNA	0.5-100 ng
Primer solution 10 pmol/µl	1 µl
10x standard PCR buffer	2.5 µl
Taq Gold (5 U/µl)	0.5 µl
dNTP (10 mM each)	1 µl
Mg_2Cl (50 mM)	0-2 µl
H_2O	x
	Σ 25 µl

The concentration of $MgCl_2$ in the PCR reaction was optimized for each primer pair. The amount of additional $MgCl_2$ added to the PCR reaction according to the primer pairs is summarized in Table 20.

Table 20. $MgCl_2$ concentration according to markers	
Primer pair for marker	**$MgCl_2$ (50 mM)**
D6S435	0 µl
D6S1274, D6S1717, D6S283, D6S1021	2 µl
D6S1589, D6S2407, D6S251, D6S1609, D6S1627, D6S1004, D6S1644, D6S1043, D6S300, D6S1682, D6S1284, D6S1716, D6S468, D6S1580, D6S447, D6S278, D6S261, D6S1657, D6S1639, D6S310	1 µl

The PCR program was retrieved from the PCR conditions as specified in GDB database (II).

Table 21. PCR program			
1x	10 min 94°C		
35x	denaturation 45 sec 94°C	annealing 45 sec 45°C	elongation 45 sec 72°C
1x	30 min 72°C		
then continuous	4°C		

The annealing temperature of the PCR program was modified according to the melting temperature of the primer sequences. The rest of the protocol was identical for all markers. The modified PCR program annealing temperatures are listed in the following table 22.

Table 22. Modifications of the annealing temperature for the different primer pairs	
Primer pair for marker	**Annealing temperature**
D6S1682	45°C
D6S251	48°C
D6S1609, D6S1644, D6S1043, D6S1274, D6S300, D6S1284, D6S1717, D6S468, D6S283, D6S1021, D6S278, D6S1657, D6S435, D6S310, D6S261	50°C
D6S1716	52°C
D6S1589, D6S2407, D6S1627, D6S1004, D6S1580, D6S447, D6S1639	53°C

PCR was performed using the following types of thermocyclers: GeneAmp PCR system 9600, Perkin-Elmer, Rodgau-Juegesheim, Germany or Tgradient 96, Biometra, Goettingen, or Tpersonal 48, Biometra, Goettingen, Germany.

3.2.2.8 Fragment length analysis

Aliquots of 1.5 µl of the PCR products were mixed with 18.5 µl HiDi-formamide and 0.5 µl size standard, denatured, and subjected to electrophoresis on a genetic analyzer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, Weiterstadt, Germany). The automatically collected data from fragment-length analysis were analyzed with GeneScan software as described in the manufacturer's manual. The conditions of fragment length analysis are summarized in Table 23.

Table 23. Conditions of fragment lengths analysis with ABI Prism 3100

Capillary	16 silicar capillaries length to detector 50 cm
Polymer	Performance Opti. Polymer6 3100 (POP-6), Applied Biosystems
Buffer	Buffer (10x) with EDTA, Applied Biosystems, Part. No. 402824
Electrophoresis	15 kV, 60°C
Size standard	Genescan™ 400 HD [ROX™] Standard, Applied Biosystems single-stranded labeled fragments: 50, 60, 90, 100, 120, 150, 160, 180, 190, 200, 220, 240, 260, 280, 290, 300, 320, 340, 360, 380, 400
Laser	Argon-ion laser Emission wave lengths 488 nm and 541.5 nm
Detection	CCD-camera (charge-coupled-device) Collected emission spectrum from 500 nm to 700 nm
Software	ABI-Prism 3100 Data collection software, Version 1.1, GeneScan 3.7

3.2.2.9 Data analysis of LOH results

Markers were regarded as informative when two different-sized fragments of the microsatellite marker were detected in germline DNA, and tumor DNA showed either a heterozygous pattern or LOH. To define LOH, ratios for the heights of peak levels of the two alleles in tumor material and in germline DNA were compared. A tumor material ratio below 50% or above 150% of the germline peak height ratio was considered indicative of LOH. An example of the definition of LOH is shown in Figure X.

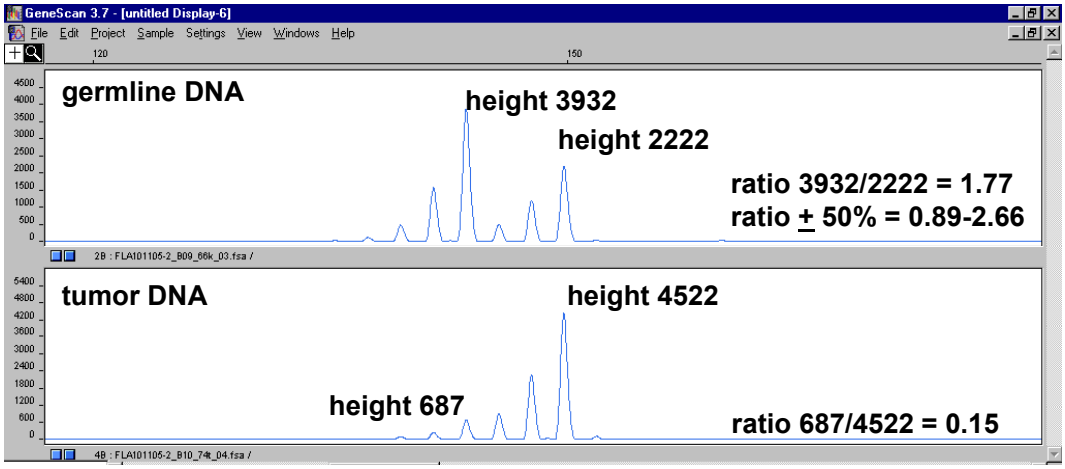


Figure X. Example of LOH

In germline DNA peak height of first allele was 3932, peak height of second allele was 2222 resulting in a ratio of 1.77 (1.77±50% range: 0.89-2.66). In tumor DNA the ratio of first allele height to second allele height was 687/4522 resulting in a ratio of 0.15, which is clearly outside the acceptable variation range for retained heterozygosity of 0.89-2.66. Therefore the present case was regarded as a finding of LOH.

A homozygous pattern in the germline control DNA or microsatellite instability rendered the microsatellite marker not informative for LOH analysis. A microsatellite marker was considered unstable if the PCR product from tumor DNA had an altered fragment length compared to alleles in the corresponding germline DNA.[261]

The most centromeric and telomeric markers with LOH defined the putative deleted region in each patient. This definition of the putative deleted region was based on the assumption that in the majority of cases with 6q aberration, there is only one deletion at 6q and only in exceptional cases two different deleted regions at chromosome 6q will have to be expected.

In contrast to the un-interpreted LOH results, the putative deleted region covers chromosomal regions with no informative markers. The putative deleted region has the advantage of not being influenced by the rate of heterozygosity or the feasibility of LOH analysis of the markers.

3.3 Diagnostics and treatment of patients

3.3.1 Diagnostics and treatment of T-LBL patients

The diagnosis was established by histopathology of the lymph node or tumor biopsy and/or by cytological[27] and immunophenotypic examination[262] of cells from malignant effusions (pleural effusion, ascites, pericardial effusion). Cases were classified according to the updated Kiel classification[10] and the WHO Classification of Hematological Malignancies.[192] Sub-classification of immunophenotypic subgroups was performed according to EGIL criteria.[263]

In more than 90% of cases, the diagnosis was centrally reviewed by one of the reference laboratories for pathology, cytomorphology and/or immunophenotyping.

Staging included PB and BM aspiration smears, cerebrospinal fluid (CSF), serum lactate dehydrogenase analysis, ultrasound, X-ray, computed tomography (CT) or magnetic resonance imaging (MRI), and skeletal scintigraphy. St. Jude staging system was used.[264]

Patients were treated according to the subsequent treatment protocols NHL-BFM 95 and EURO-LB 02. These protocols were based on the ALL-BFM treatment strategy. Patients with LBL stage III/IV received an induction protocol I followed by the extra-compartment consolidation protocol M, re-induction protocol II and maintenance up to total therapy duration of 24 months. Patients with stage I/II disease received the induction protocol I and the extra-compartment consolidation protocol M followed by maintenance up to a total therapy duration of 24 months.[77] The simplified treatment strategy is outlined in Figure XI.

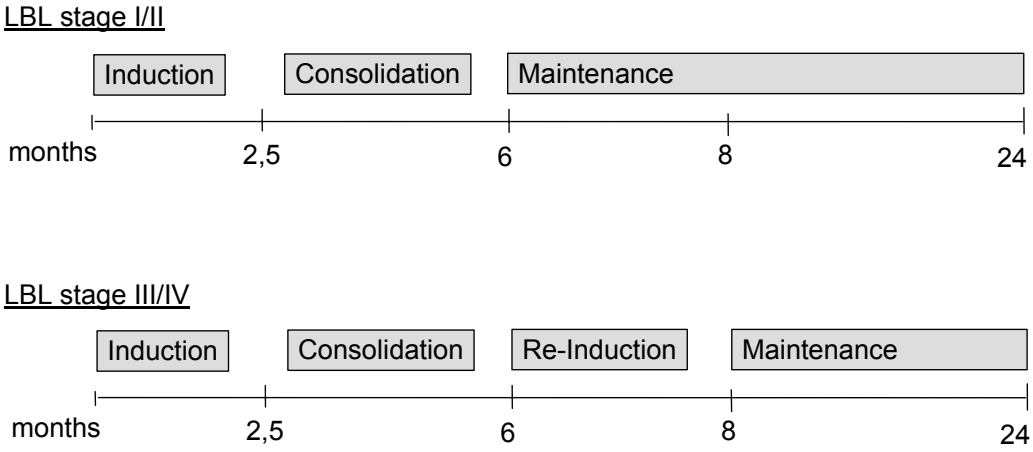


Figure XI. Overview of the treatment plan for T-LBL patients

CNS-positive patients received two additional doses of intrathecal MTX at days 18 and 27 of induction and received cranial radiotherapy (CRT) after re-induction. The dosage was 12 Gy in the second year of life and 18 Gy in older children (children <1 year of age were not irradiated). The details of the treatment are described in appendix 8.1.

Treatment was identical in the trial EURO-LB 02 with the exception for the dose of the *E.coli* L-Asparaginase in induction protocol I. In trial NHL-BFM 95 patients received *E.coli* L-Asparaginase 5,000 U/m^2 in the induction protocol I while in trial EURO-LB 02 patients received *E.coli* L-Asparaginase 10,000 U/m^2 in the induction protocol I.

Response to treatment was evaluated at day 33 of induction. Sufficient response was defined as at least 70% tumor-regression, less than 5% BM-blasts, no CNS-disease. For patients with insufficient response at day 33 treatment was to be intensified according to the high-risk branch of trial ALL-BFM 95.[265] Non-response was defined as persistence of a residual mass ≥ 30% of initial tumor volume, or persistence of ≥ 5% lymphoblasts in the bone marrow or persistence of lymphoblasts in the CSF (with ≥ 5 cells/µl) evaluated after the third course of high-risk treatment. Relapse was defined as the re-occurrence of lymphoma manifestations during or after the end of frontline treatment

3.3.2 Diagnostics and treatment of T-ALL patients

For T-ALL patients the diagnosis was established by cytomorphology of bone marrow (BM) and/or peripheral blood (PB) smears and flow cytometry analysis. Immunophenotyping was conducted as previously described[266] and the criteria for subclassification of T-ALL were adopted from EGIL criteria[263]. In more than 95% of the patients the diagnosis was centrally reviewed by the reference cytomorphological laboratory at the ALL-BFM study center in Kiel and/or the central reference laboratory for immunophenotyping at the Robert-Rössle Clinic in Berlin-Buch (head Prof. Dr. W.-D. Ludwig).

Patients were treated according to protocol ALL-BFM 2000, which is based on the ALL-BFM treatment strategy.[153;256] The treatment was comparable to the treatment of patients with T-LBL with stage III/IV disease as described above.

In T-ALL treatment was stratified according to the early in vivo response to a 7-day prednisone treatment and one intrathecal dose of methotrexate, defined by the number of peripheral blood blasts per microliter on day 8.[2;152] Based on the prednisone response, patients were classified into prednisone good responders (< 1000 blasts/µl) (PGR) and poor responders (≥ 1000 blasts/µl) (PPR). The patients were further stratified according to minimal residual disease (MRD) kinetics.[175]

Complete remission (CR) was defined as the absence of leukemia blasts in the peripheral blood and cerebrospinal fluid, less than 5% lymphoblasts in bone marrow aspiration smears, and no evidence of localized disease. Relapse was defined as recurrence of lymphoblasts or localized leukemic infiltrates at any site.

3.4 Statistical analysis

The probability of event-free survival (pEFS) was calculated according to Kaplan and Meier[267] with differences compared by the log-rank test.[268] pEFS was calculated from the date of diagnosis to the first event (death from any cause, non-response, relapse, or second malignancy) or to the date of the last follow-up. Patients lost to follow-up (LFU) were censored at the time of their last follow-up examination. Cumulative incidence functions for relapse were constructed by the method of Kalbfleisch and Prentice.[269] Functions were calculated from the date of initial diagnosis to the date of diagnosis of non-response or relapse and compared with Gray's test.[270] Differences in the distribution of individual parameters among patient subsets were analyzed using the χ^2 test or Fisher's exact test. Odds ratios were calculated using standard methods.[271]

For the calculation of the risk ratios for relapse, patients with less than two years follow-up due to death from initial complications or treatment related mortality (TRM) were excluded. For the group of 217 T-LBL patients, corrected estimates for the probabilities of relapse in patients with and without LOH and the resulting risk ratio were calculated according to Bayes' formula. This estimation was based on the relapse rate of 27/209 patients with sufficient follow-up. For the group of 186 T-ALL patients, corrected estimates for the probabilities of relapse in patients with and without LOH and the resulting risk ratio were calculated according to Bayes' formula based on a relapse rate of 27/177, excluding 9 patients who died from treatment-related toxicity.

Statistical analyses were conducted using the SAS statistical program (SAS-PC, Version 9.1, Cary, NC: SAS Institute Inc.). Follow-up data were updated as of December 2005 for T-LBL patients and March 2006 for T-ALL patients.

4 Results

4.1 Results of fluorescence in situ hybridization examinations

The design of useful FISH probes was complicated by the problem that several probes prepared from bacterial artificial chromosome clones turned out to hybridize with chromosomal sequences other than the target sequence at chromosome 6q. Hybridization and fluorescence microscopy of these particular probes revealed multiple fluorescence signals (4-10 signals) per nucleus. Experiments showing multiple signals per nucleus were retested. In case of repeated findings of multiple signals per nucleus, the clone was excluded from further examinations. The multiple fluorescence signals were interpreted as an indicator of unspecific hybridization of the probe to chromosomal regions with a high amount of homologous sequences compared with target sequence on chromosome 6q.

Testing of several clones for each of the three target sequences led to the identification of useful probes for each of the loci. After multiple testing three locus specific probes could be identified with clone RP11-602G22 (~157 kb) hybridizing to the locus specific DNA sequence flanking genetic marker D6S1601 at chromosome 6q14, clone RP11-951B10 (~190 kb) complementary to marker D6S283 and clone RP11-815N24 (~207 kb) complementary to marker D6S246. These probes showed only two fluorescence signals per nucleus; one for each chromosome 6q. The localization of the probe at the target sequence was controlled by the examination of hybridization results in metaphase nuclei in which the chromosome 6 were identifiable. The results for all clones tested are depicted in the following table 24.

Table 24. Hybridization results of the different BAC probes

Clone	Locus	Marker	Hybridization results
RP3-435K13	6q14.3	D6S1601	multiple fluorescence signals/nucleus
RP1-33L1	6q14.3	D6S1601	multiple fluorescence signals/nucleus
RP11-602G22	6q14.3	D6S1601	2 fluorescence signals/nucleus
RP11-133H4	6q16.3	D6S283	multiple fluorescence signals/nucleus
RP11-259I14	6q16.3	D6S283	multiple fluorescence signals/nucleus
RP11-487F5	6q16.3	D6S283	multiple fluorescence signals/nucleus
RP11-423D17	6q16.3	D6S283	multiple fluorescence signals/nucleus
RP11-951B10	6q16.3	D6S283	2 fluorescence signals/nucleus
RP 1-111B22	6q21	D6S246	multiple fluorescence signals/nucleus
RP3-429G5	6q21	D6S246	multiple fluorescence signals/nucleus
RP11-815N24	6q21	D6S246	2 fluorescence signals/nucleus
RP11-15H7	6q24.1		multiple fluorescence signals/nucleus

In early phase of testing, newly prepared microscope slides with fixated cells of bone marrow of blood were used for hybridization. Exemplary hybridization results in this type of target samples are shown in Figure XII.

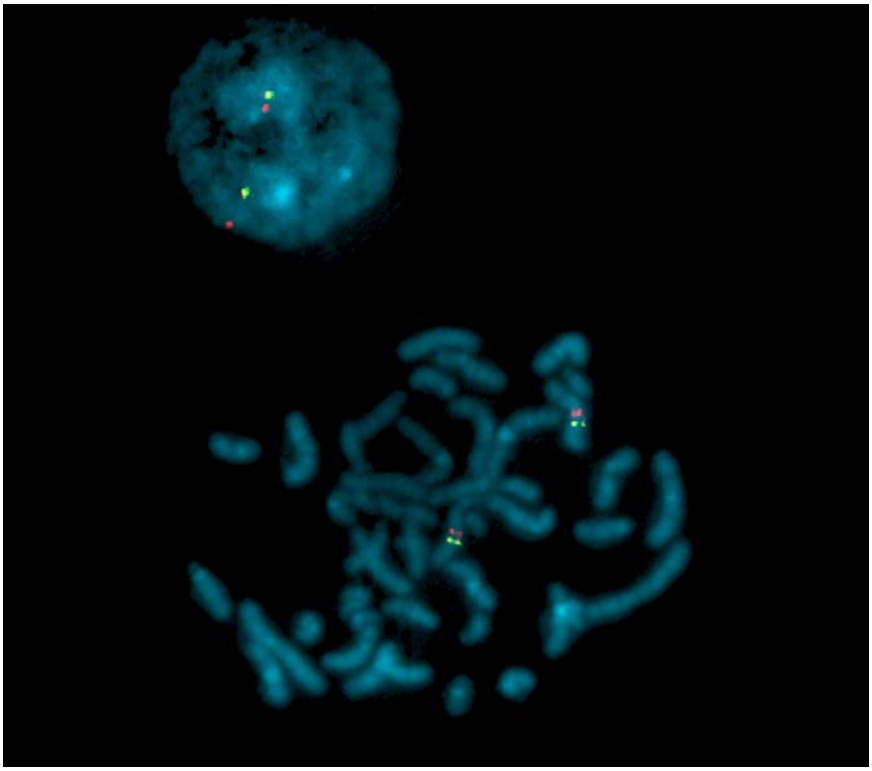


Figure XII. Results of fluorescence hybridization of nucleated blood cells after fixation. Hybridization of an interphase nucleus and a metaphase nucleus with Cy3-labeled probes from RP11-602G22 and Biotin-labeled probes from RP11-815N24

After successful and stable hybridization results in newly prepared samples of fixated cells, further optimization of the FISH technique was necessary due to the characteristics of the available tumor samples that were intended to be analyzed by FISH. These samples were mainly cytospin preparations or tumor touch imprints which were prepared at the time of diagnosis of the patients and then stored for several years at room temperature. For the necessary adaptation of the hybridization protocol according to the duration of storage, the following steps of optimization were undertaken:

1) Increase of pepsin concentration and duration of pepsin treatment to improve permeability of cell membrane
2) Modification of the temperature for the denaturation of the target DNA
3) Optimization of the amount of sample probe to allow specific hybridization signals with minimum background staining

These measures improved the hybridization results. However, several hybridization series proved, that the hybridization results were insufficient for microscopic slides that were stored for a longer time period. The quality of the results remained highly variable and critical, especially for samples after storage for two years or longer.

