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## Genetic and epigenetic characterization of pediatric high hyperdiploid acute lymphoblastic leukemia

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**GENETIC AND EPIGENETIC  
CHARACTERIZATION OF  
PEDIATRIC HIGH HYPERDIPLOID  
ACUTE LYMPHOBLASTIC LEUKEMIA**

**Akademisk Avhandling**

av

**JOSEF DAVIDSSON**

**Blekingska Nationen**

som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds universitet  
för avläggande av doktorsexamen i medicinsk vetenskap kommer att  
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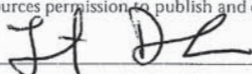
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Title and subtitle Genetic and epigenetic characterization of pediatric high hyperdiploid acute lymphoblastic leukemia			
Abstract <p>The aim of this thesis was to analyze the genetic and epigenetic characteristics of pediatric high hyperdiploid acute lymphoblastic leukemia (HeH ALL), the most common type of childhood malignancy. The three original articles presented in this thesis have addressed three major questions regarding HeH ALL: what are the genetic characteristics of the most common structural abnormality – dup(1q)? Are aberrant methylation patterns involved in leukemogenesis? How do diagnostic and relapse samples relate to each other genetically? In article I we found that the proximal breakpoints of dup(1q)-positive Burkitt lymphomas (BL) and pediatric HeH ALLs cluster close to the centromere, suggesting this region to be breakprone, and that five genes on 1q were significantly overexpressed and thus potentially pathogenetically important. In addition, the satellite II domain was not hypomethylated in either disorder, ruling out that decondensation of pericentric heterochromatin, due to hypomethylation of satellite II sequences, is the mechanism behind dup(1q) in BL and pediatric HeH ALL. In article II unsupervised cluster and principal component analyses of BAC array chromosome-wide methylome profiles accurately subgrouped the t(12;21) and HeH ALL subtypes, demonstrating that they are characterized by subtype specific methylomes. Analysis of promoter-specific CpG islands demonstrated that several B-cell- and neoplasia-associated genes were hypermethylated and underexpressed. Methylation hotspots were associated with chromosome bands predicted to harbor imprinted genes, suggesting that regions rich in imprinted genes could be associated with aberrant methylation in t(12;21)-positive and HeH ALLs. Moreover, we demonstrated that the tri-/tetrasomic chromosomes in HeH ALLs were less methylated than their disomic counterparts. Decreased methylation of gained chromosomes is a previously unknown phenomenon that may have ramifications not only for the pathogenesis of HeH ALL but also for other disorders with acquired or constitutional numerical chromosome anomalies. In article III we concluded that structural changes were significantly more common in relapse HeH samples than in diagnostic ones and that no single, recurrent aberration could be directly linked to relapse. In addition, the triple trisomies +4, +10, and +17, associated with low-risk ALL, were detected in two-thirds of cases that subsequently relapsed, thus questioning the favorable impact of these trisomies. The genetic differences between diagnostic and relapse samples suggest the presence of an ancestral HeH clone in the vast majority of patients, with structural changes and mutations being secondary to the HeH pattern.</p>			
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**GENETIC AND EPIGENETIC  
CHARACTERIZATION OF  
PEDIATRIC HIGH HYPERDIPLOID  
ACUTE LYMPHOBLASTIC LEUKEMIA**

**JOSEF DAVIDSSON**



**DEPARTMENT OF CLINICAL GENETICS**

**FACULTY OF MEDICINE**

**LUND UNIVERSITY**

**2009**

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This thesis is dedicated to my mother and father,  
for always believing in me.



What is it that we human beings ultimately  
depend on? We depend on our words. We are  
suspended in language. Our task is to  
communicate experience and ideas to others.

*Niels Bohr*

© Josef Davidsson

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## ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
BAC	bacterial artificial chromosome
BASE	bioarray software environment
BL	Burkitt lymphoma
CGH	comparative genomic hybridization
CML	chronic myeloid leukemia
COG	Children's Oncology Group
FISH	fluorescent <i>in situ</i> hybridization
GWA	genome-wide association
HeH	high hyperdiploid
MeDIP	methylated DNA immunoprecipitation
NOPHO	Nordic Society of Paediatric Haematology and Oncology
PCR	polymerase chain reaction
Ph	Philadelphia chromosome
RT	reverse transcriptase
SNP	single nucleotide polymorphism
UPD	uniparental isodisomy
WBC	white blood cell





## ORIGINAL ARTICLES

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals as listed below.