In conclusion, three specific BAC clones could be identified and establish for FISH analysis to detect deletions of the proximal part of chromosome 6q. This technique was applicable in samples with high or optimal sample quality. However, in the current study the available samples are of low quality due to long storage of the cytospin preparations or smears at room temperature for several years.
Therefore, the attempt to narrow down a minimal deleted region in pediatric T-LBL by FISH analysis turned out to be not feasible with the available sample series. In consequence, the implementation of a different methodological approach was considered. This new approach was supposed to allow analysis of the available samples with reduced amount and quality of DNA. For this purpose, the technique of loss of heterozygosity (LOH) analysis was implemented.

4.2 Characterization of the available samples for LOH analysis

LOH is an alternative method to detect deletions of chromosomal material, which allows analyzing specimens that do not fulfill the prerequisites in terms of quantity and quality necessary for other methods, e.g. FISH or cytogenetics. The samples which were available for LOH analysis are characterized in the following section.

T-LBL samples

From April 1995 to May 2004, 217 patients were diagnosed with T-LBL and registered in the NHL-BFM study center after informed consent. From this cohort, cases with available tumor and germline control DNA were chosen for the current analysis. One hundred eight of the 217 T-LBL patients had available samples for the preparation of tumor and germline DNA. The available samples in these 108 patients are summarized in Table 25. For details see appendix 8.3.

Table 25. Available tumor samples in the 108 evaluable T-LBL patients

T-LBL samples for preparation of tumor DNA	No. of pts
Cytospin(s) of malignant effusion	27
Tumor touch imprint(s)	21
Paraffin embedded tumor biopsy	20
Tumor touch imprints + paraffin embedded tumor biopsy	15
Cytospin of malignant effusion + frozen cells of malignant effusion	12

continue Table 25	
Frozen cells of malignant effusion	4
Tumor touch imprints + paraffin embedded tumor biopsy + frozen tumor cells	2
Frozen tumor cells	1
Tumor touch imprints + frozen tumor cells	1
Tumor touch imprints + cytospin of malignant effusion	1
Tumor touch imprints + frozen cells of malignant effusion	1
Cytospin of malignant effusion + tumor touch imprints + frozen cells of malignant effusion	1
Cytospin of malignant effusion + frozen cells of malignant effusion + paraffin embedded tumor biopsy	1
Cytospin of malignant effusion + paraffin embedded tumor biopsy	1

Table 26. Available samples for the preparation of control DNA in the 108 evaluable T-LBL patients

T-LBL samples for preparation of germline control DNA	**No. of pts**
Blood and/or bone marrow smears	90
Blood and/or bone marrow smears + frozen cells of blood and/or bone marrow	10
Frozen cells of blood and/or bone marrow	8

T-ALL samples

From August 1999 to July 2002, 186 pediatric T-ALL patients were consecutively enrolled in the multicenter trial ALL-BFM 2000 after informed consent. One hundred twenty-seven of the 186 T-ALL patients had available tumor DNA and germline DNA. Blast count of the initial blood or bone marrow smears was examined by central cytomorphological review in the ALL-BFM study center (Kiel, former Hannover). The initial blast count of the bone marrow or blood smears was >70% in 113 of the 127 analyzed samples. In the remaining 14 samples, the blast count in the smear preparations was 16% (additional examination of pleural effusion), 34%, 38%, 40%, 43%, 49%, 50%, 50%, 53%, 55%, 58%, and 3x 60%, respectively. However, the cytomorphologic blast count did not represent the blast count in the analyzed samples. Due to the preparation of mononuclear cells from the fluid bone marrow or blood samples, lymphoblasts were enriched in the analyzed sample probes and the blast count was certainly higher than the blast count in the smear preparations for cytomorphology. The resources for the analyzed samples are summarized in the following Tables 27 and 28. The control DNA was extracted from follow-up samples cytomorphologically proven to be free of remaining lymphoblast infiltration.

Table 27. Resource of tumor DNA in T-ALL patients	
T-ALL samples for preparation of tumor DNA	**No. of pts**
Bone marrow	106
Peripheral blood	18
Bone marrow smear	1
Pleural effusion	2

Table 28. Resource of control DNA in T-ALL patients	
T-ALL samples for preparation of germline control DNA	**No. of pts**
Follow-up samples of blood or bone marrow	127

4.3 Validation of data - quality and results control

The following aspects contribute to the validity and significance of the data obtained in the LOH analyses:

1. Regarding the number of results per patients, a median of 17 markers (9 informative markers) per patient was available in T-LBL cases. Three patients were included with three informative markers, 11 patients with four or five informative markers, 42 patients with six to nine informative markers, and 52 patients with 10 or more informative markers. In T-ALL cases a median of 25 markers (19 informative markers) was available per patient. All T-ALL cases had more than 10 informative markers. In both entities, cases with less than three informative results in LOH analyses were excluded prior to the start of the study due to insufficient DNA quality and/or quantity.

2. To rule out PCR errors, LOH findings were retested and confirmed in an independent second experiment for 69% of all LOH findings in T-LBL (at least one per LOH positive case), and for 98% of all LOH findings in T-ALL. Findings of isolated LOH of a single marker were retested and confirmed in a third experiment (cases 19, 63, 65, O, and P).

3. To test the impact of less-than-100% blast counts in tumor samples, exemplary dilution experiments were performed in two cases of LOH positive T-ALL with almost 100% blasts in initial cytomorphological differentiation of initial bone marrow smears. LOH analysis of undiluted DNA and 1:1 diluted DNA from initial BM with control germline DNA from the same patient revealed identical findings of LOH in undiluted and diluted initial BM DNA.

4. To test a possible impact of different types of samples used for the preparation of the tumor DNA in T-LBL cases, a systematic comparison of results was performed. In two representative patients with detectable LOH at chromosome 6q tumor DNA was extracted from tumor touch imprints and in parallel DNA was extracted from paraffin embedded tumor biopsies. A total of 39 informative markers were examined and the comparative analysis of the results revealed no difference for any of the markers between the two different sources of tumor DNA. Therefore it could be shown that different types of samples lead to reproducible results in the LOH analysis. In the following, the availability of different sources of tumor DNA in about one third of patients was used to increase the total number results of in these cases.

5. The following criteria for the quality of the results were used: minimal peak height 200 and optimal peak height 1000. With respect to the quality of the available samples in the current series, the criteria were more stringent than the criteria used by an European Consortium focused on quantitative analysis chimerism after allogeneic stem cell transplantation, which defined a minimal peak height of 50 and an optimal peak height of 5000 (Sandra Preuner personal communication[272]).

4.4 Deletions of chromosome 6q in T-LBL

4.4.1 Patients' characteristics in T-LBL

For the entire group of 217 T-LBL patients the probability of event-free survival (pEFS) at 5 years was 80±3%. pEFS was 77±4% for the 108 evaluable T-LBL patients. The median follow-up of was 4.4 (1.2-10.1) years for the entire group. In line with the NHL-BFM protocol, 5 of the 108 analyzed patients received intensified therapy with high-risk courses of the ALL-BFM protocol because of insufficient tumor reduction after 5 weeks of treatment.

Table 29 depicts the clinical characteristics of the 108 patients evaluable for LOH analysis compared with the 109 patients in whom no DNA specimens were available. The 108 patients evaluable for LOH analysis and the 109 non-evaluable patients were similar with respect to age, sex ratio, stage of disease, BM involvement, CNS involvement and lactate dehydrogenase (LDH) level. The group of patients not evaluable for LOH analysis was less frequently diagnosed with a mediastinal mass and the immunophenotype was more frequently not further classified. The evaluable group of 108 T-LBL patients included 21 of 27 patients in the total cohort who suffered from relapse.

Table 29. Patients' characteristics of 109 not evaluable and 108 evaluable patients with T-LBL

Characteristics		Patients not evaluable for LOH analysis (N=109)		Patients evaluable for LOH analysis (N=108)		*P* value (Fisher)
Gender	female	32	29%	36	33%	
	male	77	71%	72	67%	0.56
Age	< 10 y	54	50%	65	60%	
	10–14 y	38	35%	33	31%	
	> 14 y	17	16%	10	9%	0.20
Stage of disease						
	stage I	1	1%	0	0%	
	stage II	3	3%	2	2%	
	stage III	84	79%	82	76%	
	stage IV	19	18%	24	22%	0.61
BM involvement		18	17%	21	19%	0.60
Mediastinal tumor		93	85%	102	94%	0.04
CNS involvement		2	2%	6	6%	0.17
LDH	< 500 U/l	53	58%	45	50%	
	> 500 U/l	38	42%	45	50%	0.28
Immunophenotype						
	pro/pre-T-cell	13	12%	10	9%	
	intermediate T-cell	39	36%	66	61%	
	mature T-cell	12	11%	8	8%	
	T-cell nfc	45	41%	24	22%	0.01
Events	early death	2	2%	1	1%	
	toxic death	4	4%	1	1%	
	relapse/progress	6	6%	21	19%	
	2nd malignancy	6	6%	1	1%	
	LFU	7	7%	2	2%	
	CCR	84	77%	82	76%	
Outcome	pEFS (5y)	83±4%		77±4%		0.30 (LR)
	incidence of relapse	5.5±2.3%		20.2±4.1%		0.002 (Gray)

Data refer to patients with successful investigation of the respective criteria. LOH: Loss of heterozygosity, y: years, BM: bone marrow; CNS: central nervous system, LDH: lactate dehydrogenase, nfc: not further classified, LFU: Lost to follow-up, CCR: continuous complete remission, pEFS (5y): probability of event-free survival at 5 years; LR: log rank test

4.4.2 Frequency of LOH in T-LBL

Fragment-length analysis of germline and tumor DNA from the 108 patients was successful for a total of 1,671 marker analyses, with LOH in 104 markers, retention of heterozygous status in 991 markers, homozygous patterns in 426 markers, and microsatellite instability in 150 markers. LOH of one or more markers was detected in 21 of the 108 T-LBL patients (19%). The detailed results of all 108 patients evaluable for this analysis are illustrated in appendix 8.5.

4.4.3 Common deleted region in T-LBL

The informative results and the putative deleted regions for the 21 patients with detectable LOH are depicted in Figure XIII.

Four cases showed LOH of all informative markers (cases 1, 2, 3, and 4).

In 15 cases, interstitial deletions of chromosome 6q were detected.

Two cases showed two regions of deletion (cases 5 and 17).

In 13 of the 21 cases, the putative deleted region (grey infill) spanned marker D6S1284 and/or adjacent marker D6S1716 and/or adjacent marker D6S1717, which therefore represents the chromosomal region most frequently deleted in this analysis of pediatric T-LBL samples.

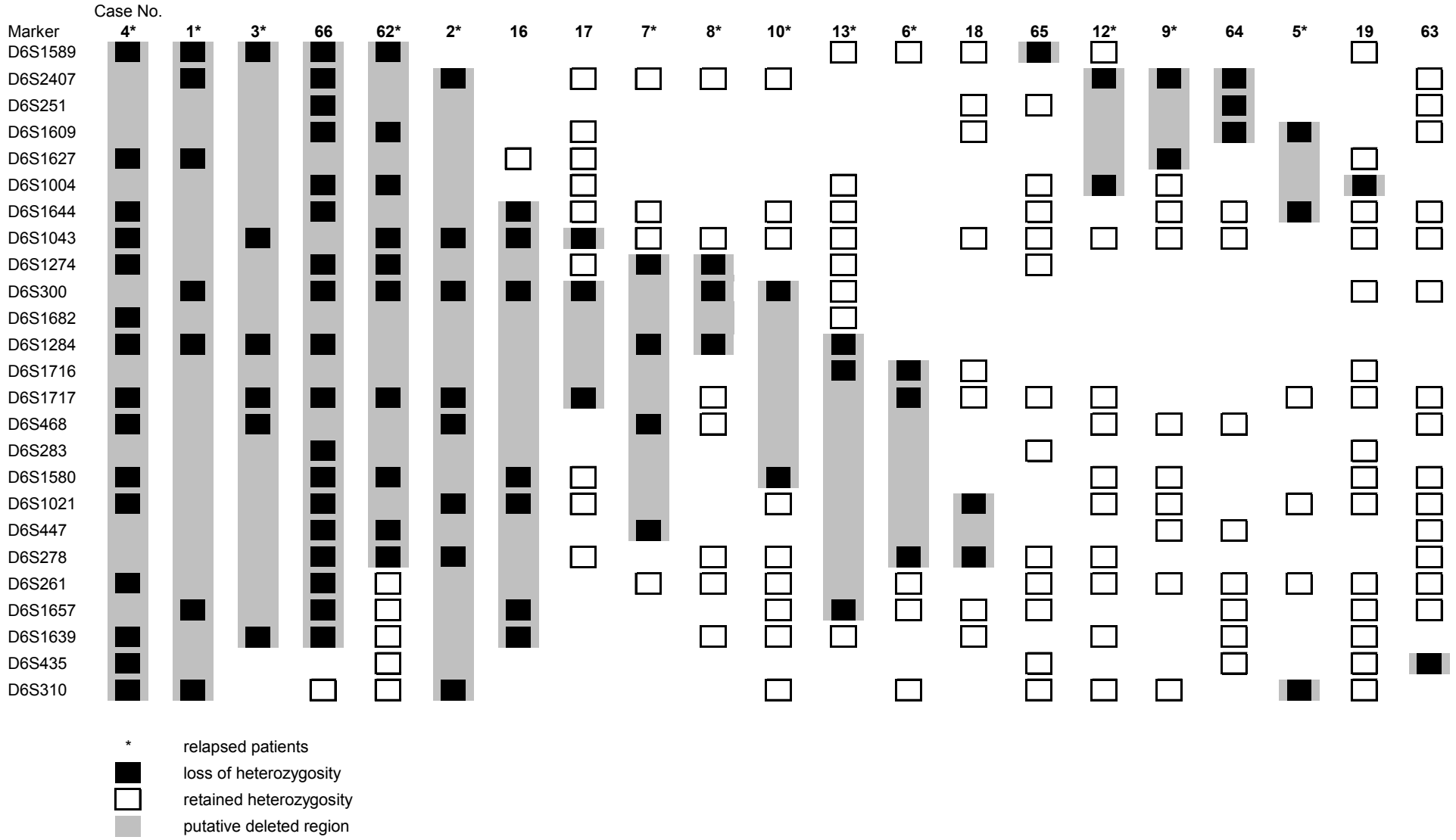


Figure XIII. Informative markers and putative deleted regions in 21 patients with T-LBL and detectable loss of heterozygosity at chromosome 6q

4.4.4 Correlation of LOH results with clinical characteristics in T-LBL

The clinical features of the 21 LOH positive T-LBL patients were not significantly different from those of the 87 T-LBL patients with no detectable LOH with respect to sex ratio, age, stage of disease, BM involvement, mediastinal involvement, CNS involvement and LDH level (Table 30).

Table 30. Clinical features of 87 patients without detectable LOH at chromosome 6q compared to 21 T-LBL patients with LOH

Characteristics		Patients without LOH (n=87)		Patients with LOH (n=21)		*P* value (Fisher)
Gender	female	29	33%	7	33%	
	male	58	67%	14	67%	1.00
Age	< 10 y	54	62%	11	52%	
	10–14 y	24	28%	9	43%	
	> 14 y	9	10%	1	5%	0.34
Stage of disease						
	stage II	1	1%	1	5%	
	stage III	67	77%	15	71%	
	stage IV	19	22%	5	24%	0.52
BM involvement		16	18%	5	24%	0.55
Mediastinal tumor		83	95%	19	91%	0.33
CNS involvement		6	7%	0	0%	0.59
LDH	< 500 U/l	37	53%	8	40%	
	> 500 U/l	33	47%	12	60%	0.45
Immunophenotype						
	pro/pre-T-cell	9	10%	1	5%	
	intermediate T-cell	57	66%	9	43%	
	mature T-cell	5	6%	3	14%	
	T-cell nfc	16	18%	8	38%	0.15

Data refer to patients with successful investigation of the respective criteria. LOH: Loss of heterozygosity, y: years, BM: bone marrow, CNS: central nervous system, nfc: not further classified

4.4.5 Prognostic impact of 6q-LOH in T-LBL

In the outcome analysis, detectable LOH at 6q was associated with a significantly higher relapse rate in LOH positive patients compared to LOH negative patients as depicted in Table 31.

Table 31. Outcome of 87 T-LBL patients without detectable LOH at chromosome 6q compared to 21 T-LBL patients with LOH

Characteristics		Patients without LOH (n=87)		Patients with LOH (n=21)		*P* value
Events	early death	1	1%	0	0%	
	toxic death	0	0%	1	5%	
	relapse/progress	8	9%	13	61%	
	2nd malignancy	1	1%	0	0%	
	LFU	2	2%	0	0%	
	CCR	75	86%	7	33%	
Outcome	pEFS (5y)	88±3%		33±10%		<0.0001 (LR)

LOH: Loss of heterozygosity, LFU: Lost to follow-up, CCR: continuous complete remission, pEFS (5y): probability of event-free survival at 5 years, LR: log rank test

In the group of patients with sufficient follow-up, LOH was detected in 13 of the 21 analyzed patients with relapse compared to 7 of the 85 patients without relapse (odds ratio 16.3, 95% confidence interval 5.2-50.1).The 5-year cumulative incidence of relapse was 9±3% for LOH negative T-LBL patients compared with a cumulative incidence of relapse of 63±12% for LOH positive T-LBL patients (Gray $P < 0.001$) as shown in Figure XIV.