I. Davidsson J, Andersson A, Paulsson K, Heidenblad M, Isaksson M, Borg Å, Heldrup J, Behrendtz M, Panagopoulos I, Fioretos T and Johansson B (2007). Tiling resolution array comparative genomic hybridization, expression and methylation analyses of dup(1q) in Burkitt lymphomas and pediatric high hyperdiploid acute lymphoblastic leukemias reveal clustered near-centromeric breakpoints and overexpression of genes in 1q22-32.3. *Hum Mol Genet* **16**: 2215-2225.

II. Davidsson J, Lilljebjörn H, Andersson A, Veerla S, Heldrup J, Behrendtz M, Fioretos T and Johansson B (2009). The DNA methylome of pediatric acute lymphoblastic leukemia. *Hum Mol Genet* **18**: 4054-4065.

III. Davidsson J, Paulsson K, Lindgren D, Lilljebjörn H, Chaplin T, Forestier E, Andersen MK, Nordgren A, Rosenquist R, Fioretos T, Young BD and Johansson B (2009). Relapsed childhood high hyperdiploid acute lymphoblastic leukemia: presence of preleukemic ancestral clones and the secondary nature of microdeletions and RTK-RAS mutations. *Submitted*.

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## PREFACE

The treatment of childhood acute lymphoblastic leukemia (ALL) is one of the true success stories of modern medicine. Before 1950, childhood leukemia was uniformly fatal, usually within a period of 3 months. Death from hemorrhages and severe infections was routine and the only treatment available, blood transfusions, offered little help. However, during the 1950's, treatment radically changed with the introduction of drugs like methotrexate, 6-mercaptopurine and cortisone; chemotherapy had been born. Since 1975, the refinements of existing protocols have resulted in a survival rate of nearly 80%. Although this is remarkable, many problems linger. Except for Imatinib, used to treat Philadelphia chromosome (Ph) positive ALL, no new major therapeutic breakthrough has been made during the last 30 years and therapy still remains complex, expensive and very toxic to the patient. In addition, the disease etiology is unknown and there is thus no way of preventing ALL.

The topic of this thesis is the most common form of pediatric ALL, namely high hyperdiploid (HeH) ALL, a malignancy with a favorable outcome, but unknown pathogenesis. The thesis is divided in four sections; the first introducing hematologic malignancies. In the second, section a review of pediatric HeH ALL is given. The third comprises the aims of the thesis, the material and methods used, results and a discussion, as well as the conclusions drawn. The final section includes the articles upon which the thesis is based.

*Lund, October 2009*



## INTRODUCTION

### **Hematologic malignancies**

Hematologic malignancies comprise all the clinically, morphologically and immunophenotypically heterogeneous neoplastic disorders of the bone marrow, involving both the myeloid and lymphoid lineages of the hematopoietic system. Acute proliferative disorders, known as either acute myeloid leukemia (AML) or ALL depending on their lineage, are characterized by an accumulation of malignant immature white blood cells (blasts) in the bone marrow and, in most cases, also the peripheral blood. This causes dysfunction of the normal hematopoiesis resulting in anemia, leukocytopenia and thrombocytopenia and leading to infection and excessive bleeding in the patient (Jandl, 1996).

Leukemia is a malignant disorder with a clonal origin from one single hematopoietic progenitor cell that has acquired somatic genetic changes causing neoplastic transformation. At present, the diagnosis and prognosis of the acute leukemias are based on the white blood cell (WBC) count, age of the patient, immunophenotype and morphology of the blasts and the presence of specific genetic abnormalities in the neoplastic cells (Pui and Evans, 2006). According to the Association of the Nordic Cancer Registries approximately 1000 cases of acute leukemia occur in the Nordic countries each year (<http://www.ancr.nu>).

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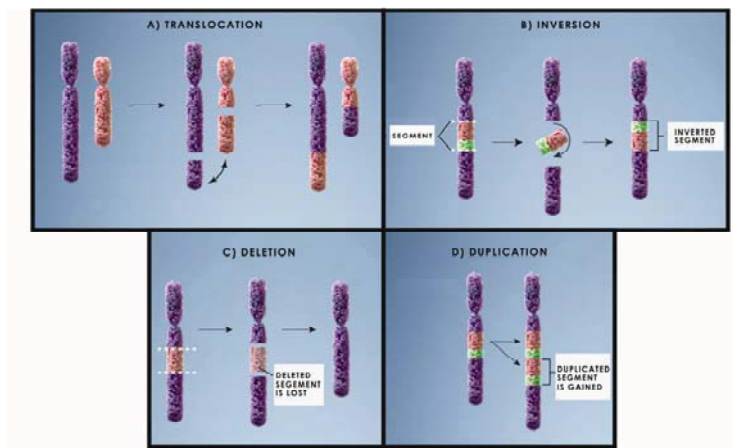
AML predominates in adults, whereas ALL primarily is a pediatric disease (Jandl, 1996). With a characteristic age peak around 2-5 years (Pui *et al.*, 2008) and an incidence of 5 cases per 100 000 and year in the Nordic countries (Hjalgrim *et al.*, 2003a), ALL constitutes the most common childhood malignancy. Therefore, both when investigating pathogenesis as a scientist and when managing the leukemia in the clinic as a practitioner, ALL primarily pose a pediatric challenge. Moreover, this special setting as a childhood disease is important to keep in mind also when investigating the genetic and epigenetic origin of ALL, which is the topic of the present thesis.

### **Genetic aberrations in ALL**

Acquired genetic aberrations have, during the last decades, been catalogued and associated with so many tumor types that they now count by the thousands (Mitelman *et al.*, 2009). In some sense, it could be argued that “the riddle of cancer” to a large extent is solved. The evidence that neoplasia essentially is a genetic disease caused by a dysregulation of cellular systems due to somatic mutations in one or many founder cells is now overwhelming (Hanahan and Weinberg, 2000). This knowledge has had a major impact on the clinical management of patients with ALL and has played a significant role in helping us understand the biology underlying leukemogenesis.

Historically, and also at present, the most characteristic cytogenetic feature of hematologic malignancies has been balanced chromosome translocations (**Figure 1A**) (Rowley, 2008). Two main functional consequences are associated with chromosome translocations. First, a balanced rearrangement can place a gene’s coding sequences under the transcriptional control of a regulatory element in close vicinity of the other

breakpoint, causing overexpression of the sequence from the first mentioned gene. Second, a translocation may result in a fusion of two different genes, generating chimeric transcripts with novel functions in the cell (Rabbitts and Stocks, 2003). Other cytogenetic aberrations commonly found in hematologic malignancies are inversions, deletions and duplications (**Figure 1B-D**). Inversions are associated with the same functional outcome as translocations, whereas duplications and deletions traditionally are regarded as imbalances causing gene copy-number gain or loss on a whole chromosome- or individual segment level. Moreover, in many different hematologic malignancies somatic point mutations have been described for a wide variety of genes, causing both gene up-regulation by activating mutations or haploinsufficiency by loss-of-function mutations (Bamford *et al.*, 2004).



**Figure 1. Common chromosome abnormalities in neoplastic disorders.**

A) A translocation is a reciprocal exchange of genetic material between two chromosomes. B) An inversion is the reversal of position of a chromosomal segment. C) A deletion is when a part of the chromosome is lost, resulting in copy-number loss. D) A duplication is when a part of the chromosome is repeated, resulting in copy-number gain.

---

Cytogenetic groups may also be classified according to ploidy, based on the number of chromosomes the tumor cell population harbors (Shaffer *et al.*, 2009). In ALL, they comprise diploidy (46 chromosomes), HeH (51-67 chromosomes), near-haploidy (25-29 chromosomes), low hypodiploidy (31-39 chromosomes), near-triploidy (66-79 chromosomes) and near-tetraploidy (84-100 chromosomes) (Harrison and Johansson, 2009). HeH is by far the most common aberrant group in ALL; in fact, it is the overall most common cytogenetic group, also when including subgroups defined by specific structural aberrations. The other aneuploidy groups are rather rare entities in ALL when making the same comparison (Johansson *et al.*, 2004). The functional outcome of these massive chromosomal imbalances has long been debated, something that I will return to in this thesis.