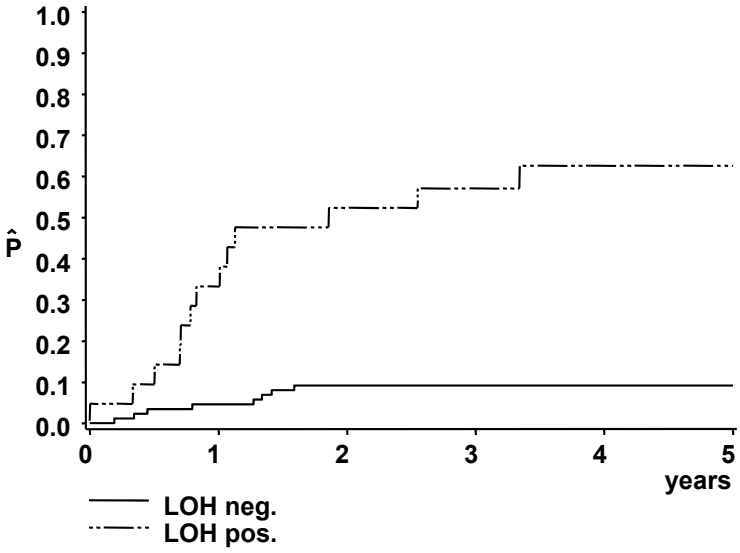
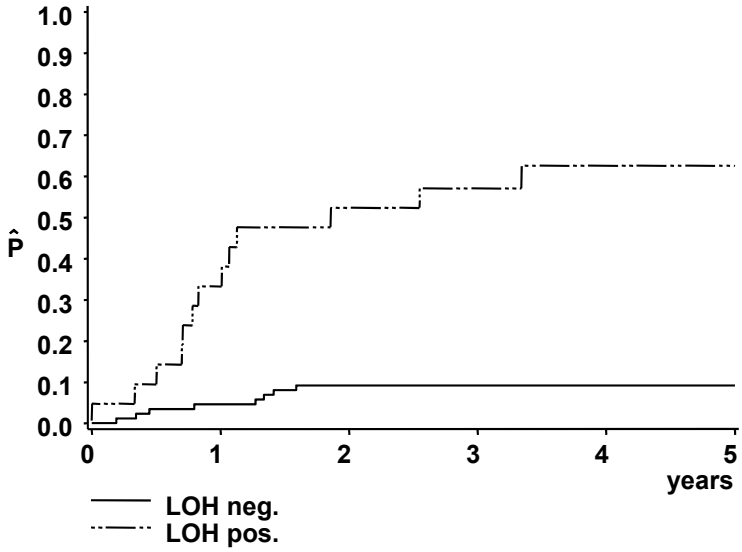


Figure XIV. Cumulative incidence of relapse in 21 patients with T-LBL and detectable LOH at chromosome 6q compared with LOH negative T-LBL cases

Because of the over-representation of relapsed patients in the analyzed group, this group was not representative of the total group of 209 T-LBL patients with sufficient follow-up. Using



Bayes' formula, this selection bias for relapsed patients was corrected to allow estimation of the probability of relapse in patients with and without LOH and the resulting risk ratio. Given the relapse rate of 27/209 for the total cohort of T-LBL patients, the corrected probability of relapse in patients without detectable LOH was 6%, while the probability of relapse in patients with detectable LOH was 50%. The corrected risk ratio for relapse in patients with detectable LOH was 8.6.

4.4.6 Correlation of LOH results with available cytogenetic data

Cytogenetic data from diagnostic tumor samples were available in 16 of the 108 evaluable T-LBL patients (Table 32). In one case (case 3) cytogenetic data were available from the time of initial diagnosis and from the time of relapse.

In 13 cases, neither cytogenetics nor LOH analysis revealed structural abnormalities in chromosome 6q (cases 1041, 1015, 1029, 1026, 26, 45, 34, 55, 43, 54, 1055, 11, and 15).

In case 2, a cytogenetically described deletion del(6)(q1?2q1?6) was confirmed by LOH analysis; however, LOH was also detected for markers D6S278 and D6S310, which were allocated to chromosomal bands 6q21 and 6q24.

For two cases (cases 6 and 3), normal karyotypes were reported in diagnostic tumor samples. LOH analysis, however, detected deletions of chromosome 6q in both cases: in case 6, there was an interstitial deletion of markers assigned to bands 6q16-q21, with LOH at markers D6S1716, D6S1717 and D6S278. The distance between markers D6S1716 and D6S278 is about 10 Mb. An interstitial deletion of that small size might be undetectable with standard cytogenetic methods.

In case 3, LOH analyses of the initial tumor sample identified LOH for all informative markers, while the cytogenetic report stated normal karyotype. Interestingly, at the time of relapse cytogenetic analysis revealed a deletion of chromosome 6q. In this case, the metaphases analyzed at initial karyotyping might not have been representative for the lymphoma cells.

Table 32. Correlation of available cytogenetic data and LOH data in 16 of the 108 patients evaluable for LOH analysis

case	LOH at 6q	karyotype
case 1041	-	47,XY,+mar
case 1015	-	46,XY
case 1029	-	46,XY
case 1026	-	46,XY
case 26	-	46,XY
case 45	-	46,XY
case 34	-	46,XX
case 55	-	45,XY,t(1;14)(p33;q11),dic(9;13)(p1?3;p13),t(10;12)(q21;p12)
case 43	-	46,XX,t(7;10)(q35;q24)
case 54	-	47,XX,t(9;17)(q34;q22),+20
case 1055	-	46,XY,del(21)(q22)
case 11	-	46,XX,t(6;17)(q24;q12),del(11)(q23)
case 15	-	47,XY,+8,i(9)(q10),t(10;11)(p13;q14)
case 6	+ (3 markers)	46,XX
case 2	+ (8 markers)	46,XX,**del(6)(q1?2q1?6)**,t(9;17)(q34;q22)
case 3	+ (6 markers in diagnostic sample)	at diagnosis: 46,XY subsequent analysis at time of relapse: 45~47,XY,**del(6)(q1?4q2?1)**,t(9;14)(q34;q11),+?20,+mar

4.5 Deletion of chromosome 6q in T-ALL

One hundred twenty-seven of the 186 T-ALL patients had available tumor DNA and germline DNA. Except for the distribution of the immunophenotypes, this subgroup of patients did not differ from the non-evaluable group in terms of basic clinical characteristics such as age, sex ratio, mediastinal involvement, CNS involvement, LDH level, and white blood cell count at diagnosis (WBC) (Table 33).

The evaluable group of 127 T-ALL patients included 22 of the 27 patients in the total cohort who suffered from relapse.

Table 33. Patients' characteristics of 59 not evaluable and 127 evaluable patients with T-ALL

Characteristics		Patients not evaluable for LOH analysis (N=59)		Patients evaluable for LOH analysis (N=127)		*P* value (Fisher)
Gender	female	17	29%	31	24%	
	male	42	71%	96	76%	0.59
Age	< 10 y	35	59%	68	54%	
	10–14 y	18	31%	36	28%	
	> 14 y	6	10%	23	18%	0.38
Mediastinal tumor		34	59%	79	64%	0.57
CNS involvement		8	16%	9	8%	0.17
LDH	< 500 U/l	13	26%	21	18%	
	> 500 U/l	37	74%	94	82%	0.30
Phenotype	pro/pre-T-cell	21	36%	25	20%	
	intermediate T-cell	23	39%	81	64%	
	mature T-cell	9	15%	19	15%	
	T-cell, nfc	6	10%	2	2%	0.001
WBC	<10 000	16	28%	14	11%	
	10 000 <20 000	5	9%	14	11%	
	20 000<100 000	16	28%	53	42%	
	100 000<200 000	11	19%	23	18%	
	>=200 000	10	17%	23	18%	0.06
Prednisone response	PGR	41	72%	78	62%	
	PPR	16	28%	47	38%	0.24
Events	early death	0	0%	0	0%	
	toxic death	2	3%	7	6%	
	relapse/progress	5	9%	22	17%	
	2nd malignancy	0	0%	0	0%	
	LFU	1	2%	0	0%	
	CCR	51	86%	98	77%	
Outcome	pEFS (5y)	88±4%		77±4%		0.09 (LR)
	incidence of relapse	8.7±3.8%		17.4±3.5%		0.12 (Gray)

Data refer to patients with successful investigation of the respective criteria. LOH: Loss of heterozygosity, y: years, CNS: central nervous system, LDH: lactate dehydrogenase, WBC: white blood cell count per microliter, PGR: prednisone good response (day 8), PPR: prednisone poor response (day 8), LFU: Lost to follow-up, CCR: continuous complete remission, pEFS (5y): probability of event-free survival at 5 years; LR: log rank test

4.5.1 Frequency of LOH in T-ALL

Fragment-length analysis of germline DNA and corresponding tumor DNA was successful for a total of 3,109 marker analyses, with LOH of 168 markers, retention of heterozygous status in 2,132 markers, homozygous patterns in 786 markers, and microsatellite instability in 23 markers. LOH of one or more markers was detected in 16 patients (13%). The detailed results of all 127 T-ALL patients evaluable for this analysis are illustrated in appendix 8.6.

4.5.2 Common deleted region in T-ALL

The informative results and putative deleted regions for the 16 patients with detectable LOH are depicted in Figure XV.

None of the cases showed LOH of all informative markers at chromosome 6q.

In 15 cases interstitial deletions were detected.

One case showed two regions of deletion (case J).

In 15 of the 16 cases, the putative deleted region spanned marker D6S1004. Thus, the chromosomal region between adjacent markers D6S1627 and D6S1644 represents the common deleted region in the T-ALL samples of this analysis.

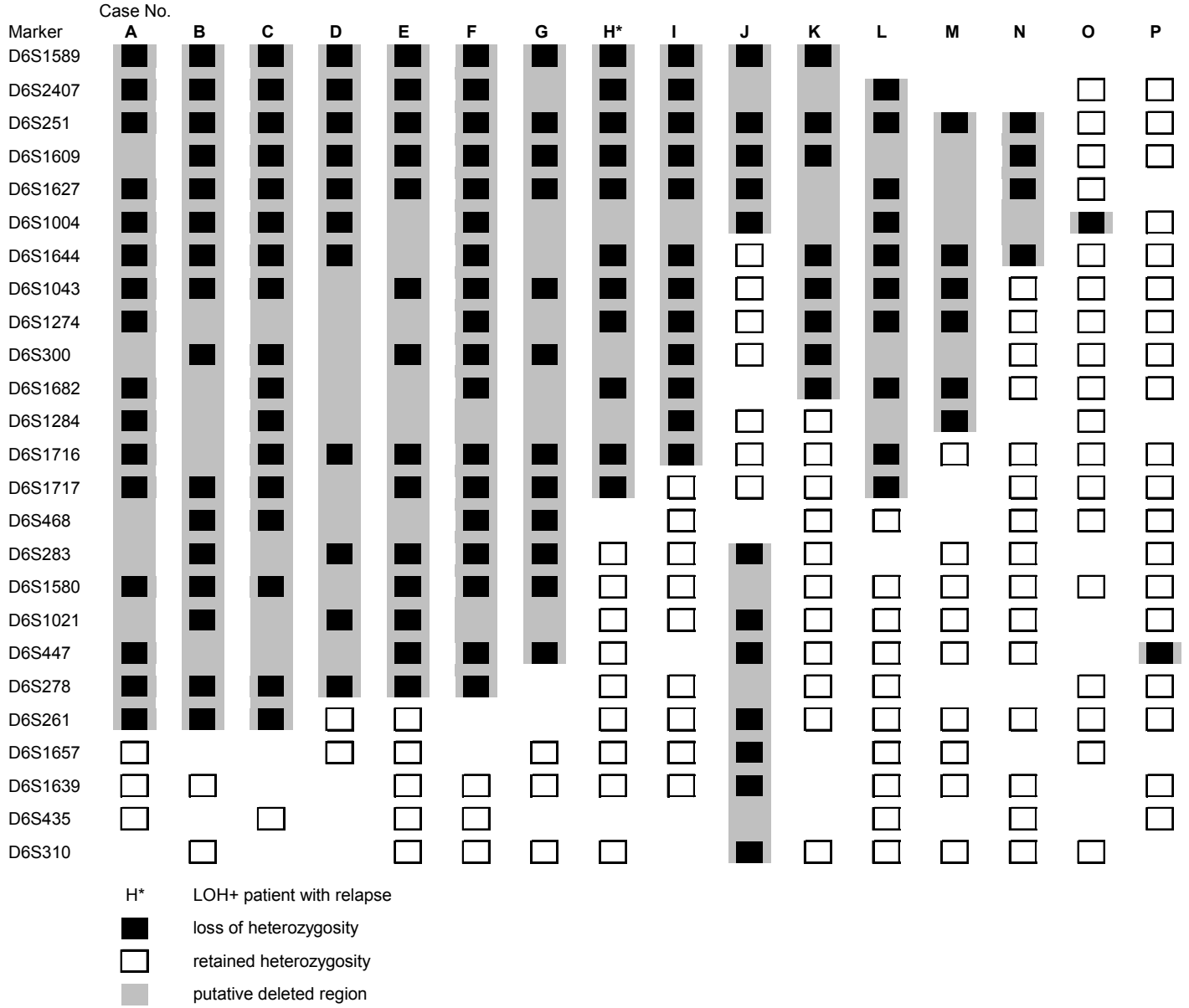


Figure XV. Informative markers and putative deleted regions in 16 patients with T-ALL and detectable loss of heterozygosity at chromosome 6q

4.5.3 Additional markers to delineate the centromeric breakpoint

In the marker set used, marker D6S1589 at chromosomal band 6q14 was the most centromeric marker analyzed. Interestingly, this marker showed findings of LOH in 11 cases of T-ALL (cases A-K) and in additionally three cases the most centromeric marker with informative results showed LOH (cases L, M, and N). This indicates that in these cases the proximal break point of the interstitial deletion was located proximal to the analyzed region. To narrow down the proximal break point in theses cases, additional microsatellite markers were introduced. The localization of the additional markers D6S1638, D6S294, D6S1557 and D6S1596 are depicted in Figure XVI.

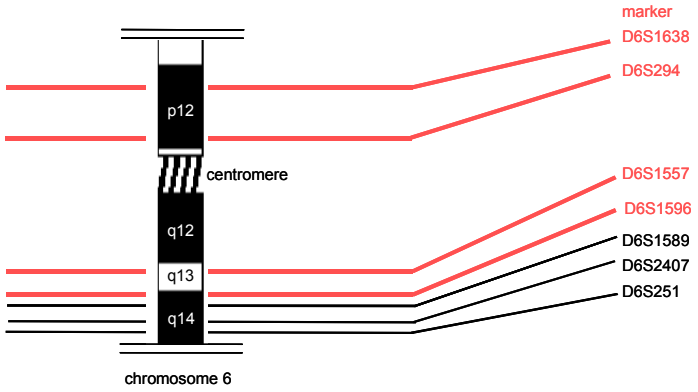


Figure XVI. Localization of the additional markers at chromosome 6 (red color)

The results of the LOH analysis of the additional markers are depicted in Figure XVII. In two cases the analysis of the additional markers did not reveal additional information as the markers were not informative (cases M and N). In the remaining 12 cases, six cases showed LOH of the centromeric markers at chromosome 6q but retained heterozygosity at chromosome 6p (cases E, L, I, K, G, and D). Six cases showed retained heterozygosity of the centromeric markers at chromosome 6q (cases F, B, A, C, H, and J).

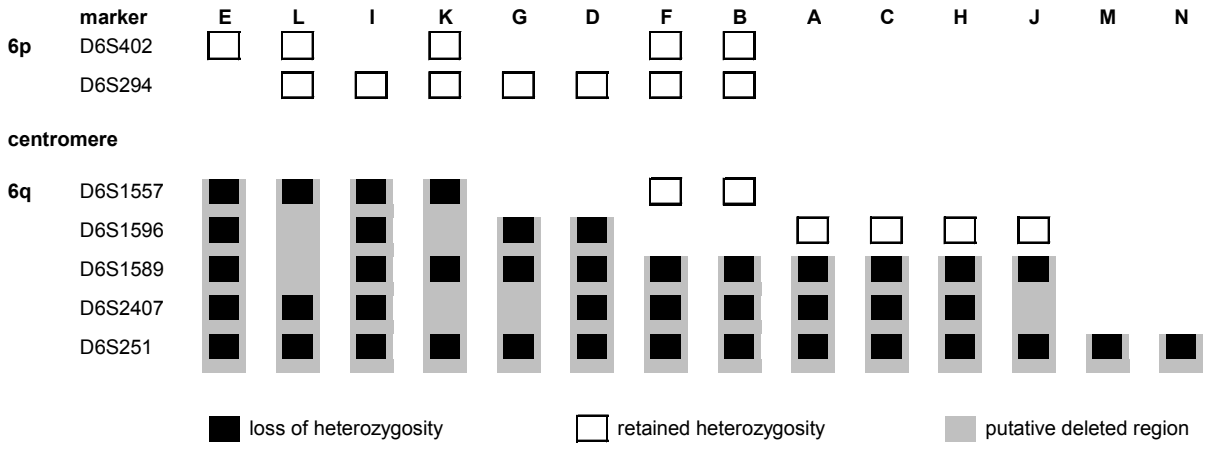


Figure XVII. Informative results of additional markers in T-ALL cases with proximal LOH

These findings indicate, that in 14 of the 16 T-ALL patients with detectable LOH at chromosome 6q the proximal break point of the interstitial deletion was localized close to the centromere. However, in none of the informative cases the putative deleted region spanned chromosome 6p. In four cases the localization of the break point could be narrowed down to be localized between the adjacent marker D6S1596 and D6S1557 (cases A, C, H, J). In two cases the localization of the break point was between the 6q markers D6S1589 and D6S1557. In four cases the breakpoint was proximal to the most centromeric 6q marker D6S1557 (cases E, L, I, K). And in two cases the available informative results indicate the break point between marker D6S1596 at 6q and marker D6S294 at chromosome 6p.

Therefore, in the majority of the cases the proximal break point was localized within the chromosomal region flanked by markers D6S402 and D6S1589. However, the present LOH analysis did not allow identifying a common break point localization in pediatric T-ALL.

4.5.4 Correlation of LOH results with clinical characteristics in T-ALL

The clinical features of the 16 LOH positive T-ALL patients were not significantly different from those of the 111 LOH negative patients with respect to sex ratio, white blood cell count, mediastinal involvement, CNS involvement, and immunophenotype. There was a trend to younger age and differences in the distribution of immunophenotypes in LOH positive patients compared with LOH negative patients (Table 34).

Table 34. Clinical features of 111 T-ALL patients without detectable LOH at 6q compared to 16 T-ALL patients with LOH

Characteristics		Patients without LOH (n=111)		Patients with LOH (n=16)		*P* value (Fisher)
Gender	female	26	23%	5	31%	
	male	85	77%	11	69%	0.54
Age	< 10 y	55	50%	13	81%	
	10–14 y	34	31%	2	13%	
	> 14 y	22	20%	1	6%	0.06
Mediastinal tumor		69	65%	10	63%	0.91
CNS involvement		6	6%	3	20%	0.10
LDH	< 500 U/l	21	21%	0	0%	
	> 500 U/l	79	79%	15	100%	0.07
Phenotype	pro/pre-T-cell	25	23%	0	0%	
	intermediate T-cell	70	63%	11	69%	
	mature T-cell	15	14%	4	25%	
	T-cell nfc	1	1%	1	6%	0.06
WBC	<10 000	13	12%	1	6%	
	10 000<20 000	13	12%	1	6%	
	20 000<100 000	44	40%	9	56%	
	100 000<200 000	22	20%	1	6%	
	>=200 000	19	17%	4	25%	0.47

Data refer to patients with successful investigation of the respective criteria. LOH: Loss of heterozygosity, y: years, CNS: central nervous system, LDH: Lactate dehydrogenase, nfc: not further classified, WBC: white blood cell count per microliter

4.5.5 Correlation of LOH results and early treatment response

Early treatment response (evaluated by prednisone response at day 8 in PB) and the stratification of patients into risk arms showed no differences between LOH positive T-ALL patients and LOH negative T-ALL patients. In the LOH negative group 48 of the 111 patients were stratified to receive intensified high risk treatment compared with 5 patients in the group of 16 LOH positive patients. The allocation of the patients in prednisone good responder and prednisone poor responder according to LOH status is depicted in Table 35.

Table 35. Early treatment response of 111 T-ALL patients without detectable LOH at chromosome 6q compared to 16 T-ALL patients with LOH

Characteristics	Patients without LOH (n=111)		Patients with LOH (n=16)		*P* value (Fisher)
Prednisone good response	67	60%	11	69%	
Prednisone poor response	42	38%	5	31%	
Prednisone response not evaluated	2	2%	-	-	0.78

LOH: Loss of heterozygosity

4.5.6 Prognostic impact of 6q-LOH results in T-ALL

In the outcome analysis of T-ALL patients, there was no significant difference between 16 LOH positive and the 111 LOH negative T-ALL patients. (Table 36).