The chromosome morphology is known to be notoriously poor in ALL. In spite of this, structural chromosome abnormalities are detected in approximately 50% of all cases (Harrison and Foroni, 2002), a number that is likely to rise with the entry of novel high resolution techniques into research and clinics. The discovery of recurring chromosomal abnormalities associated with patient outcome has both revealed key genes involved in leukemogenesis as well as enabled cytogenetic subclassification of ALL. Today, such cytogenetic classification contributes to risk stratification of the patients into different treatment regimens (Pui and Evans, 2006; Pui *et al.*, 2008). The most common cytogenetic subgroups in ALL are shortly reviewed below (HeH will be discussed separately):

The t(12;21)(p13;q22) translocation generating the *ETV6/RUNX1* fusion occurs in about 25% of all pediatric ALLs and the resulting protein seems to lead to hampered early lymphocyte development (Pui *et al.*, 2008).

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This aberration is not visible by conventional cytogenetics and hence have to be detected by fluorescent *in situ* hybridization (FISH) or reverse transcriptase (RT) polymerase chain reaction (PCR) (Romana *et al.*, 1994; Golub *et al.*, 1995). The outlook for patients harboring this translocation has been considered favorable; however, some studies indicate that this abnormality is associated with late relapses (Harbott *et al.*, 1997; Forestier *et al.*, 2008a). Secondary aberrations, such as deletions of 6q21-27, 8p11-23, 9p13-24, 11q23-25, 12p11-13, 13q14-34 and the whole X chromosome and gains of chromosomes 10, 16 and 21 are common in t(12;21)-positive ALL but do not seem to affect prognosis (Forestier *et al.*, 2007).

t(1;19)(q23;p13) [*TCF3/PBX1*] occurs in a balanced form as well as an unbalanced der(19)t(1;19). This translocation is found in approximately 5% of all B cell precursor ALLs and the resulting protein product leads to cell differentiation arrest (Hunger, 1996). For pediatric patients this aberration is risk stratifying in some parts of the world (Kager *et al.*, 2007). In 60% of the cases secondary aberrations are found, most often +21, +8, +4, i(7)(q10) and i(9)(q10), but they do not seem to affect prognosis (Pui *et al.*, 1994). The der(19)t(1;19) may be associated with slightly better prognosis than the t(1;19); however, various studies have reported conflicting results (Uckun *et al.*, 1998; Schultz *et al.*, 2007).

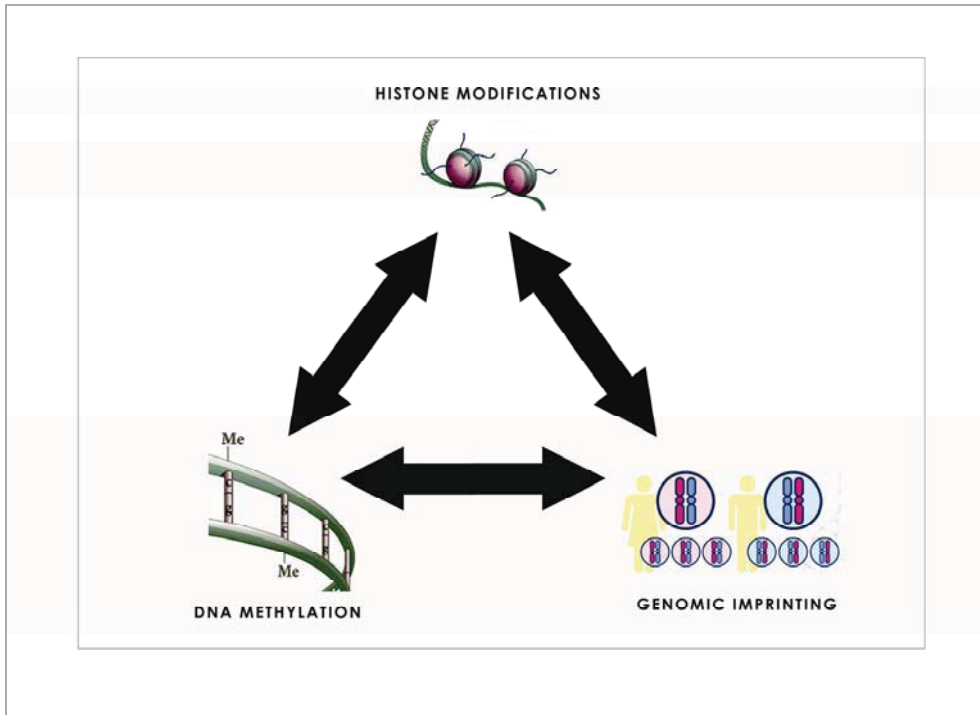
The *MLL* gene at 11q23 is highly promiscuous, having several different translocation partners. Aberrations involving *MLL* occur in the majority of children  $\leq 1$  year of age affected by ALL and constitute approximately 8% of all pediatric ALL and about 10% of all older ALL patients (Pui *et al.*, 2004). The two most common gene fusions involving *MLL* in ALL are *MLL/AFF1* [t(4;11)(q21;q23)] and *MLL/MLLT1* [t(11;19)(q23;p13)] both associated with

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an unfavorable prognosis (Pui *et al.*, 2008). Prognostically insignificant secondary aberrations are found in 25% of the t(4;11) cases (often +X, i(7)(q10) and +8) and in 35% of the t(11;19) cases (often +X and +8) (Moorman *et al.*, 2005).

The t(9;22)(q34;q11) [*BCR/ABL1*] resulting in the Ph characteristic for chronic myeloid leukemia (CML) is found in 2-3% of pediatric ALLs and in about 25% of adult ALLs (Pui *et al.*, 2004). The aberration is associated with very poor prognosis in both children and adults, with allogeneic stem cell transplantation being the only curative treatment (Pui and Evans, 2006). Secondary aberrations are found in 50% of the cases, commonly +der(22)t(9;22), -7, +21 and +8. Monosomy of chromosome 7 is associated with an even worse prognosis (Heerema *et al.*, 2004; Li *et al.*, 2009). Two common *BCR/ABL1* transcripts have been described: P190 and P210. The P210 transcript is a hallmark of CML, whereas the P190 is primarily (but not exclusively) associated with ALL (Clark *et al.*, 1987). The P190 transcript is found in nearly 90% of children diagnosed with Ph-positive ALLs (Suryanarayan *et al.*, 1991) whereas adult Ph-positive ALL cases can present with either a P190 or a P210 oncoprotein, or both (Klco *et al.*, 2008).





**Figure 2. Epigenetic mechanisms.** Histone modifications, genomic imprinting and DNA methylation constitute different branches of the field of cancer epigenetics. But in reality these are in fact closely intertwined and simultaneous processes, together interacting and contributing to the regulation of gene transcription.

### Epigenetic changes in ALL

The term epigenetics means heritable alterations in gene expression not due to a physical change in the DNA sequence (Bird, 2007). Today, three main types of, closely interacting, epigenetic levels are recognized: histone modifications, genomic imprinting and DNA methylation (**Figure 2**). During the last two decades, epigenetic alterations have gained recognition as important contributors to tumorigenesis and neoplastic progression (Feinberg and

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Tycko, 2004). This may be particularly relevant for ALL, since some translocations arise in utero, but without cooperative secondary genetic or epigenetic events they fail to produce overt leukemia (Greaves, 2005). In addition, since epigenetic events are reversible, they constitute interesting targets for therapeutic intervention, not least in hematologic malignancies (Shaker *et al.*, 2003; Fiskus *et al.*, 2009; Issa and Kantarjian, 2009).

The fact that histone modifications affect transcription has been known for long; however, this epigenetic event was in fact the last one to be linked to cancer. To date, several histone modifications have been described, such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ribosylation, deamination and proline isomerization. These modifications mainly have two functional outcomes, either they regulate transcription by inducing looser or more tightly packaged chromatin or they serve as docking sites for other chromatin-remodeling protein complexes. A growing number of studies is now emerging, focusing on elucidating the chromatin maps and histone modifications specific for different type of hematologic malignancies (Neff and Armstrong, 2009).