Table 36. Outcome of 111 T-ALL patients without detectable LOH at chromosome 6q compared to 16 T-ALL patients with LOH

Characteristics		Patients without LOH (n=111)		Patients with LOH (n=16)		*P* value (Fisher)
Events	toxic death	7	6%	0	0%	
	relapse/progress	21	19%	1	6%	
	CCR	83	75%	15	94%	
Outcome	pEFS (5y)	75±4%		94±6%		0.10 (LR)

LOH: Loss of heterozygosity, CCR: continuous complete remission, pEFS (5y): probability of event-free survival at 5 years, LR: log rank test

Comparing patients with sufficient follow-up, LOH was detected in 1 of the 22 analyzed patients with relapse compared to 15 of the 98 patients without relapse (odds ratio 0.29, 95% confidence interval 0.04-2.3) The cumulative incidence of relapse at 5 years was 6±6% for LOH positive T-ALL patients and 19±4% for LOH negative T-ALL patients. The difference was statistically not significant different *(Gray* P = 0.21) (Figure XVIII).

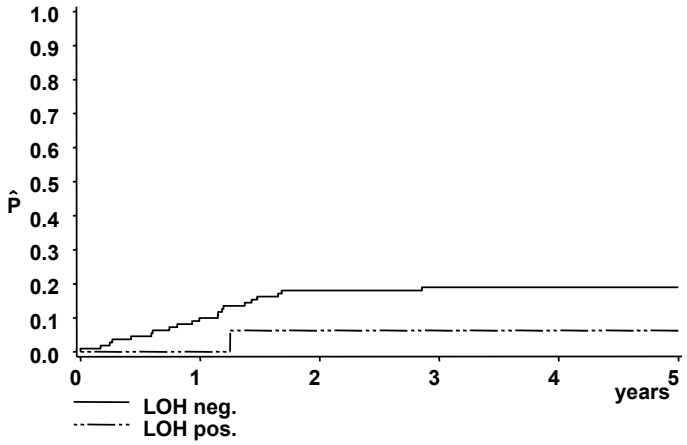


Figure XVIII. Cumulative incidence of relapse in 16 patients with T-ALL and detectable LOH at chromosome 6q compared with LOH negative T-ALL cases

With a relapse rate of 27 relapses in 177 patients for the total cohort of T-ALL patients and after correction for over-representation of relapse patients in the analyzed group using Bayes' formula, the corrected probability of relapse in patients with no detectable LOH was 17%, while the probability of relapse in patients with detectable LOH was 6%. The corrected risk ratio for relapse in patients with LOH was 0.3.

4.6 Delineation of a critical region of deletion

To determine the prognostic impact of LOH at the different markers, the LOH rate and the deletion rate were analyzed for each marker. The **LOH rate** was defined as the number of findings of LOH in all informative results per marker. The **deletion rate** of a marker was defined as the percentage of cases with putative deleted regions spanning the particular marker. The putative deleted region in the particular patient was defined by the most centromeric and telomeric markers with LOH. The estimates for the LOH rate and the deletion rate are illustrated exemplarily for 7 markers in the T-ALL series in Figure XIX.

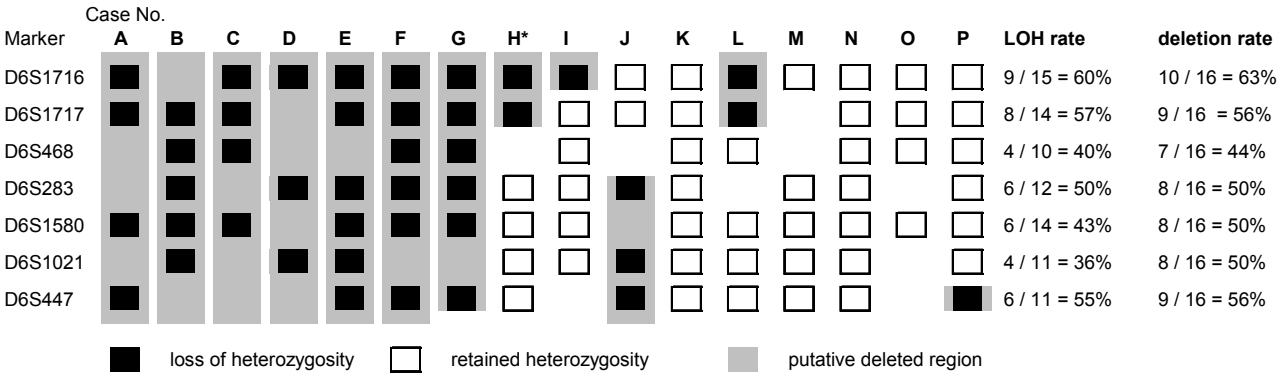


Figure XIX. Definition of the putative deleted region comparing the LOH rate and the deletion rate

The definition of the putative deleted region was based on the assumption that in the majority of cases with 6q aberration, there is only one detectable deletion. In contrast to the LOH rate, the putative deleted region covers chromosomal regions with no informative markers. The putative deleted region has the advantage of not being influenced by the rate of heterozygosity or the feasibility of LOH analysis of the markers. Therefore the deletion rate might be advantageous for the analysis of markers with only a limited number of informative results.

For the determination of the prognostic impact of LOH at the different markers, both, the LOH rate and the deletion rate were analyzed. The estimated rates were compared for

1) T-ALL versus T-LBL

2) T-LBL patients with and without relapse

3) T-ALL patients with and without relapse.

For the comparison of T-ALL and T-LBL, the deletion rates are illustrated in Figure XX. Due to the higher number of markers without results in T-LBL compared with T-ALL, the illustrative comparison of the deletion rates must be analyzed with certain caution. Therefore the comparison of the LOH rates are depicted in Table 37.

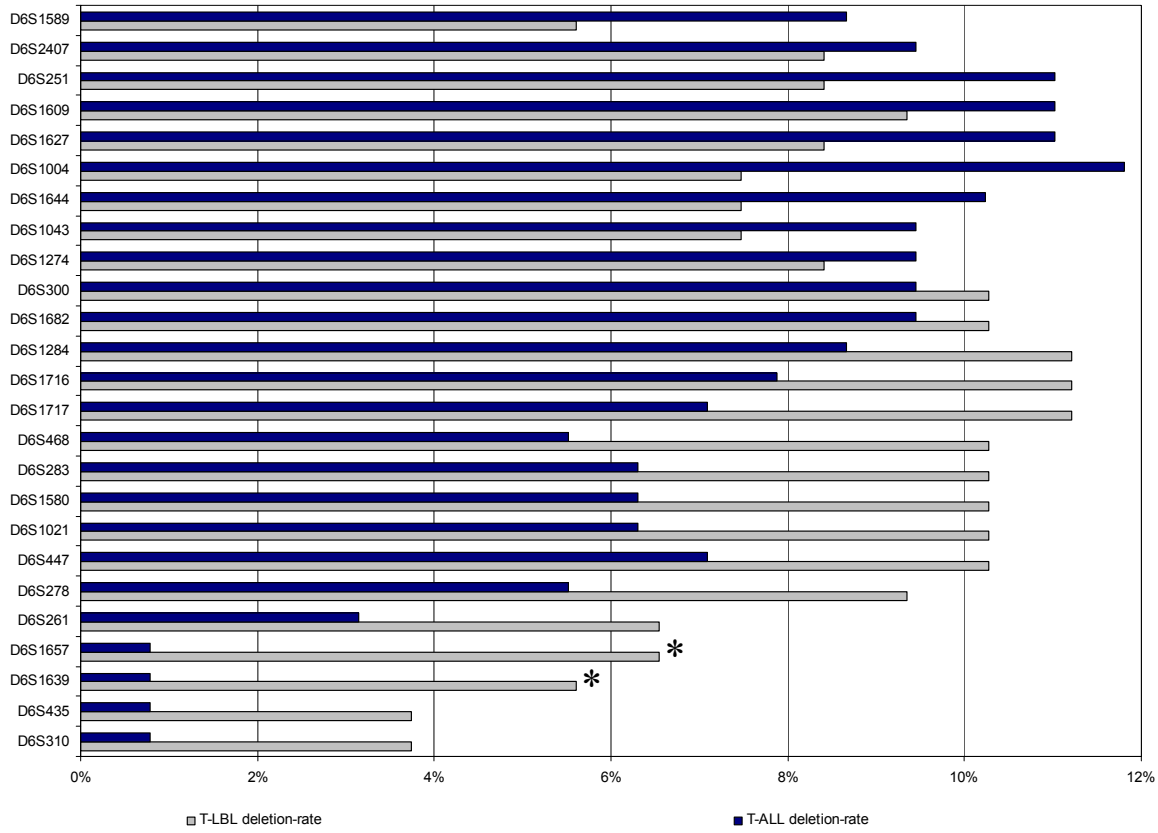


Figure XX. Deletion rates of particular markers in chromosome 6q of T-ALL (black bars) and T-LBL patients (light grey bars). For the markers telomeric to marker D6S1682, the deletion rate differed between T-LBL and T-ALL patients with statistically significant differences for markers D6S1657 and D6S1639. *: statistically significant differences

Table 37. LOH rates per marker in patients with T-ALL and T-LBL

	T-ALL patients (n=127)				T-LBL patients (n=108)			
Marker	No. of results	No. of informative results	No. of LOH	LOH rate (%)	No. of results	No. of informative results	No. of LOH	LOH rate (%)
D6S1589	123	96	11	11%	84	53	6	11%
D6S2407	123	81	9	11%	81	51	6	12%
D6S251	126	101	14	14%	39	31	2	6%
D6S1609	123	84	11	13%	39	27	4	15%
D6S1627	116	96	12	13%	76	59	3	5%
D6S1004	126	84	8	10%	74	45	4	9%
D6S1644	125	110	11	10%	65	53	4	8%
D6S1043	125	104	11	11%	94	74	6	8%
D6S1274	127	86	7	8%	54	32	5	16%
D6S300	121	101	7	7%	54	39	8	21%
D6S1682	126	78	8	10%	48	19	1	5%
D6S1284	126	48	4	8%	57	38	7	18%
D6S1716	127	96	9	9%	64	41	2	5%
D6S1717	124	98	8	8%	81	50	7	14%
D6S468	127	88	4	5%	92	50	4	8%
D6S283	119	98	6	6%	34	25	1	4%
D6S1580	126	97	6	6%	92	55	5	9%
D6S1021	123	93	4	4%	69	48	5	10%
D6S447	126	88	6	7%	84	48	3	6%
D6S278	123	85	6	7%	48	32	5	16%
D6S261	127	100	4	4%	78	53	2	4%
D6S1657	126	104	1	1%	77	49	4	8%
D6S1639	125	111	0	0%	59	45	4	9%
D6S435	122	75	0	0%	48	19	2	11%
D6S310	127	98	1	1%	80	59	4	7%

The deletion rates according to outcome of the patients were analyzed separately for T-ALL and T-LBL patients and illustrated in Figures XXI and XXII. For this analysis only patients with relapse or patients with follow-up of at least two years were included. Of the 108 T-LBL patients, two patients could not be included in this analysis because of toxic death (case 58) or death after initial complications (case 19; LOH positive for marker D6S1004). Of the 127 T-ALL patients, seven cases (all LOH negative) were excluded due to toxic death.

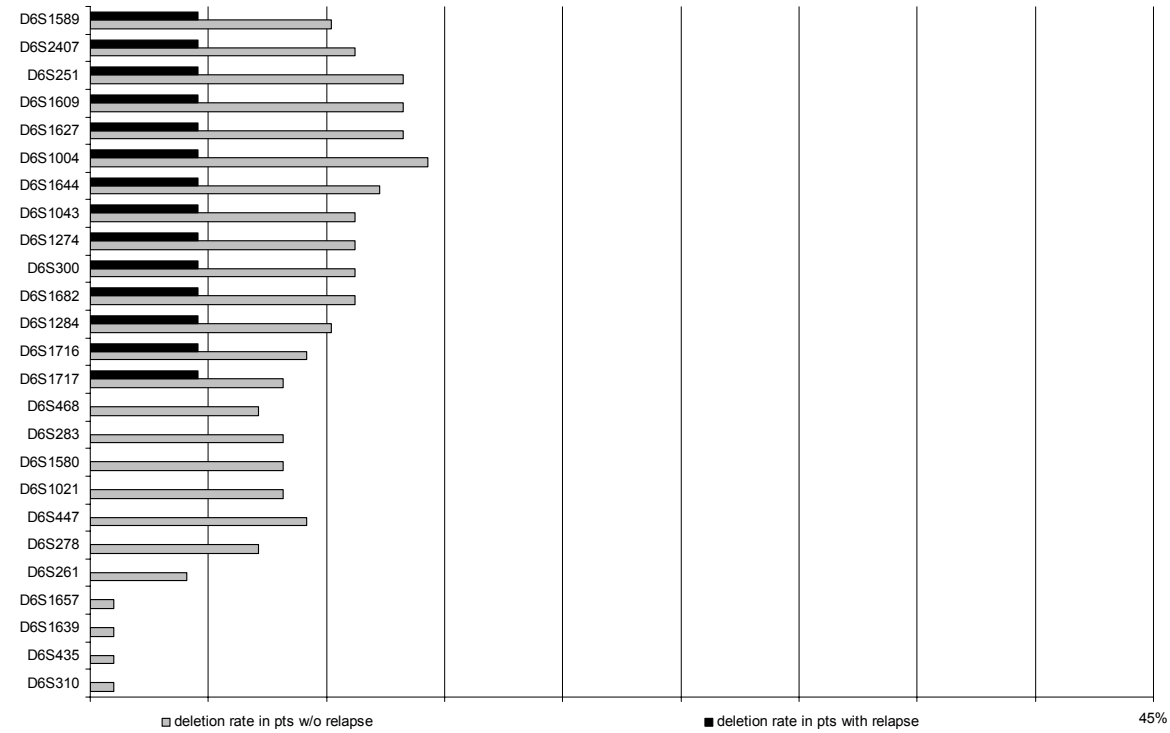


Figure XXI. Deletion rates of particular markers in relapsed T-ALL patients (black bars) and in T-ALL patients without relapse (light grey bars). All *P* values not signficant

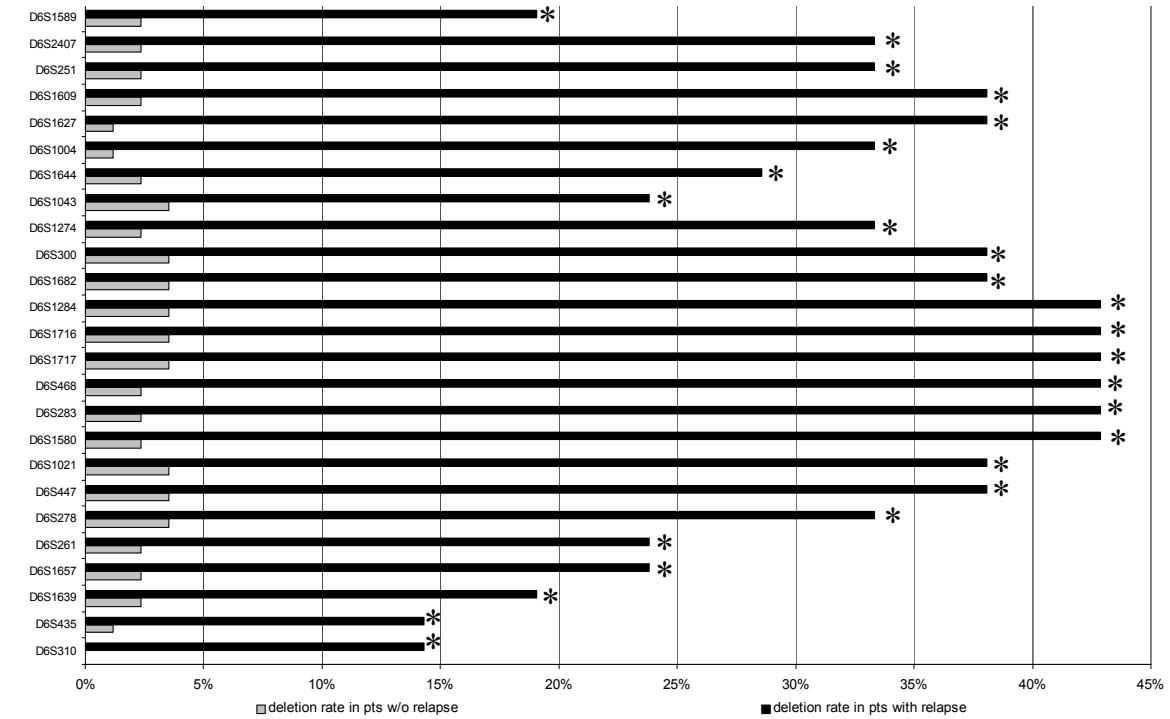


Figure XXII. Deletion rates of particular markers in relapsed T-LBL patients (black bars) and in T-LBL patients without relapse (light grey bars). *: statistically significant differences of the deletion rates in patients with and without relapse (*P* value ≤ 0.05)

In T-ALL, statistical analysis using Fisher's exact test comparing the deletion rates according to outcome of the patients did not reveal significant differences for any of the markers. In contrast, in T-LBL patients, the deletion rates were significantly different for all markers with higher rates in relapsed patients compared with clearly lower deletion rates in patients without relapse.

Combining the findings described above, led to the identification of markers, the deletion of which was associated with high risk of relapse in T-LBL patients. The adjacent markers D6S1284, D6S1716, D6S1717, D6S468, D6S283 and D6S1580 showed a deletion rate of 43% in the 21 relapsed T-LBL patients. In contrast, the deletion rate for each of these markers was less than 5% for T-LBL patients without relapse and less than 15% for T-ALL cases with or without relapse.

Therefore, the centromeric marker D6S1682 and the telomeric marker D6S1021 flank a chromosomal region, that was frequently deleted in relapsed T-LBL patients, but not in T-LBL patients without relapse or in T-ALL patients. Thus, the results of the current study might be a first step for the identification of a chromosomal region which might be a "critical region" for determining the risk of relapse in T-LBL patients.

5 Discussion

Knowledge about genetic alterations in T-cell lymphoblastic lymphoma (T-LBL) is very limited. The main reason for this is the scarcity of adequate tumor material for cytogenetic or molecular genetic analysis. Almost all T-LBL patients are diagnosed with a large mediastinal mass and a significant number of patients suffer from severe respiratory impairment at the time of their admission to the hospital. In these critically ill patients invasive procedures to obtain diagnostic samples are limited to the absolute minimum, in order to not aggravate the clinical condition of the patients. Under these circumstances, the available samples are usually sufficient for obligatory examinations to assure the diagnosis but usually do not allow additional research experiments. In addition, the remaining samples are often processed and stored in a way that limits possible techniques for molecular biological and genetic analyses.

This is in certain contrast to pediatric acute T-cell lymphoblastic leukemia (T-ALL), where initial bone marrow and/or blood samples provide large amounts of leukemic cells. Although these samples are primarily used for diagnostic procedures, in almost all cases parts of the samples remain unused and can be stored. These samples have been collected and used for extensive research during the last decades, and the findings of this research allow certain insights into the genetic alterations and deregulated pathways in leukemic cells.