Imprinting, which refers to the selective methylation of maternal and paternal alleles during gametogenesis in such a way that a specific parental allele is exclusively expressed in the embryo (Surani *et al.*, 1984), has also been linked to human neoplasia. Loss of imprinting, leading to aberrant biallelic expression of genes with oncogenic potential, or selective loss of uniquely paternally or maternally expressed tumor suppressor genes has been shown in a number of human neoplasias (Feinberg and Tycko, 2004). In addition, it has been reported that the frequent deletions of chromosome arm 9p seen in ALL leads to preferential loss of maternal alleles, suggesting a germ-line event

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promoting leukemogenesis (Morison *et al.*, 2002); however, this has as yet not been confirmed in any subsequent study.

DNA methylation, which is the most well studied branch of epigenetics, involves methyl group addition to the cytosine ring of CpG nucleotides. Although CpG dinucleotides are under-represented in the total genome they are frequently found within the promoter region of human genes. These regions, named promoter-associated CpG islands, usually lack methylation in normal cells; however, this may vary from cell to cell type. This lack of methylation allows transcription to occur (Suzuki and Bird, 2008). Abnormal methylation patterns have been associated with neoplasia for a long time, with cancer cells displaying global hypomethylation and gene promoter-specific CpG hypermethylation. Methylation of promoters is associated with a closed chromatin structure and transcriptional silencing of the associated gene (Feinberg and Vogelstein, 1983). A large number of genes has been implicated as hypermethylated in hematologic malignancies in general (Boulwood and Wainscoat, 2007) and in ALL specifically (Garcia-Manero *et al.*, 2009). Hematopoietic progenitors generally are free from this type of gene silencing (Herman *et al.*, 1997); however, dynamic changes in promoter methylation levels in order to control growth factor and cytokine expression during myeloid development have been demonstrated (Lubbert *et al.*, 1997).



## REVIEW OF PEDIATRIC HeH ALL

The HeH subgroup was first described as an entity of its own in the early 1980's when Kaneko and coworkers (1981) reported that modal chromosome numbers ranging from 50 to 59 only occurred in pediatric ALLs and not in adult ALL. Closely thereafter, several independent studies reported the same finding and by the mid 1980's several additional studies had confirmed HeH as a cytogenetic subgroup of childhood ALL (Paulsson and Johansson, 2009). However, it should be stressed that already 1967 Fritz Lampert described that high DNA content was associated with sensitivity to chemotherapy and longer survival in childhood acute leukemias; the favorable prognosis of HeH ALL is now well recognized (see below) (Lampert, 1967).

### **Cytogenetic features**

The HeH subgroup in pediatric ALL is characterized by a nonrandom pattern of gains of chromosomes X, 4, 6, 10, 14, 17, 18 and 21, resulting in modal numbers ranging from 51-67. Chromosome 21 is frequently tetrasomic, even pentasomic in some cases, whereas the remaining commonly gained chromosomes usually are trisomic. Monosomies are rarely seen. In addition to the chromosomal gains, approximately 50% of all HeH cases carry structural chromosome changes identifiable by conventional cytogenetics (Groupe Francais de Cytogénétique Hématologique, 1993; Raimondi *et al.*, 1996;

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Forestier *et al.*, 2000a; Moorman *et al.*, 2003; Paulsson and Johansson, 2009). The most common ones are listed below:

About 5% of HeH ALL carries cytogenetically identifiable balanced abnormalities. These are mainly translocations, half of which are common ALL-associated ones, such as the previously mentioned t(1;19) and t(9;22). The other half is non-recurrent balanced rearrangements. To date, no recurring balanced translocation resulting in a novel fusion-gene specific for HeH ALL has been identified (Mitelman *et al.*, 2009).