Both T-LBL and T-ALL derive from malignant thymocytes corresponding to defined stages of intrathymic T-cell maturation. T-LBL and T-ALL are often considered to be different manifestations of one and the same disease.[4] Several study groups, including the BFM group, distinguish between leukemia and lymphoma based on the primary site of involvement. Patients with less than 25% lymphoblasts in the bone marrow and no lymphoblasts in the peripheral blood are diagnosed with lymphoblastic lymphoma (T-LBL), while patients with 25% or more blasts in the bone marrow are diagnosed with lymphoblastic leukemia (T-ALL).

Both diseases share common characteristics such as immunophenotypic features and lymphoblast morphology, as well as clinical characteristics such as median age at diagnosis and favorable outcome after ALL-type chemotherapy (our own data and [4;18;20;84]).

However, there are also differences between the two diseases. In lymphoma patients an anterior mediastinal mass represents the primary site of disease in the vast majority of cases, while in leukemia patients bone marrow involvement is the predominant site of manifestation, although mediastinal involvement is commonly seen in T-ALL. Interestingly, the typical sites of relapse differ, with predominantly local relapse in lymphoma patients[36;80] and systemic relapse in T-ALL.[153] In addition, involvement of the central nervous system (CNS) at the time of diagnosis is more frequent in T-ALL compared with T-LBL.[18;84] These differences in the site of disease manifestation might indicate differences in the homing of lymphoblasts between lymphoma cells and leukemia cells.

This raises the question of whether T-LBL and T-ALL are really one and the same disease with two different manifestations, or whether the cells of the two diseases carry different biologic potential. The resolution of this question is hampered by the scarcity of available T-LBL specimens for detailed genetic and immunophenotypic characterization. It is stated in the literature and in current textbooks that the typical chromosomal aberrations reported in T-ALL can also be found in T-LBL.[20;192] However, larger series of cytogenetic and molecular genetic data for T-LBL are lacking. Frequently the knowledge obtained in T-ALL is transferred onto T-LBL without any experimental prove.

Three recent reports addressed the comparison of genetic features in T-LBL and T-ALL. Raetz and colleagues compared gene expression profiles of nine T-LBL and ten T-ALL samples. They reported significant and clear distinctions in the expression profiles of T-LBL and T-ALL using unsupervised and supervised methods of data analysis. Though the number of samples was limited.[198]

In a retrospective series of 44 T-LBL cases (12 patients < 16 years), Baleydier and colleagues performed T-cell receptor rearrangement genotyping by Southern blot, paraffin tissue microarray immunophenotyping, and PCR and RQ-PCR quantification of pTa, RAG1, HOXA5/A9, HOX11, HOX11L2, LMO1/2, LYL1, TAL-1, SIL-TAL, CALM-AF10 and NUP214-ABL (fusion-) transcripts. The authors concluded that pediatric T-LBL is a disease of T-lymphoblasts arrested during beta-selection of T-cell maturation but with different oncogenic profiles compared to those of T-ALL arrested at the same stage of maturation.[199]

The same group compared the incidence of NOTCH1 mutations. NOTCH1 plays a fundamental role in physiological T-cell maturation and has been shown to be mutated in the heterodimerisation (HD) and/or PEST domains in 50% of pediatric T-ALLs. Comparing T-ALL and T-LBL the findings differed insofar as HD and PEST mutations were seen in 6/22 T-ALL and 6/18 T-LBL with mutations and HD-only mutations in 16/22 and 7/18 respectively, whereas PEST-only mutations were not seen in T-ALL compared to 5/18 T-LBL cases.[200]

These three recent reports encourage the hypothesis that T-ALL and T-LBL might be closely related albeit with differences in the biologic potential of the cells. These biological distinctions might be reflected in the differences of the clinical manifestations of the diseases.

The present study is specifically focused on one genetic alteration: deletions in chromosome 6q. The rationale for this focus is two-fold:

First, a very small series of T-LBL patients with available cytogenetic data indicated that 6q deletions might be associated with poor outcome. All four patients with 6q deletion suffered a relapse of their disease. Since the prognosis for patients with T-LBL who failed frontline

treatment is still very poor, parameters that allow early adaptation of frontline treatment to different relapse risks are urgently needed. For this reason further investigation of the impact of 6q deletions seemed worthwhile, even though the number of index patients was very small and there were no published data on 6q deletion in pediatric T-LBL.

The second rationale for the study was derived from analysis of the available published data on 6q deletions in T-ALL, which is considered to be a closely related disease. The reports in the literature on 6q deletions in pediatric T-ALL patients are inconsistent. So far, neither the role in pathogenesis nor the impact on treatment outcome of patients has been clarified. Because of inconsistent findings the clinical impact of chromosome 6q deletions remains unclear and it was supposed that these deletions might have different effects in different biological entities. In addition, the prognostic value might depend on the respective treatment applied.

5.1 Methods and samples

T-LBL samples of tumor material were available in the NHL-BFM study center (Non-Hodgkin Lymphoma Berlin-Frankfurt-Münster group) for 108 T-LBL patients. These samples were mostly tumor touch imprints or cytospin preparations of malignant effusions. Given this limitation, the first attempt to obtain additional information on 6q deletion in T-LBL was performed with fluorescence in situ hybridization (FISH) analysis. In general, FISH analysis is an adequate technique for the analysis of cells transferred onto microscopic slides, which offers the opportunity to analyze tumor touch imprints and cytospin preparations. However, FISH analysis of the available sample series was hampered by the long period of storage of the slides, which led to complete and irreversible dehydration of the cells, making the slides unusable for FISH analysis.

In a second attempt loss of heterozygosity (LOH) analysis was used, which is an alternative method for the detection of deletions of chromosomal material and allows analyzing specimens that do not fulfill the prerequisites in terms of quantity and quality necessary for other methods, e.g. FISH or cytogenetics. Therefore this technique was optimal for the examination of the available series of T-LBL samples.

The samples used showed a certain variety in quality and quantity. For optimal utilization of the available resources different protocols were used for DNA preparation in order to allow maximum amount of DNA recovery according to the type of sample.

5.2 Common deleted regions and prognostic impact of 6q deletions

A total of 108 T-LBL and 127 T-ALL patients were evaluable for this study. In these groups of patients, LOH analysis was successfully performed for more than 4,500 microsatellite marker

analyses. These data represent the largest comparative analysis of T-LBL and T-ALL. In addition, all patients had been treated with an ALL-BFM treatment regimen, meaning that direct comparison and detailed analysis of outcome data of the patients was possible.

5.2.1 Common deleted regions and prognostic impact of 6q deletions in T-ALL

LOH of at least one marker was detected in 16 of 127 analyzed T-ALL cases (13%). The frequency of LOH at 6q observed in the present study was comparable to those reported in the literature.[92;93;95;204;227;228;240;255]

Regarding clinical characteristics, there was a trend in LOH positive cases for younger age, more frequent CNS involvement and less frequent immature T-cell immunophenotype, even though these findings did not reach statistical significance. There were no differences with respect to sex ratio, the incidence of mediastinal tumor, the lactate dehydrogenase (LDH) level and the white blood cell count at diagnosis. Therefore, no specific association of 6q deletions with other clinical characteristics could be assessed.

Regarding the identification of a common deleted region in the present T-ALL series, it was interesting to observe that the vast majority of LOH positive cases showed LOH of the microsatellite markers at the proximal part of chromosome 6q, while the more telomeric markers retained heterozygosity. Due to the fact that in 14 of the 16 LOH positive patients the most centromeric informative marker showed LOH, the proximal break points of 6q could not be identified with the set of 25 markers used in this analysis. To narrow down the location of the proximal break points, additional microsatellite markers were implemented. Twelve cases could be analyzed successfully with these additional markers, showing either retained heterozygosity at more centromeric marker on 6q or retained heterozygosity at the most centromeric marker localized on chromosome 6p. These results confirm the finding of interstitial deletions with proximal break points close to the centromere. The telomeric break points varied but were almost all within the chromosomal bands 6q15, 6q16 and 6q21. Only one patient with two regions of deletion showed LOH of the more telomeric markers.

Regarding the minimal common deleted region of the 16 LOH positive T-ALL cases, a small region of 4.3 Mb at the passage of chromosomal bands 6q14 and 6q15 could be identified. This region was spanned by the putative deleted region in 15 of the 16 LOH positive patients (94%). Since the centromeric limitation of this common deleted region was defined by only one individual case (case O; isolated LOH of marker D6S1004; shown in three independent PCR and fragment length analysis reactions), further analysis will be necessary to confirm this finding. The telomeric limitation of the common deleted region was defined by a total of three cases (cases J, N, and O), which might provide certain evidence for the telomeric limitation of the minimal common deleted region in our series of pediatric T-ALL cases.

Interestingly, the common deleted region in the current sample series does not overlap with most of the common deleted regions described in the literature. Figure XXIII summarizes the relative positions of common deleted regions in ALL or in some studies ALL plus NHL samples identified by fluorescence in situ hybridization or LOH in eight published studies.[204-206;226;229;231;240;254]

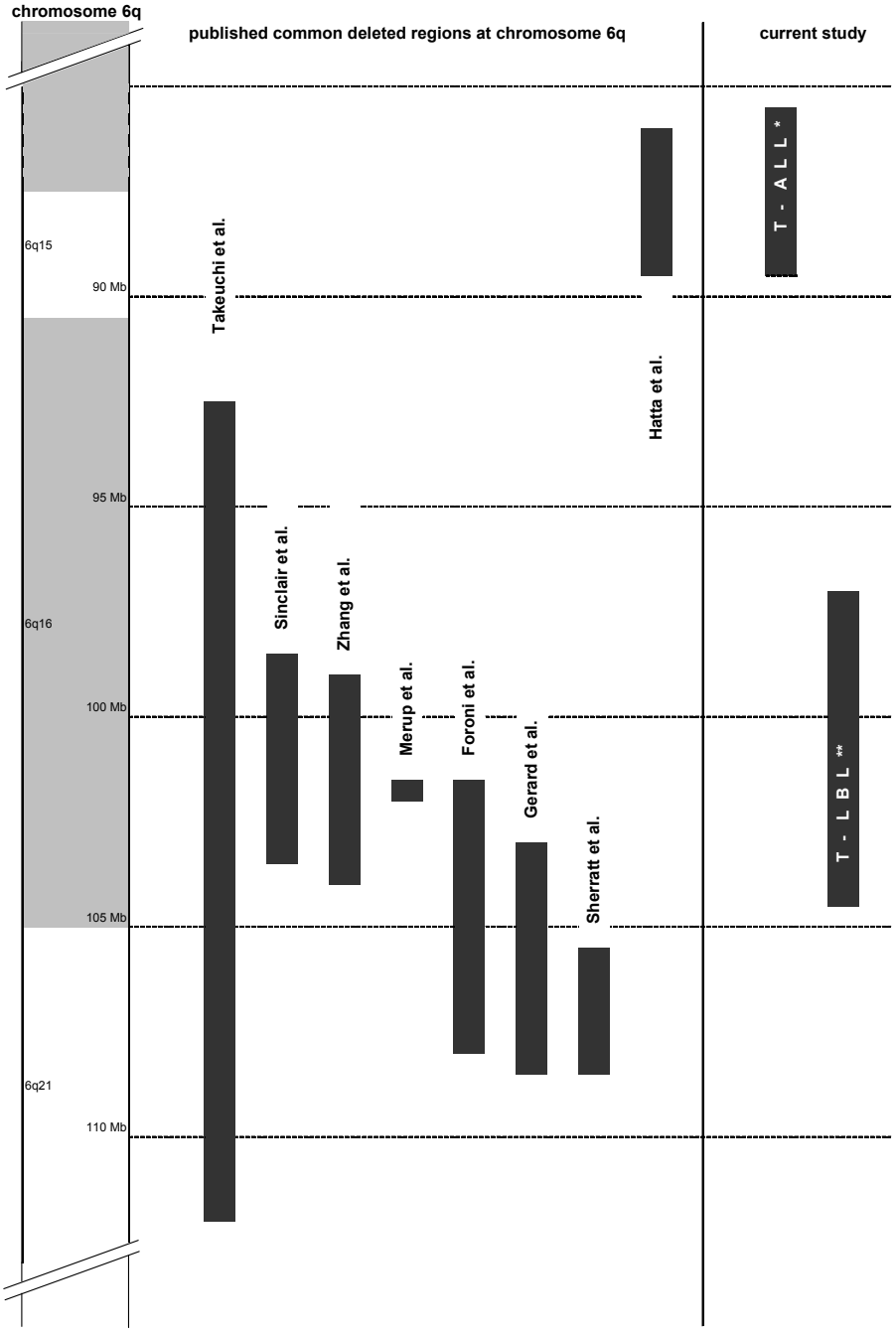


Figure XXIII. Regions of minimal deletion in ALL (+NHL) identified in published studies mapping 6q deletion with fluorescence in situ hybridization or loss of heterozygosity. T-ALL* common deleted region in T-ALL samples of the present study; T-LBL** critical region of deletion in T-LBL samples of the present study

Only one of the eight identified common deleted regions overlaps with the common deleted region detected in our T-ALL series. Interestingly, the study of Hatta et al. was the only published series that was restricted to leukemia samples of T-cell immunophenotype, however, the population were HTLV-I positive adult T-cell leukemia patients, which is biologically different from pediatric T-ALL. The other studies included precursor B-cell and T-cell ALL and cases of Non-Hodgkin lymphoma. The almost complete overlap of the common deleted regions identified in our T-cell-restricted study and that of Hatta et al. supports the theory that, although 6q deletions are observed in a variety of hematological malignancies, the pattern of 6q deletion might be specific for the immunophenotype of the cells. Such subtype-specific minimal regions of deletion were described earlier for different subtypes of B-cell non-Hodgkin lymphoma[213;216] and were reported to allow discrimination of Waldenstrom macroglobulinemia from IgM monoclonal gammopathy of undetermined significance[273].

In the outcome analysis of the T-ALL patients there was no association between clinical characteristics or incidence of relapse and the LOH status of the patients. Due to better options of response evaluation in T-ALL compared with T-LBL, in general more T-ALL patients are stratified into the high-risk arm of treatment. However, the initial response to treatment was not associated with the LOH status, and the percentage of patients stratified to receive high-risk treatment was similar for LOH positive and LOH negative T-ALL patients. To further rule out any possible effect of the intensified high-risk treatment on the prognostic impact of 6q deletions in T-ALL, high-risk patients were excluded in a separate analysis (data not shown). However, this analysis, too, did not show any prognostic impact of LOH at 6q in T-ALL patients. Therefore, the higher rate of patients receiving high-risk treatment in T-ALL compared with T-LBL cannot explain the difference between the two diseases regarding prognostic impact of 6q deletions.

5.2.2 Common deleted regions and prognostic impact of 6q deletions in T-LBL

Findings of LOH at chromosome 6q were detected in 21 of the 108 evaluable T-LBL patients (19%). There was no association of LOH status with clinical features such as sex, age, stage of disease, BM involvement, mediastinal involvement, and CNS involvement.

Correlated with the available cytogenetic data, 6q deletions were detected more frequently with LOH analysis than by standard karyotyping. This can be explained by the higher resolution of the results obtained with the current set of microsatellite markers compared to the characteristic resolution of cytogenetic analysis. In the proximal part of chromosome 6q the markers were chosen to lie within less than 2 Mb (million base-pairs) distance from each other, while

cytogenetic analysis only allows a resolution of > 10 Mb, depending on the quality of the metaphase preparation.

In the 21 LOH positive T-LBL cases the putative deleted regions showed only a partial overlap, which complicated the definition of a common deleted region. The chromosomal region most frequently deleted in this analysis spanned marker D6S1284 and/or adjacent marker D6S1716 and/or adjacent marker D6S1717 in 13 of the 21 LOH positive cases.

In the current sample series, the most interesting result in T-LBL was not primarily the description of a common deleted region but the strong association between detectable LOH at 6q and the occurrence of relapses. Outcome analysis revealed that detectable LOH at 6q was associated with a significantly higher relapse rate compared to LOH-negative patients. The 5-year cumulative incidence of relapse was 9±3% for LOH-negative patients compared with 63±12% for LOH-positive patients ($P < 0.001$). A total of 21 relapsed patients were included in the current analysis and LOH was detected in 13 of the 21 patients. This LOH rate was significantly higher than in the group of 85 patients without relapse, where only 7 patients were LOH positive (odds ratio 16.3, 95% confidence interval 5.2-50.1).

These results must be discussed with a certain caution, since the evaluable group of 108 patients was not fully representative for the whole group of 217 patients. Comparison of patients' characteristics showed more frequent mediastinal involvement and an intermediate immunophenotype in the evaluable group compared to the patients from whom no samples were available. In addition, patients with relapse were over-represented in the analyzed group. Using Bayes' formula, this selection bias for relapsed patients was corrected to allow estimation of the probability of relapse in patients with and without LOH and the resulting risk ratio. Given the relapse rate of 27/209 for the total cohort of T-LBL patients with sufficient follow-up, the corrected probability of relapse in patients without detectable LOH was 6%, while the probability of relapse in patients with detectable LOH was 50%. The corrected risk ratio for relapse in patients with detectable LOH was 8.6.

Yet the findings concerning the prognostic impact of 6q deletions contrast with our own data of the T-ALL series and some reports on lymphoid malignancies, including pediatric ALL, which could not detect a predictive value of 6q deletions for poor outcome.[93;240;252] These discrepancies might be explained by our hypothesis that the deletion of a circumscribed region of the q-arm of chromosome 6 is associated with poor outcome, whereas 6q deletions not involving this critical region do not affect outcome.

Aiming to identify this critical chromosomal region, the deletion of which confers a high risk of relapse on the patients, detailed case-by-case analysis was performed. This analysis revealed a critical region on chromosomal band 6q16, for which LOH was detected in nine out of 21

analyzed relapsed T-LBL patients (deletion rate 43%). This region included the markers D6S1284, D6S1716, D6S1717, D6S468, D6S283, and D6S1580. The deletion rate of these particular markers was less than 5% for T-LBL patients without relapse and less than 15% for T-ALL patients with or without relapse.

Interestingly Foroni et al. reported an association of del6q16-q21 with poor outcome in childhood and adult T-ALL-patients.[254] Unfortunately the report provided only rare data on the patients' characteristics. Therefore the data could not be read separately for children and adults. In addition the provided data do not allow checking whether cases with T-LBL according to the BFM-definition were included in the study of Foroni et al. This differentiation of T-LBL cases would be have been relevant for further discussion, as the minimal region of deletion in the study of Foroni et al. (as indicated for ALL samples in Sinclair et al.[226]) does not overlap with the common deleted region of the T-ALL samples in our study. But the minimal deleted region of Foroni et al. partly overlaps with the critical region of deletion identified in our T-LBL patients (Figure XXIII). These preliminary data might support our hypothesis of a critical region in the chromosomal band 6q16, the deletion of which might be associated with a high risk of relapse. In our series this region was frequently deleted in T-LBL, while in T-ALL the markers of the critical region were retained in the majority of cases. Whether the deletion of the critical region confers high risk of relapse in although in T-ALL needs to be analyzed in a larger series of cases.

The group of Foroni et al. mapped a minimal region of deletion in ALL between markers D6S1510 and D6S1692 at chromosomal band 6q16. Expression and mutational analyses identified GRIK2 as a candidate tumor suppressor gene. In patients with T-lineage ALL, 6q deletions were associated with a statistically significant reduction in GRIK2 expression.[226] Data on the expression level of this postulated candidate tumor suppressor gene in ALL are currently lacking for lymphoblastic lymphoma samples. However, one might speculate that either GRIK2 or another gene located in the critical 6q region identified from our T-LBL samples might influence the treatment response and chemosensitivity of lymphoma cells.

The critical region within chromosome 6q has been fully sequenced.[274] The genes, predicted genes and open reading frames allocated to the critical region between markers D6S1682 and D6S1021 are listed in Table 38.

Table 38. Genes, predicted genes and open reading frames in the critical region of chromosome 6q

KIAA0776	KIAA0776
LOC442237	similar to 40S ribosomal protein S7 (S8)
LOC642420	hypothetical protein LOC642420

continue Table 38	
KIAA1900	KIAA1900
FHL5	four and a half LIM domains 5
GPR63	G protein-coupled receptor 63
C6orf66	chromosome 6 open reading frame 66
C6orf167	chromosome 6 open reading frame 167
POU3F2	POU domain, class 3, transcription factor 2
DHRS6P1	dehydrogenase/reductase (SDR family) member 6 pseudogene 1
COQ3	coenzyme Q3 homolog, methyltransferase (*yeast*)
USP45	ubiquitin specific peptidase 45
CCNC	cyclin C
GPR145	G protein-coupled receptor 145
LOC442238	similar to hypothetical protein FLJ20619
SIM1	single-minded homolog 1 (*Drosophila*)
LOC153893	similar to RP42 homolog
ASCC3	activating signal cointegrator 1 complex subunit 3
LOC653171	similar to MAPK-interacting and spindle-stabilizing protein
GRIK2	glutamate receptor, ionotropic, kainate 2
FBXL4	F-box and leucine-rich repeat protein 4
C6orf168	chromosome 6 open reading frame 168
C6orf111	chromosome 6 open reading frame 111
LOC642765	hypothetical protein LOC642765
PRDM13	PR domain containing 13
LOC642491	hypothetical protein LOC642491
LOC442239	similar to Peroxiredoxin 2 (Thioredoxin peroxidase 1)

Several possible candidate genes in addition to GRIK2 were allocated to that region, e.g., the transcription factors POU3F2 and SIM1 and the putative ubiquitin processing enzymes FBXL4 and USP45. Candidate genes cyclin C and open reading frame 111 (C6orf111) have been shown to be expressed in hematopoetic cells,[226;275] and cyclin C is a known negative regulator of cell growth.[276]

An unselected prospective study with a larger series of T-LBL patients is necessary in order to characterize the patterns of 6q deletions in T-LBL in further detail. Also, further experimental studies will be necessary to clarify whether deletion of the suggested critical region of 6q leads to altered expression of one or more of the above mentioned genes and to identify the mechanism responsible for the altered chemosensitivity of lymphoma cells.

5.2.3 Comparison of LOH findings in T-ALL and T-LBL

Interestingly, the results of LOH analysis were similar in T-LBL and T-ALL considering only the naïve data of all analyzed markers. This comparison is shown in Figure XXIV.

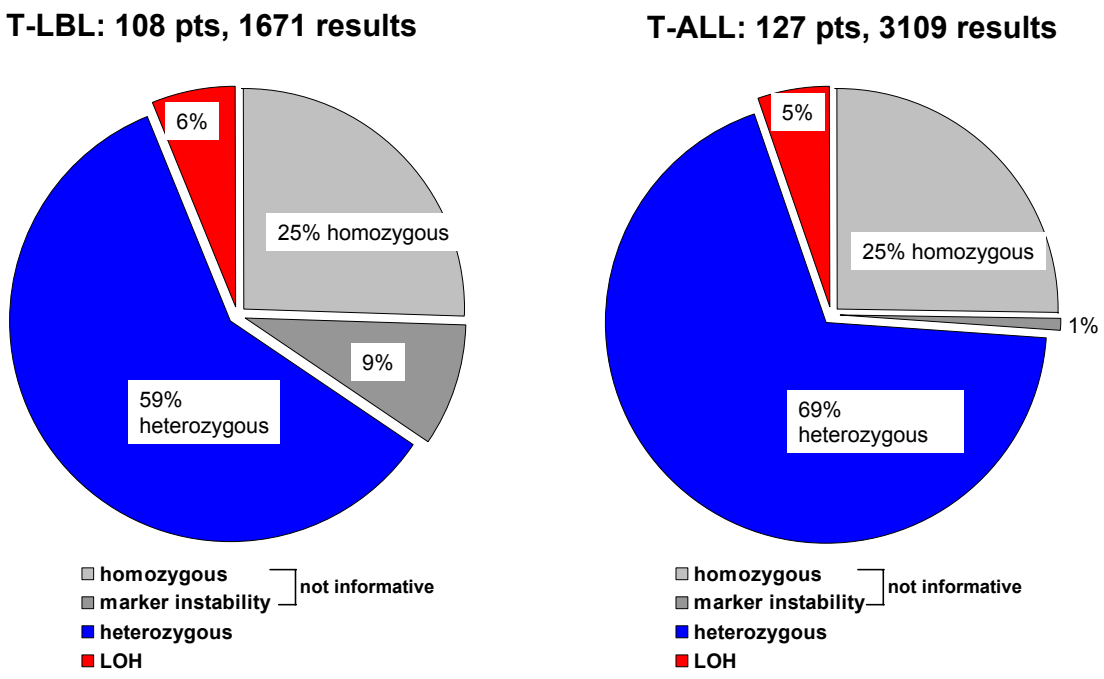


Figure XXIV. Comparison of the sum of LOH results in the group of T-LBL patients versus the group of T-ALL patients

The rate of LOH findings was almost identical, with 6% of LOH in T-LBL compared to 5% of LOH in T-ALL. Also, the number of homozygous markers was identical in both T-ALL and T-LBL. This confirms the validity of the results, because analysis of patients belonging to an identical population (Germany/Switzerland) is expected to result in identical rates of homozygous markers. The rate of markers with retained heterozygosity was higher in T-ALL, while the rate of instable markers was higher in T-LBL. It is the objective of a subsequent ongoing research project to rule out, whether this reflects a reproducible difference between the two diseases and not an artificial phenomenon e.g. caused by the differences of the sample types.

Although the rate of markers with detectable LOH was similar, going into detail revealed clear differences in the patterns of LOH between T-LBL and T-ALL samples. In T-ALL, cases showed interstitial 6q deletions which were – except one case – restricted to the proximal part of chromosome 6q. The centromeric and telomeric breakpoints were not identical for the cases, but the breakpoint regions could be narrowed down to relatively small chromosomal regions.

The homogenous breakpoint regions might indicate a common mechanism introducing the interstitial deletions of proximal chromosome 6q in T-ALL.

Different mechanisms are discussed to cause interstitial deletions. In general, interstitial deletions are supposed to be caused by DNA double strand breaks (DSB) and subsequent illegitimate joining of DNA ends. One possible mechanism is the inappropriate activity of V(D)J recombinases which mediate the rearrangement of the T-cell receptor gene segments (TCR) during lymphoid maturation. The lymphoid-specific RAG1 and RAG2 proteins introduce double-strand breaks at specific recognition sequences (recombination signal sequences, RSSs) in the process of the rearrangement of the T-cell receptor genes. It was reported, that these proteins were also capable of transposing RSS-ended fragments into new DNA sites.[277] In addition, the access of RAG proteins to the DNA is strongly regulated so that particular chromosomal sites are available only in certain developmental stages.[278] In a preliminary hypothesis, one might speculate, whether the common breakpoint regions of chromosome 6q in T-ALL might be marked by increased RAG accessibility during certain stage of T-cell maturation. Such a mechanism of illegitimate RAG activity has been reported to be involved e.g. in the deletions of chromosome 9q21 in lymphoid leukemia and in the translocations of BCL-2 and follicular lymphoma.[279-281] Further structural analyses are necessary to elucidate the mechanism of the interstitial 6q deletion in T-ALL.

In T-LBL, the findings of LOH were not as homogeneous as in T-ALL. Neither the size of the deleted regions nor the breakpoints clustered in a recognizable muster. Interestingly, the rate of markers with marker instability was higher in T-LBL compared with T-ALL. These alterations of the length of simple repetitive genomic sequences often indicate deficiency of the human mismatch repair genes hMSH2, hMSH6 and hMLH1.[282] These mismatch repair (MMR) proteins are capable of recognizing and processing single base-pair mismatches and insertion-deletion loops that occur during DNA replication. Impairment of mismatch repair function is postulated to play a role in mutations of DNA double-strand breaks repair genes e.g. ATM.[283] In a recent report it was shown, that impairment of the MMR system caused aberrant transcripts of the DSB repair genes ATM and MRE11 in leukemia and lymphoma cell lines. This might lead to decreased function of the DSB repair system, inducing the accumulation of genetic damage.[284] It was reported earlier, that germline or acquired ATM alterations are associated with lymphoid malignancies, especially of T-cell origin.[137;138;140;285] Further efforts will be undertaken to analyze the possible association of microsatellite instability in T-LBL patients with defects in the MMR system.

5.2.4 Conclusions of the study

The present study describes differences in the patterns of 6q deletion between pediatric T-cell lymphoblastic leukemia and T-cell lymphoblastic lymphoma patients. These differences were not only in the localization of the common deleted region, but also in the prognostic impact of 6q deletions, with a strong association with poor outcome in T-LBL but not in T-ALL patients. The pattern of 6q deletions in hematological malignancies might partly depend on the subtype and the immunophenotype of the malignant cells. Comparing uniformly treated and well-characterized groups of patients might be an important step in clarifying the impact of 6q deletions in different hematological diseases. Conversely, knowledge of 6q deletion patterns might be helpful in the differentiation of subtypes of these diseases. The data obtained in the current analysis support the hypothesis that there are indeed genotypic differences between pediatric T-cell lymphoblastic leukemia and T-cell lymphoblastic lymphoma.

The detailed analysis of 6q deletions in T-ALL revealed a common deleted region that was altered in 15 of the 16 LOH positive cases. It might be worthwhile to spend further efforts on the characterization of the genes located in this common deleted region in order to identify their possible contribution to the pathogenesis of pediatric T-ALL.

In addition, for T-LBL patients a critical region of deletion could be suggested; a region, the deletion of which was associated with a higher risk of relapse. A larger prospectively evaluated series of patients and comprehensive biological and functional studies of the genes in this critical region will be necessary to confirm this result. However, the result of the current study might be a first step in identifying specific genetic alterations in T-LBL cells to distinguish low- and high-risk patients, enabling successful risk adaptation of frontline treatment.

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6.2 Internet

I http://www.ensembl.org/Homo_sapiens/index.html

II http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=genome

III http://genome.ucsc.edu/

IV http://bacpac.chori.org

7 List of abbreviations

A

ABL1	v-abl Abelson murine leukemia viral oncogene homolog 1 gene
AF10	myeloid/lymphoid or mixed-lineage leukemia translocated to 10 gene (MLLT10)
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ATM	Ataxia teleangiectasia mutated gene

B

BAC	Bacterial artificial chromosome
BCL-2	B-cell CLL/lymphoma 2 gene
BFM	Berlin-Frankfurt Münster
BM	Bone marrow
BMT	Bone marrow transplantation
bp	Base-pairs

C

°C	Degree Celsius
CCD	Charge coupled device
CRR	Constant complete remission
CD	Cluster of differentiation
CDKN2A	cyclin-dependent kinase inhibitor 2A gene (ARF, INK4a, p14, p14ARF, p16, p16INK4a, p19)
CDKN2B	cyclin-dependent kinase inhibitor 2B gene (INK4B, p15)
CNS	Central nervous system
CR	Complete remission
CRT	Cranial radiotherapy
C segments	Constant segments
CSF	Cerebrospinal fluid
CT	Computed tomography

D

ddH_2O	Double distilled H_2O
dATP	2-Desoxyadenosin-5-triphosphate
dCTP	2-Desoxycytidine-5-triphosphate
dGTP	2-Desoxyguanosine-5-triphosphate
dUTP	2-Deoxyuridine 5-triphosphate
dTTP	2-Desoxythymidine-5-triphosphate

dNTP	2-Desoxyribonucleosid–5-triphosphate
DNA	Deoxyribonucleic acid
DSB	Double strand breaks
D segments	Diversity segments

E

E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EFS	Event free survival
e.g.	For example "*exempli gratia"*

F

6-FAM	6-Carboxyfluorescein
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
FLA	Fragment length analysis

G

g	Gram
G 1 phase	G1 phase is a period in the cell cycle during interphase
GRIK2	Glutamate receptor, ionotropic, kainate 2 gene

H

H	Hour(s)
H_2O	Water
HD	Heterodimerisation domain
hMLH1	MutL homolog 1, colon cancer, nonpolyposis type 2 (*E. coli*) gene
hMSH2	MutS homolog 2, colon cancer, nonpolyposis type 1 (*E. coli*) gene
hMSH6	MutS homolog 6 (*E. coli*) gene
HOX11	T-cell leukemia homeobox 1 gene (TLX1)
HOX11L2	T-cell leukemia homeobox 3 gene (TLX3)
HR	High risk arm
HTLV-I	Human T-lymphotropic virus type I

I

IL	Interleukin
Iv	Intravenously
It	intrathecally

J

J segments	Joining segments

K

kb	Kilo base-pairs (1 000 base-pairs)

L

LBL	Lymphoblastic lymphoma
LCK	Lymphocyte-specific protein tyrosine kinase gene
LDH	Lactate dehydrogenase
LFU	Lost to follow up
LMO1	LIM domain only 1 gene (rhombotin 1) (RBNT1)
LMO2	LIM domain only 2 gene (rhombotin-like 1) (RBNT2)
LOH	Loss of heterozygosity
LYL1	Lymphoblastic leukemia derived sequence 1 gene

M

Mb	Million base-pairs
MHC	Major histocompatibility complex
min	Minute(s)
MMR	Mismatch repair
MML	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, *Drosophila*) gene
MRE11	MRE11 meiotic recombination 11 homolog A (*S. cerevisiae*) gene
MRD	Minimal residual disease
MRI	Magnetic resonance imaging
MTX	Methotrexate
MYC	v-myc myelocytomatosis viral oncogene homolog gene

N

N	Number(s)
NaCl	Natriumchloride
NHL	Non-Hodgkin Lymphoma
NK cell	Natural killer cell
NOTCH1	Notch homolog 1 gene
NUP214	Nucleoporin 214kDa gene

O

OD	Optical density

P

P	Probability (of statistical test)
p14	cyclin-dependent kinase inhibitor 2A
p15	cyclin-dependent kinase inhibitor 2B
p16	cyclin-dependent kinase inhibitor 2A
p53	tumor protein p53 gene
PB	Peripheral Blood
pB-ALL	Precursor B-cell acute lymphoblastic leukemia
pB-LBL	Precursor B-cell acute lymphoblastic l lymphoma
PCR	Polymerase chain reaction
pEFS	Probability of event free survival
PGR	Prednisone good responder
PPR	Prednisone good responder
Pre-Tα	Pre T-cell receptor alpha chain
Pt	Patient
Pts	Patients

R

RAG1	Recombination activating gene 1
RAG2	Recombination activating gene 2
Rb	Retinoblastoma 1 gene (RB1)
RBTN1	LIM domain only 1 (rhombotin 1) (LMO1)
RBTN2	LIM domain only 2 (rhombotin-like 1) (LMO2)
REAL	Revised European American Lymphoma Classification
rpm	Revolutions per minute
RR	Risk ratio
RSS	Recombination signal sequences

S

SD	Standard deviation
SDS	Sodium dodecyl sulfate
Sec	seconds
SIL	SCL/TAL-1 interrupting locus (STIL)
STR	Short tandem repeat

T

Tab.	Table
TAL-1	T-cell acute lymphocytic leukemia 1 gene (SCL)
Taq	*Thermus aquaticus*
T-ALL	Precursor T-cell acute lymphoblastic leukemia
TCR	T-cell receptor
TCRαβ	T-cell receptor alpha beta
TCRα	T-cell receptor alpha chain
TCRβ	T-cell receptor beta chain
TCRγδ	T-cell receptor gamma delta
TCRγ	T-cell receptor gamma chain
TCRδ	T-cell receptor delta chain
TdT	Terminal deoxynucleotidyl transferase
T-LBL	Precursor T-cell lymphoblastic lymphoma
TRM	Treatment related mortality

U

U	Units
UV	Ultraviolett

V

V	Volt
V segments	Variable segments

W

WHO	World Health Organization

Y

y	Year(s)

8 Appendix

8.1 Detailed treatment plan NHL-BFM 95 for lymphoblastic lymphoma

Drug	Dose	Given on days
Induction Protocol I (week 1-9)		
Prednisone (orally)	60 mg/m^2	1-28, then taper over 3x3 days
Vincristine (iv)	1.5 mg/m^2 (max 2 mg)	8, 15, 22, 29
Daunorubicin (iv over 1 h)	30 mg/m^2	8, 15, 22, 29
E. coli L-Asparaginase (iv over 1h)	5,000 IU/m^2	12, 15, 18, 21, 24, 27, 30, 33
Cyclophosphamide & (iv over 1 h)	1000 mg/m^2	36, 64
Cytarabine (iv)	75 mg/m^2	38-41, 45-48, 52-55, 59-62
6-Mercaptopurine (orally)	60 mg/m^2	36-63
Methotrexate (it)**	12 mg	1, 12, 33, 45, 59*
Protocol M (starting two weeks after the end of protocol I)		
6-Mercaptopurine (orally)	25 mg/m^2	1-56
Methotrexate***	5 g/m^2	8, 22, 36, 50
Methotrexate (it)***	12 mg	8, 22, 36, 50
Re-Induction Protocol II (starting two weeks after the end of protocol M)		
Dexamethasone (orally)	10 mg/m^2	1-21, then taper over 3x3 days
Vincristine (iv)	1.5 mg/m^2 (max 2mg)	8, 15, 22, 29
Doxorubicin (iv over 1 h)	30 mg/m^2	8, 15, 22, 29
E.coli L-Asparaginase (iv over 1h)	10,000 IU/m^2	8, 11, 15, 18
Cyclophosphamide (iv over 1 h)	1000 mg/m^2	36
Cytarabine (iv)	75 mg/m^2	38-41, 45-48
6-Thioguanine (orally)	60 mg/m^2	36-49
Methotrexate (it)**	12 mg	38, 45
Maintenance		
6-Mercaptopurine (orally)	50mg/m²	daily
Methotrexate (orally)	20mg/m²	weekly

iv = intravenously; it = intrathecally

* additional doses at days 18, 27 for CNS-positive patients

** MTX intrathecally was given 2 hours after the start of the MTX iv infusion (6 mg for age < 1 year; 8 mg for age 1 year to <2 years, 10 mg for age 2 years to <3 years, 12 mg for age ≥ 3 years)

*** 10% of the dose over 30 minutes, and 90% as a 23.5-hour continuous iv infusion. Leucovorin rescue: 15 mg/m^2 iv at hours 42, 48 and 54. Serum levels of MTX should be <3 µmol/l at hour 36 after the start of the MTX infusion, $\leq$1 µmol/l at hour 42, and $\leq$ 0.4 µmol/l at hour 48