The most frequent structural changes in HeH are unbalanced, resulting in net gain or loss of chromosomal material. Most common are partial gain of 1q, deletions involving 6q and isochromosome 7q and 17q (Pui *et al.*, 1992; Raimondi *et al.*, 1996; Moorman *et al.*, 2003; Paulsson and Johansson, 2009). Gain of 1q through duplication, isochromosome formation or unbalanced translocations is one of the most frequent acquired genetic abnormalities in neoplasia and has been reported in all major diagnostic subgroups of hematologic malignancies (Mitelman *et al.*, 2009). Despite the high prevalence very little is known about its origin, molecular genetic characteristics and functional outcome. This stimulated us to undertake the first study (*Article I*) of this thesis, a genomic and epigenetic investigation of dup(1q) in pediatric HeH ALL. This work is reviewed later in this thesis. Deletions on chromosome arm 6q occur in about 5% of all HeH cases and are usually independent of dup(1q) (Moorman *et al.*, 2003). Isochromosome 7q are present in about 1-2% of the cases, with the functional outcome being net loss of 7p and 7q gain (Pui *et al.*, 1992; Martineau *et al.*, 1996). Trisomy 17 is one of the characteristic whole chromosomal gains of HeH and 2-5% of all cases harbor i(17q) (Martineau *et al.*, 1996; Raimondi *et al.*, 1996; Moorman *et al.*,

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2003). However, gain of chromosome 17 and i(17q) are mutually exclusive indicating that the functional outcome of both aberrations could be gain of 17q (Mitelman *et al.*, 2009; Paulsson and Johansson, 2009).

With the introduction of high resolution copy-number arrays it has become evident that microdeletions are relatively frequent in pediatric HeH ALL. Recurring loss of individual genes such as *CDKN2A*, *ETV6*, *IKZF1*, *PAX5*, *RB1* and *TCF3* results from these submicroscopic aberrations (Mullighan *et al.*, 2007). Compared with other genetic ALL subgroups the overall frequency of microdeletions is lower in HeH; however, this could be explained by potential masking in single nucleotide polymorphism (SNP) arrays of submicroscopic aberrations on trisomic chromosomes by the additional homologue (Paulsson and Johansson, 2009).

## **Mutations**

The RTK-RAS signaling pathway involves, among others, four genes that have been demonstrated to be frequently (~30%) mutated in pediatric HeH ALL: *FLT3*, *KRAS*, *NRAS* and *PTPN11* (Armstrong *et al.*, 2004; Taketani *et al.*, 2004; Tartaglia *et al.*, 2004; Wiemels *et al.*, 2005; Paulsson *et al.*, 2007; Stam *et al.*, 2007). Probably due to the fact that these genes are involved in the same signaling cascade, mutations in these genes are usually mutually exclusive in HeH ALL (Paulsson *et al.*, 2007). In addition, among childhood ALLs harboring *PTPN11* mutations, HeH seem to have a significantly higher prevalence compared to other genetic subtypes, with the mutation being secondary to the HeH karyotype and more common at relapse (Molteni *et al.*, 2009).

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## Epigenetic changes

The focus of epigenetic studies in childhood ALL has been entirely on delineation of DNA methylation; to date, no studies investigating histone modifications have been published. Furthermore, the DNA methylation studies of HeH ALL have mainly focused on promoter hypermethylation of individual genes. Several tumor suppressor gene promoters have been shown to have an aberrant methylation status, with *CADM1*, *ESR1*, *FHIT*, *RARB*, and *WNT5A* being hypermethylated in more 50% of HeH cases (Paulsson *et al.*, 2009).

To date, we know of only two studies utilizing array-based methodologies to identify differential methylation of a larger number of genes in B-lineage ALL and these studies utilized low-resolution platforms (60 and 2300 genes, respectively) and included mainly adult patients (Scholz *et al.*, 2005; Taylor *et al.*, 2007). Thus, next to nothing is known about the complete DNA methylome of childhood ALL. This led us to investigate methylation on a genome-wide level investigating primary patient samples from t(12;21)-positive and HeH ALL (*Article II*). We analyzed large-scale global methylation profiles of each chromosome using bacterial artificial chromosome (BAC) arrays as well as direct gene silencing through hypermethylation by the use of a microarray platform covering all promoter-associated CpG islands. In addition, global gene expression data were used to investigate correlations between hypermethylated target genes and their expression levels. Furthermore, tiling resolution array comparative genomic hybridization (CGH) was used to detect genomic imbalances that might affect gene expression and/or be associated with large scale methylation changes. This work is reviewed in the *The Present Study* section (*Article II*).