8.2 Solutions, buffers, and media

10x NT-buffer	1 M Tris-HCl, pH 8.8, Roth, Karlsruhe, Germany	2.5 ml
	0.5 M $MgCl_2$, Merck, Darmstadt, Germany	0.5 ml
	10 mg/ml Bovine Serum Albumin, acetylated, Promega, Madison, USA	0.25 ml
20x SSC	NaCl, Roth, Karlsruhe, Germany	175.3 g
	Tri-Na-Citratdihydrate, Roth, Karlsruhe, Germany	88.2 g
	ddH_2O	ad 1000 ml
	adjustment of pH 7.0	
	autoclave	
4x SSC+Tween 0.05%	20x SSC	200 ml
	ddH_2O	800 ml
	Tween 20, Merck-Schuchardt, Hohenbrunn, Germany	0.5 ml
	adjustment of pH 7.0	0.5 ml
2x SSC	20x SSC (pH 7.0)	100 ml
	ddH_2O	900 ml
	adjustment of pH 7.0	
2x SSC+Tween 0.1%,	20x SSC	100 ml
	ddH_2O	900 ml
	Tween 20, Merck-Schuchardt, Hohenbrunn, Germany	1 ml
	adjustment of pH 7.0	1 ml
1x SSC	20x SSC (pH 7.0)	50 ml
	ddH_2O	950 ml
	adjustment of pH 7.0	
0.1x SSC	2x SSC (pH 7.0)	50 ml
	ddH_2O	950 ml
	adjustment of pH 7.0	
10x PBS	NaCl, Roth, Karlsruhe, Germany	80 g
	KCl, Merck, Darmstadt, Germany	2 g
	Na_2HPO4, Merck, Darmstadt, Germany	14.4 g
	KH_2PO4, Merck, Darmstadt, Germany	2.4 g
	ddH_2O	ad 1000 ml
	autoclave	

1x PBS	10x PBS	100 ml
	ddH_2O	900 ml
3 M Sodium Acetate	Sodium acetate, Roth, Karlsruhe, Germany	408 g
	ddH_2O	ad 1000 ml
Ethanol series	Ethanol 30%, 50%, 70%, 90% dilutions of Riedel de Haen, Sigma, Seelze, Germany	
Fixative methanol:acetic acid	Methanol, JT Baxter, Unterschleissheim, Germany	80 ml
	Acetic acid, JT Baxter, Unterschleissheim, Germany	20 ml
HCl solution	1 N HCl, Merck, Darmstadt, Germany	1 ml
	ddH_2O	99 ml
Formamide solution	Formaldehyde solution 99%, Sigma, Steinheim, Germany	7 ml
	20x SSC	1 ml
	ddH_2O	2 ml
	adjustment of pH 7.0	
Pepsin solution (10%)	Pepsin (3,200-4,500 units/mg protein, No. P-6887), Sigma, Steinheim, Germany,	250 mg
	ddH_2O	2.5 ml
Lysogeny Broth (LB) Medium	Trypton/Pepton from Casein, Roth, Karlsuhe, Germany	10 g
	Bacto-Yeast-Extract, Becton Dickinson, Heidelberg, Germany	5g
	NaCl, Roth, Karlsruhe, Germany	10 g
	H_20	ad 1000 ml
	adjustment of pH 7.0, autoclave	
	Chloramphenicol, 25 mg/ml	800 µl/1000 ml medium
0.1 M ß-Mercaptoethanol	14.4 M ß-Mercaptoethanol, Roth, Karlsruhe, Germany	35 µl
	ddH_2O	4.965 ml
dNTP, Roche, Roche, Mannheim, Germany	100 mM dATP, Roche, Mannheim, Germany	5 µl
	100 mM dCTP, Roche, Mannheim, Germany	5 µl
	100 mM dGTP, Roche, Mannheim, Germany	5 µl
	100 mM dTTP, Roche, Mannheim, Germany	5 µl
	ddH_2O	984 µl

8.3 DNA preparation from T-LBL samples

DNA preparation kits	
PeqLab Forensic	PeqGOLD Forensic DNA Kit, PeqLab, Erlangen, Germany
Invitrogen Forensic	ChargeSwitch® Forensic DNA Purification kit, Invitrogen, Karlsruhe, Germany
PeqLab E.Z.N.A. Blood	E.Z.N.A. Blood DNA Kit, PeqLab, Erlangen, Germany
TriFast	PeqGOLD TriFast, PeqLab, Erlangen, Germany
QIAamp DNA mini kit	QIAamp DNA mini kit, Qiagen, Erlangen, Germany
Roche High Pure PCR	High Pure PCR Template Preparation Kit, Roche, Mannheim, Germany

case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as PCR template [ng]
70	pleural effusion cytospin	PeqLab Forensic	148	20-50
	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	14	10
	BM smear	PeqLab Forensic	151	5-40
	germ line DNA*	Roche High Pure PCR	105	10
71	tumor touch imprint	PeqLab Forensic	159	50
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	397	10
	PB smear	PeqLab Forensic	90	20
	germ line DNA*	Roche High Pure PCR	26	10
25	pleural effusion cytospin	PeqLab Forensic	137	40-80
	BM smear	PeqLab Forensic	121	20
	germ line DNA*	Roche High Pure PCR	37	5
26	pleural effusion cytospin	PeqLab Forensic	132	20-40
	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	88	5
	BM smear	PeqLab Forensic	47	5
	BM frozen cells in DMSO	PeqLab E.Z.N.A. Blood	51	5
21	tumor touch imprint	PeqLab Forensic	392	20
	BM smear	PeqLab Forensic	42	5
69	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	1427	10
	tumor touch imprint	PeqLab Forensic	137	20-80
	BM smear	PeqLab Forensic	67	5-50
	PB smear	PeqLab Forensic	162	10-100
	germ line DNA*	Roche High Pure PCR	37	10
5	tumor touch imprint	PeqLab Forensic	212	100
	BM smear	PeqLab Forensic	185	100
22	tumor touch imprint	PeqLab Forensic	903	100
	BM smear	PeqLab Forensic	304	100
28	pleural effusion cytospin	PeqLab Forensic	200	40
	BM smear	PeqLab Forensic	247	20-40

case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as PCR template [ng]
72	pleural effusion cytospin	PeqLab Forensic	185	20-100
	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	62	5
	PB smear	PeqLab Forensic	165	5-50
	germ line DNA*	Roche High Pure PCR	16	10
29	pleural effusion cytospin	PeqLab Forensic	154	20
	pleural effusion cytospin	Invitrogen Forensic	25	5
	BM smear	PeqLab Forensic	47	5-100
	germ line DNA*	Roche High Pure PCR	97	10
2	pleural effusion cytospin	PeqLab Forensic	342	40
	BM smear	PeqLab Forensic	155	5-20
	germ line DNA*	Roche High Pure PCR	92	10
30	tumor touch imprint	PeqLab Forensic	259	40
	BM smear	PeqLab Forensic	103	20-80
	germ line DNA*	Roche High Pure PCR	65	5
31	tumor touch imprint	PeqLab Forensic	53	20-40
	tumor touch imprint	Invitrogen Forensic	13	1
	BM smear	PeqLab Forensic	142	5
32	pleural effusion cytospin	PeqLab Forensic	70	20-100
	PB smear	PeqLab Forensic	79	5-100
	germ line DNA*	Roche High Pure PCR	17	10
73	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	712	10
	PB smear	Invitrogen Forensic	21	2.5
4	tumor touch imprint	PeqLab Forensic	207	20
	BM smear	PeqLab Forensic	40	5-20
	germ line DNA*	Roche High Pure PCR	21	10
74	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	228	10
	BM smear	Invitrogen Forensic	7	5
11	pleural effusion cytospin	PeqLab Forensic	42	20-50
	tumor touch imprint	PeqLab Forensic	142	20
	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	79	25
	BM smear	PeqLab Forensic	54	2.5
33	tumor touch imprint	PeqLab Forensic	204	80
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	47	10-20
	BM smear	PeqLab Forensic	152	
	PB smear	PeqLab Forensic	144	
	germ line DNA*	Roche High Pure PCR	6	10
34	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	73	20
	BM frozen cells in DMSO	PeqLab E.Z.N.A. Blood	75	20
	PB smear	PeqLab Forensic	112	20-50
	germ line DNA*	Roche High Pure PCR	109	10

case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as PCR template [ng]
35	pleural effusion cytospin	PeqLab Forensic	289	20-50
	pleural effusion cytospin	PeqLab Forensic	86	20-50
	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	38	25-50
	BM smear	PeqLab Forensic	68	10-80
	BM smear	PeqLab Forensic	80	10-80
75	tumor touch imprint	PeqLab Forensic	61	20-40
	tumor cells frozen	PeqLab E.Z.N.A. Blood	17	20-40
	PB smear	Invitrogen Forensic	12	2.5-20
	PB smear	PeqLab Forensic	40	2.5-20
1	tumor touch imprint	PeqLab Forensic	146	60-100
	tumor touch imprint	Invitrogen Forensic	93	60-100
	BM smear	PeqLab Forensic	24	2.5-50
36	pleural effusion cytospin	PeqLab Forensic	176	40-80
	BM smear	PeqLab Forensic	273	100
63	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	500	10
	BM smear	PeqLab Forensic	61	10
76	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	1936	10
	BM froze cells	PeqLab E.Z.N.A. Blood Kit	43	10
77	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	1258	10
	BM smear	Invitrogen Forensic	17	2.5
20	pleural effusion cytospin	PeqLab Forensic	731	20-60
	PB smear	PeqLab Forensic	121	5-40
	germ line DNA*	Roche High Pure PCR	35	20
14	pleural effusion cytospin	PeqLab Forensic	118	50
	pleural effusion cytospin	PeqLab Forensic	106	50
	BM smear	PeqLab Forensic	105	5
12	pleural effusion cytospin	PeqLab Forensic	151	50
	BM smear	PeqLab Forensic	71	5-30
	germ line DNA*	Roche High Pure PCR	93	20
37	tumor touch imprint	PeqLab Forensic	60	20-40
	BM frozen cells in DMSO	PeqLab E.Z.N.A. Blood	86	5
	BM smear	PeqLab Forensic	68	20-50
3	pleural effusion cytospin	PeqLab Forensic	125	20-60
	pleural effusion cytospin	PeqLab Forensic	114	40
	BM smear	PeqLab Forensic	104	5
38	tumor cells frozen in DMSO	PeqLab E.Z.N.A. Blood Kit	81	5-10
	BM smear	PeqLab Forensic	121	50
60	pleural effusion cytospin	PeqLab Forensic	201	20-100
	pleural effusion frozen cells in DMSO	PeqLab DNA mini	92	10
	PB smear	PeqLab Forensic	144	5-100
	germ line DNA*	Roche High Pure PCR	171	10

case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as PCR template [ng]
78	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	649	10
	BM smear	Invitrogen Forensic	17	2.5
79	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	1513	10
	BM smear	Invitrogen Forensic	34	2.5
80	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	1598	5
	BM frozen cells	PeqLab E.Z.N.A. Blood	50	5
64	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	368	10-20
	germ line DNA*	Roche High Pure PCR	23	10
39	tumor touch imprint	PeqLab Forensic	177	20
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	682	10
	BM smear	PeqLab Forensic	46	5
	germ line DNA*	Roche High Pure PCR		5
40	pleural effusion cytospin	PeqLab Forensic	40	5-40
	pleural effusion cytospin	Invitrogen Forensic	28	0.75
	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	82	20-50
	BM smear	PeqLab Forensic	63	2.5-20
41	pleural effusion cytospin	PeqLab Forensic	107	20-40
	BM smear	PeqLab Forensic	31	20
42	pleural effusion cytospin	PeqLab Forensic	275	50
	BM smear	PeqLab Forensic	223	20
	germ line DNA*	Roche High Pure PCR	48	10
43	pericardial effusion cytospin	PeqLab Forensic	163	20-60
	pericardial effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	101	20
	BM smear	PeqLab Forensic	73	5
13	tumor touch imprint	PeqLab Forensic	140	20-100
	BM smear	PeqLab Forensic	95	5
10	tumor touch imprint	PeqLab Forensic	229	100
	BM smear	PeqLab Forensic	293	100
44	tumor touch imprint	PeqLab Forensic	218	100
	BM smear	PeqLab Forensic	248	100
45	tumor touch imprint	PeqLab Forensic	538	40
	BM smear	PeqLab Forensic	36	10-20
	germ line DNA*	Roche High Pure PCR	34	10
46	tumor touch imprint	PeqLab Forensic	300	40-80
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	453	10
	BM smear	PeqLab Forensic	312	20
	germ line DNA*	Roche High Pure PCR	47	10
47	pleural effusion cytospin	PeqLab Forensic	159	70
	BM smear	PeqLab Forensic	36	5

case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as PCR template [ng]
65	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	1874	10
	germ line DNA*	Roche High Pure PCR	10	10
48	tumor touch imprint	PeqLab Forensic	138	20-100
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	104	10
	PB smear	PeqLab Forensic	67	5-50
	germ line DNA*	Roche High Pure PCR	33	10
81	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	629	10
	BM smear	Invitrogen Forensic	21	2.5
82	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	877	10
	PB smear	Invitrogen Forensic	9	2.5
83	pleural effusion cytospin	Invitrogen Forensic	27	10
	germ line DNA*	Roche High Pure PCR	85	10
49	tumor touch imprint	PeqLab Forensic	482	60
	BM smear	PeqLab Forensic	105	10-40
	germ line DNA*	Roche High Pure PCR	6	5
84	pleural effusion cytospin	PeqLab Forensic	189	40-60
	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	113	5
	BM smear	PeqLab Forensic	396	20-40
	germ line DNA*	Roche High Pure PCR	40	5
50	pleural effusion cytospin	PeqLab Forensic	171	20-40
	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	28	30
	BM smear	PeqLab Forensic	85	5
85	tumor touch imprint	PeqLab Forensic	136	20-40
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	544	10
	BM smear	PeqLab Forensic	38	5-20
	germ line DNA*	Roche High Pure PCR	27	10
51	tumor touch imprint	PeqLab Forensic	143	20-50
	tumor touch imprint	PeqLab Forensic	366	20-50
	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	68	5
	BM smear	PeqLab Forensic	58	5-10
52	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	141	5
	germ line DNA*	Roche High Pure PCR	36	50
86	pleural effusion cytospin	PeqLab Forensic	83	20-50
	pleural effusion frozen cells	PeqLab E.Z.N.A. Blood	574	5-10
	BM smear	PeqLab Forensic	36	5-20
	PB smear	PeqLab Forensic	104	10-40
	BM frozen cells	PeqLab E.Z.N.A. Blood	111	5-10
87	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	2227	10
	BM smear	Invitrogen Forensic	15	2.5

case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as PCR template [ng]
88	pleural effusion cytospin	PeqLab Forensic	256	40-100
	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	453	10
	BM smear	PeqLab Forensic	276	20-100
	germ line DNA*	Roche High Pure PCR	16	10
66	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	1051	5-10
	germ line DNA*	Roche High Pure PCR	42	5
89	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	983	10
	PB smear	Invitrogen Forensic	12	2.5
90	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	1506	10
	PB smear	Invitrogen Forensic	16	2.5
53	pleural effusion cytospin	PeqLab Forensic	187	20
	PB smear	PeqLab Forensic	26	5-100
54	pleural effusion cytospin	PeqLab Forensic	152	40-100
	pleural effusion cytospin	PeqLab Forensic	207	20
	PB smear	PeqLab Forensic	90	5-50
	PB smear	PeqLab Forensic	90	5-50
	germ line DNA*	Roche High Pure PCR	135	5
18	tumor touch imprint	PeqLab Forensic	22	20-100
	tumor touch imprint	PeqLab Forensic	208	20-100
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	643	10
	BM smear	PeqLab Forensic	64	5-30
	germ line DNA*	Roche High Pure PCR		10
91	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	2289	10
	BM smear	PeqLab Forensic	108	10
55	pleural effusion cytospin	PeqLab Forensic	269	20
	BM smear	PeqLab Forensic	200	5-40
	BM smear	PeqLab Forensic	135	5-40
	germ line DNA*	Roche High Pure PCR	101	10
56	tumor touch imprint	PeqLab Forensic	284	40-80
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	527	10
	BM smear	PeqLab Forensic	236	20-100
	germ line DNA*	Roche High Pure PCR	-	5
92	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	2550	10
	BM smear	Invitrogen Forensic	31	2.5
93	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	622	10
	BM frozen cells	PeqLab E.Z.N.A. Blood	109	5
23	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	59	5
	germ line DNA*	Roche High Pure PCR	41	20

case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as PCR template [ng]
17	pleural effusion cytospin	PeqLab Forensic	305	40-100
	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	10	40
	BM smear	PeqLab Forensic	306	100
57	tumor touch imprint	PeqLab Forensic	97	20
	BM smear	PeqLab Forensic	44	2.5-5
19	pleural effusion cytospin	PeqLab Forensic	112	20-50
	pleural effusion cytospin	Forensic Invitrogen	10	20-40
	BM smear	PeqLab Forensic	51	2.5
7	pleural effusion cytospin	PeqLab Forensic	45	20-40
	pleural effusion cytospin	Invitrogen Forensic	139	50
	germ line DNA*	Roche High Pure PCR	76	15
	BM smear	PeqLab Forensic	177	5-20
	PB smear	PeqLab Forensic	67	
8	pleural effusion cytospin	PeqLab Forensic	135	60
	PB smear	PeqLab Forensic	69	5-60
	germ line DNA*	Roche High Pure PCR	28	40
9	pleural effusion cytospin	PeqLab Forensic	338	20
	BM smear	PeqLab Forensic	77	10
24	tumor touch imprint	PeqLab Forensic	471	80
	BM smear	PeqLab Forensic	287	20-40
	germ line DNA*	Roche High Pure PCR	92	5
59	tumor touch imprint	PeqLab Forensic	306	20-100
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	221	10
	BM smear	PeqLab Forensic	174	100
15	tumor touch imprint	PeqLab Forensic	257	20
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	92	no results
	BM smear	PeqLab Forensic	26	5-20
	BM smear	PeqLab Forensic	121	5-20
	germ line DNA*	Roche High Pure PCR	17	5
58	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	27	50
	BM frozen cells in DMSO	PeqLab E.Z.N.A. Blood	73	2.5
16	pleural effusion cytospin	PeqLab Forensic	220	50
	BM smear	PeqLab Forensic	62	10-20
61	tumor touch imprint	PeqLab Forensic	147	20-50
	tumor touch imprint	Invitrogen Forensic	39	20
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	595	10
	BM smear	PeqLab Forensic	138	5-20
	germ line DNA*	Roche High Pure PCR		10
94	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	325	10
	germ line DNA*	Roche High Pure PCR	16	10
6	tumor touch imprint	PeqLab Forensic	532	100
	BM smear	PeqLab Forensic	447	100

case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as PCR template [ng]
95	pleural effusion cytospin	PeqLab Forensic	112	20-60
	pleural effusion cytospin	Invitrogen Forensic	17	2.5
	pleural effusion frozen cells	TriFast	205	10
	paraffin embedded tumor biopsy**	xylol treatment, proteinase K digestion, QIAamp DNA mini kit	92	1
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	1017	20
	BM smear	PeqLab Forensic	144	5-100
	BM smear	Invitrogen Forensic	12	2
	BM smear	Invitrogen Forensic	40	2
96	ascites cytospin	PeqLab Forensic	90	20
	ascites cytospin	Invitrogen Forensic	88	2.5
	BM smear	PeqLab Forensic	17	5
	BM frozen cells	PeqLab E.Z.N.A. Blood	215	5
97	pleural effusion cytospin	PeqLab Forensic	133	20-40
	pleural effusion cytospin	Invitrogen Forensic	32	2.5-5
	PB smear	PeqLab Forensic	73	10
	PB smear	Invitrogen Forensic	34	2.5-5
98	pleural effusion cytospin	PeqLab Forensic	53	10-40
	paraffin embedded tumor biopsy**	xylol treatment, proteinase K digestion, QIAamp DNA mini kit	160	1
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	1134	20
	PB smear	PeqLab Forensic	39	5-15
	BM frozen cells	PeqLab E.Z.N.A. Blood	135	1-5
99	tumor touch imprint	PeqLab Forensic	41	20-40
	tumor touch imprint	Invitrogen Forensic	53	10-20
	tumor cells frozen	TriFast	57	5-10
	paraffin embedded tumor biopsy**	QIAamp DNA mini kit	231	1
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	1283	20
	BM smear	PeqLab Forensic	89	5-10
	BM smear	PeqLab Forensic	180	5-10
	BM smear	Invitrogen Forensic	109	2.5
100	tumor touch imprint	PeqLab Forensic	102	20-50
	paraffin embedded tumor biopsy**	xylol treatment, proteinase K digestion, QIAamp DNA mini kit	82	1
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	149	20
	PB smear	PeqLab Forensic	54	5-30

case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as PCR template [ng]
67	tumor touch imprint	PeqLab Forensic	97	20
	paraffin embedded tumor biopsy**	xylol treatment, proteinase K digestion, QIAamp DNA mini kit	69	1
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	538	20
	isolated tumor DNA	-	223	20
	BM smear	PeqLab Forensic	32	2.5-5
	BM smear	PeqLab Forensic	46	2.5-5
	BM cells frozen	PeqLab E.Z.N.A. Blood	145	2.5-5
68	pleural effusion cytospin	PeqLab Forensic	58	20-40
	BM cells frozen	PeqLab E.Z.N.A. Blood	46	2.5-20
101	pleural effusion cytospin	PeqLab Forensic	275	20
	BM cells frozen	PeqLab E.Z.N.A. Blood	84	5
102	pleural effusion cytospin	PeqLab Forensic	61	10-20
	BM cells frozen	PeqLab E.Z.N.A. Blood	34	2.5-5
103	pleural effusion cytospin	PeqLab Forensic	57	20
	pleural effusion cytospin	Invitrogen Forensic	16	20
	BM cells frozen	PeqLab E.Z.N.A. Blood	201	2.5-20
104	tumor touch imprint	PeqLab Forensic	107	20-50
	tumor touch imprint	PeqLab Forensic	150	20-50
	PB smear	PeqLab Forensic	129	5-20
	PB smear	PeqLab Forensic	126	5-20
105	tumor touch imprint	PeqLab Forensic	68	20-50
	paraffin embedded tumor biopsy**	xylol treatment, proteinase K digestion, QIAamp DNA mini kit	37	1
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	198	20
	BM smear	PeqLab Forensic	14	5-10
	BM cells frozen	PeqLab E.Z.N.A. Blood	238	2.5-10
106	tumor touch imprint	PeqLab Forensic	34	10-50
	tumor touch imprint	PeqLab Forensic	53	10-50
	tumor touch imprint	Invitrogen Forensic	369	10-50
	paraffin embedded tumor biopsy**	xylol treatment, proteinase K digestion, QIAamp DNA mini kit	98	1
	paraffin embedded tumor biopsy**	xylol treatment and proteinase K digestion	652	20
	tumor cells frozen	TriFast	53	5-10
	BM smear	PeqLab Forensic	62	5-10
	BM smear	Invitrogen Forensic	63	2
	BM cells frozen	TriFast	34	5-10
107	tumor touch imprint	PeqLab Forensic	283	20
	BM smear	PeqLab Forensic	95	5-10
62	tumor touch imprint	PeqLab Forensic	87	10-50
	tumor touch imprint	PeqLab Forensic	130	10-50
	tumor touch imprint	PeqLab Forensic	197	10-50
	BM smear	PeqLab Forensic	51	5-20
	BM smear	PeqLab Forensic	28	5-20

case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as PCR template [ng]
108	tumor touch imprint	PeqLab Forensic	54	20-40
	tumor touch imprint	Invitrogen Forensic	133	20-40
	PB smear	PeqLab Forensic	97	5-100
	BM smear	Invitrogen Forensic	29	2.5
72	pleural effusion cytospin	PeqLab Forensic	36	20-40
	tumor touch imprint	PeqLab Forensic	286	20-40
	tumor touch imprint	Invitrogen Forensic	266	20-40
	BM smear	PeqLab Forensic	6	5-100
	BM smear	PeqLab Forensic	183	5-100
	BM smear	Invitrogen Forensic	141	2.5
	BM cells frozen	PeqLab E.Z.N.A. Blood	242	5-100

8.4 DNA preparation from T-ALL samples

DNA preparation kits	
QIAamp DNA blood midi	QIAamp DNA blood mini, Qiagen, Erlangen, Germany
Invitrogen Forensic	ChargeSwitch® Forensic DNA Purification kit, Invitrogen, Karlsruhe, Germany
PeqLab E.Z.N.A. Blood	E.Z.N.A. Blood DNA Kit, PeqLab, Erlangen, Germany

case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as template for PCR [ng
Q	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
R	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
D	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
S	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
T	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
U	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
V	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
W	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
X	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
Y	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
DC	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
Z	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
AA	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
AB	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 52	QIAamp DNA blood midi*	50	0.5-0.75
AC	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
AD	PB smear day 0	Invitrogen Forensic	27	0.75
	PB smear day 0	Invitrogen Forensic	25	0.75
	BM smear prior to protocol III	Invitrogen Forensic	15	0.75
	BM smear prior to protocol III	Invitrogen Forensic	57	0.75
AE	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75

case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as template for PCR [ng
AF	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
AG	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 52	QIAamp DNA blood midi*	50	0.5-0.75
AH	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
DD	pleural effusion day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
DE	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
AI	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
AJ	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
P	BM smear day 0	Invitrogen Forensic	18	0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
AK	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 35	QIAamp DNA blood midi*	50	0.5-0.75
AL	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
E	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
DF	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
O	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
AM	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
AN	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
AO	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
AP	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
DG	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
AQ	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
C	pleural effusion frozen cells in DMSO	PeqLab E.Z.N.A. Blood	111	0.75
	BM day 0	QIAamp DNA blood midi*	50	0.75-1
	BM day 33	QIAamp DNA blood midi*	50	0.75-1
G	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
AR	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
DH	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75

case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as template for PCR [ng
AS	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
AT	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
A	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
AU	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
AV	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior 2nd HR	QIAamp DNA blood midi*	50	0.5-0.75
AW	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
AX	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
AY	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
AZ	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
BA	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior 2nd HR	QIAamp DNA blood midi*	50	0.5-0.75
BB	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
BC	BM smear (Tu)	Invitrogen Forensic	30	0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
BD	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
DI	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
BE	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 44	QIAamp DNA blood midi*	50	0.5-0.75
BF	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
DJ	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
DK	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior 3rd HR	QIAamp DNA blood midi*	50	0.5-0.75
BG	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
DL	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
BH	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 52	QIAamp DNA blood midi*	50	0.5-0.75
BI	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
BJ	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
BK	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
DM	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior 3rd HR	QIAamp DNA blood midi*	50	0.5-0.75

case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as template for PCR [ng
BL	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
BM	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
BN	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
BO	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 52	QIAamp DNA blood midi*	50	0.5-0.75
DN	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior 1st HR	QIAamp DNA blood midi*	50	0.5-0.75
BP	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
BQ	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
B	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 37	QIAamp DNA blood midi*	50	0.5-0.75
DO	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
BR	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
BS	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
I	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
BT	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
BU	BM smear day 0	Invitrogen Forensic	63	0.75
	PB prior Prot. III	QIAamp DNA blood midi*	50	0.5-0.75
BV	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 52	QIAamp DNA blood midi*	50	0.5-0.75
BW	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
BX	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
DP	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 52	QIAamp DNA blood midi*	50	0.5-0.75
DQ	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
BY	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
BZ	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
J	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
CA	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
M	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
CB	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75

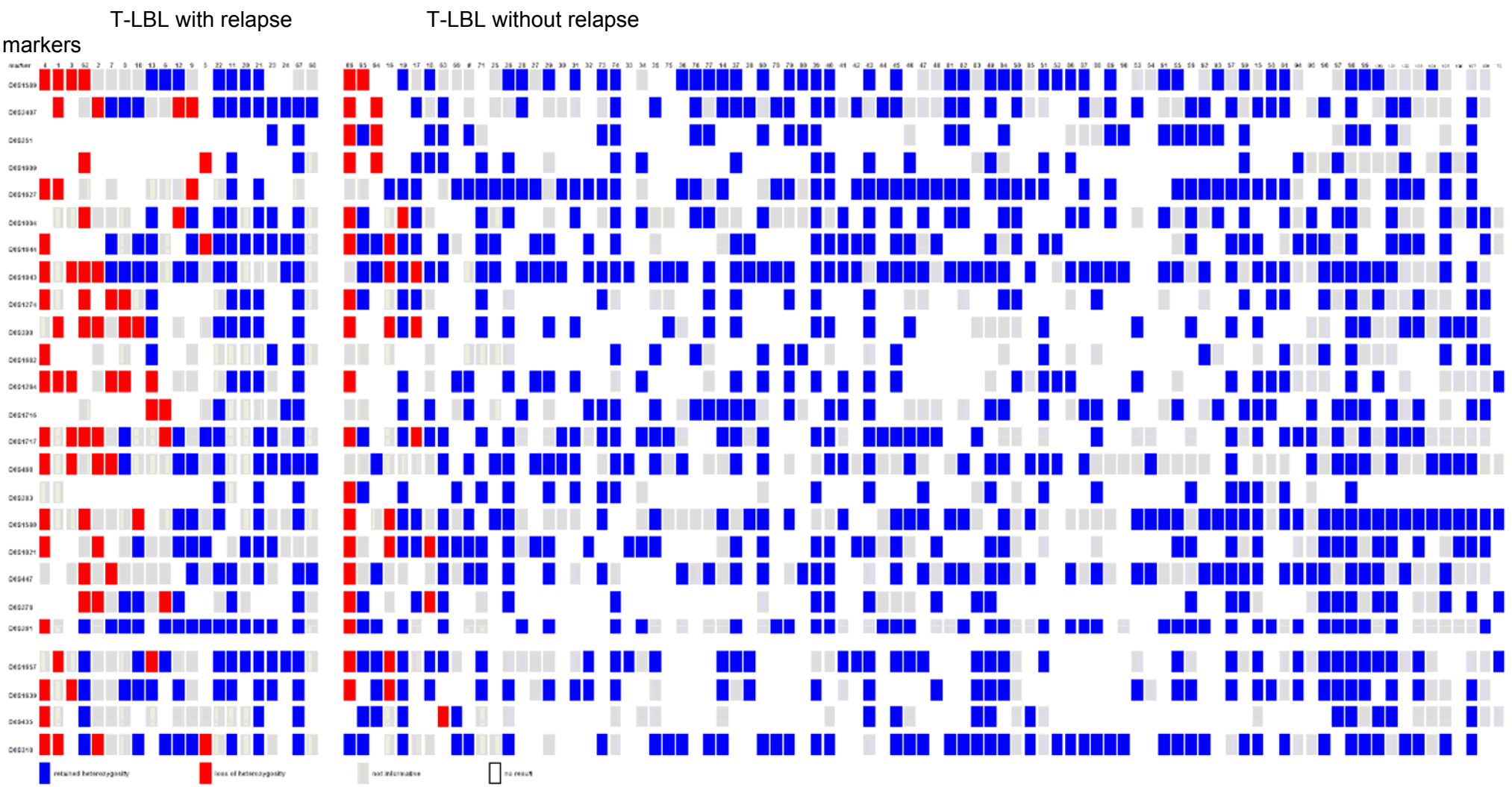
case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as template for PCR [ng
CC	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
DR	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
F	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
CD	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
H	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
CE	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 52	QIAamp DNA blood midi*	50	0.5-0.75
CF	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 52	QIAamp DNA blood midi*	50	0.5-0.75
DS	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM smear prior to 1st HR	Invitrogen Forensic	19	0.75
	BM day 52	QIAamp DNA blood midi*	50	0.5-0.75
CG	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
DT	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior 1st HR	QIAamp DNA blood midi*	50	0.5-0.75
DU	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
CH	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
K	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
CI	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
CJ	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
CK	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
DV	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	prior SCT	QIAamp DNA blood midi*	50	0.5-0.75
CL	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
CM	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
CN	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
CO	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
CP	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 41	QIAamp DNA blood midi*	50	0.5-0.75
CQ	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
CR	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75

case	type of sample	DNA preparation kit	DNA concentration [ng/µl]	DNA amount as template for PCR [ng
CS	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
CT	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
CU	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior 1st HR	QIAamp DNA blood midi*	50	0.5-0.75
CV	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
CW	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
CX	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
L	PB day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
CY	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
CZ	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
DA	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 52	QIAamp DNA blood midi*	50	0.5-0.75
N	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM prior Prot. M	QIAamp DNA blood midi*	50	0.5-0.75
DW	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75
DB	BM day 0	QIAamp DNA blood midi*	50	0.5-0.75
	BM day 33	QIAamp DNA blood midi*	50	0.5-0.75

* All DNA preparations using the QIAamp DNA blood midi kit were performed by the staff of the ALL-BFM Study center in Hannover/Kiel. Forty microliter aliquots with 50 ng DNA/µl were provided by the ALL-BFM study center.

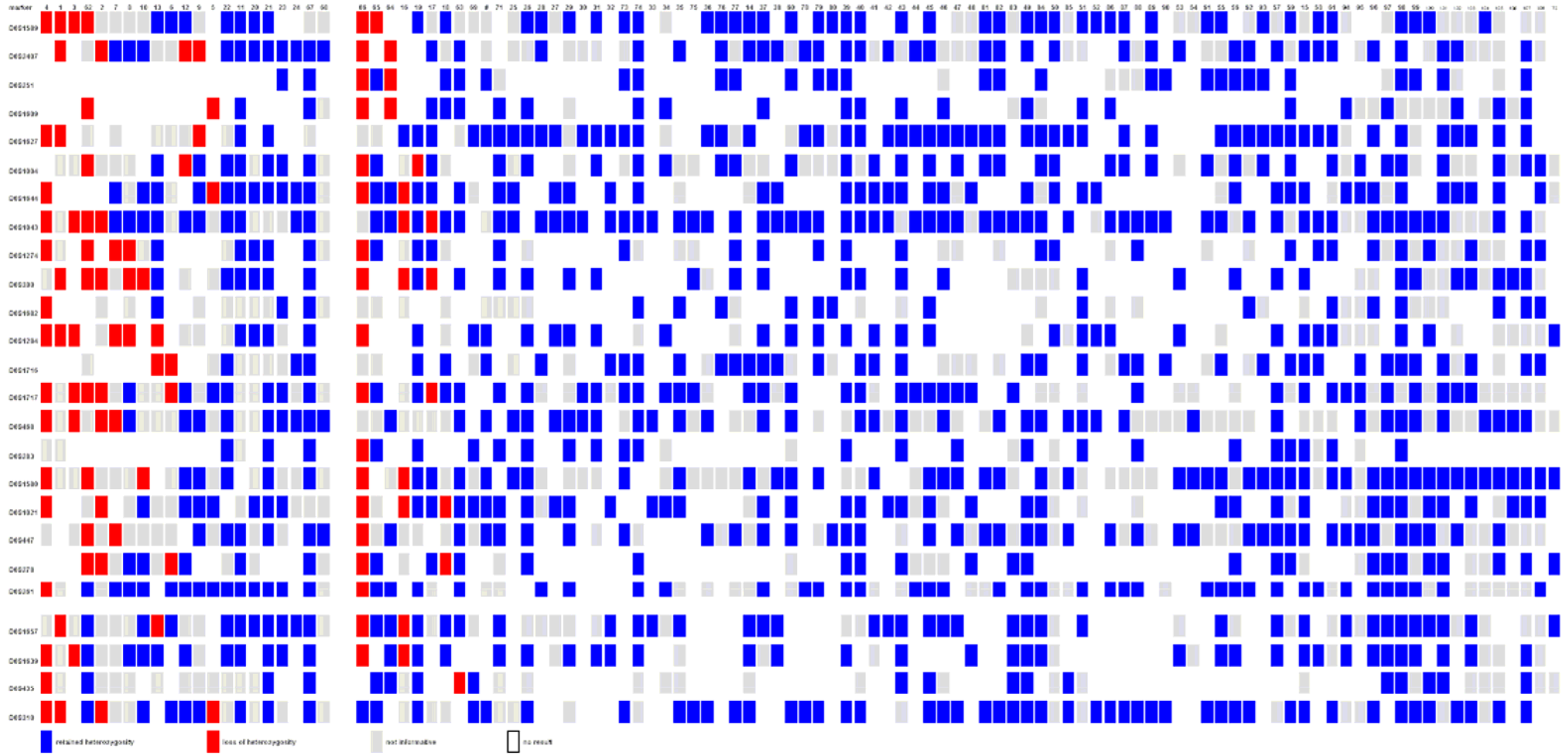
8.5 Results of LOH analyses in T-LBL patients

Each column represents the results for one individual patient; each line represents the results for one particular marker. The markers are shown in centromere to telomere direction as indicated in Figure IX.



8.6 Results of LOH analyses in T-ALL patients

Each column represents the results for one individual patient; each line represents the results for one particular marker. The markers are shown in centromere to telomere direction as indicated in Figure IX.



8.7 Acknowledgment

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8.8 Curriculum vitae

Surname	Burkhardt born Pusill-Wachtsmuth
First name	Birgit
Date of birth	July 21, 1975
Place of birth	Stuttgart/Germany
Home address	Riegelpfad 50 35392 Gießen, Germany
Office address	Justus Liebig University Giessen, Dept. of Pediatric Hematology and Oncology, Feulgenstr. 12, 35385 Giessen, Germany

Academic education

11/1997 until 12/2002	Research project and M.D. thesis in emergency medicine, Doctor of Medicine 12/02 (summa cum laude): "Analyse der integrierten Leitstellen in Meckenburg-Vorpommern und Vorschläge zur Umstrukturierung mit dem Ziel einer verbesserten Effizienz"
04/2002	Medical state exam
10/1995-04/2002	Studies of Medicine at • Martin-Luther Universität Halle/Wittenberg (10/95-07/97) • Ernst-Mortitz-Arndt-Universität Greifswald (10/97-03/01) • Technische Universität München (04/01-03/02)
09/1986-05/1995	Grammar school, graduate with Abitur
09/1982-07/1986	Primary school

Professional experience and research

since 07/2002	Physician at the Children's Hospital, University Giessen Department of Pediatric Hematology/Oncology
since 2002	Experimental research (PhD thesis): Chromosome 6q deletion in precursor T-cell lymphoblastic lymphoma and leukemia of childhood and adolescence
since 07/2002	Clinical research: Co-worker of the NHL-BFM study center, coordination of multicenter international clinical trials for the treatment of children and adolescents with Non-Hodgkin Lymphoma (EURO-LB 02, B-NHL BFM Rituximab, B-NHL BFM 04, ALCL 99, ALCL-Relapse)

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8.9 Declaration

I declare that I have completed this dissertation single-handedly without unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced text passages that are derived literally from or are based on the content published or unpublished work of others, and all information that relates verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.