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## Clinical features

Pediatric HeH ALL is associated with a low WBC count; the median in published data is  $<10 \times 10^9/l$  (Paulsson and Johansson, 2009). Thus, based on WBC count alone these patients are rarely stratified into other than the standard risk group of the Nordic Society of Paediatric Haematology and Oncology (NOPHO) protocol used in the Nordic countries (Schmiegelow *et al.*, 2009). The patients are often moderately anemic and thrombocytopenic at the time of diagnosis; however, a subset displays normal platelet levels.

The immunophenotype of HeH is that of a typical B-cell precursor ALL: CD10<sup>+</sup>, CD13<sup>-</sup>, CD19<sup>+</sup>, CD22<sup>+</sup>, CD24<sup>+</sup>, CD33<sup>-</sup>, CD34<sup>+</sup>, CD45<sup>-</sup>, CD65<sup>-</sup>, CD66c<sup>+</sup>, HLA-DR<sup>+</sup>, cIg<sup>-</sup>, sIg<sup>-</sup> and TdT<sup>+</sup> (Hrusák and Porwit-MacDonald, 2002). However, the use of immunophenotyping to diagnose HeH is limited, since it cannot distinguish HeH from some other B-cell precursor ALL subtypes. The bone marrow morphology, displaying small and uniformly shaped cells, are more often FAB L1 than L2, and never L3; but the morphology is not used for diagnosis of HeH due to lack of usable characteristics (Smets *et al.*, 1995). Patients with HeH ALL usually harbors a high bone marrow blast percentage (Paulsson and Johansson, 2009).

The outcome of HeH is among the most favorable within ALL, not surpassed by any other cytogenetic subgroup (Kaneko *et al.*, 1981; Pui and Evans, 2006). Initial treatment failure is very rare and subsequently complete morphological remission is reached in the great majority of cases. The event-free survival is above 80% and the overall survival currently reaches approximately 90% (Forestier *et al.*, 2008a; Pui *et al.*, 2008). It is therefore apparent that treatment reaches a second successful remission in many of the relapsed patients.



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## **Incidence**

HeH are found in about 25% of all pediatric ALLs, but in less than 10% of adult ALLs (Mitelman *et al.*, 2009). This makes it the most common ALL subgroup in children. All major studies of pediatric HeH ALL, derived from different parts of the world, have reported a prevalence around 25-30%, indicating that there is no major geographic incidence heterogeneity (Paulsson and Johansson, 2009). However, it should be noted that most studies did not include patients from developing countries. Whether the abovementioned frequency is influenced by socioeconomic factors thus remains unknown. Ethnic background could have an influence HeH ALL. It has been reported that HeH ALL is underrepresented among African-Americans, compared to other ethnic groups (Pui *et al.*, 1995; Raimondi *et al.*, 1996; Pollock *et al.*, 2000; Bhatia *et al.*, 2002). Whether this is due to genetic or environmental factors is unknown; perhaps genome-wide association (GWA) studies of this ALL subtype in different ethnic groups could shed some light on this issue.

HeH displays a pronounced and very characteristic age peak at 2-4 years of age, being very rare in infant leukemia and uncommon above the age of seven years. HeH together with t(12;21)-positive ALL comprise about 80% of all ALLs occurring in the 2-7 year age span (Forestier and Schmiegelow, 2006). The reasons for this specific age peak of the two most common forms of pediatric ALLs are discussed below, in relation to HeH leukemogenesis. The ratio of male/female incidence based on all cases of HeH ALL below the age of 18 years present in the Mitelman Database of Chromosome Aberrations in Cancer (Mitelman *et al.*, 2009) is 1.3, revealing a slight male preponderance. However larger individual series have demonstrated a sex ratio close to 1.0,

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making it uncertain whether HeH is really more common in boys (Paulsson and Johansson, 2009).

## **Etiology**

Considering that HeH is the most common subgroup in pediatric ALL our knowledge about its etiology is surprisingly poor. Looking at the spectrum of genetic risk factors, several Mendelian disorders have been associated with increased risk of developing acute leukemias; however, not specifically HeH ALL (Benson and Horwitz, 2006). Among constitutional genetic conditions, only Down syndrome has been associated with an elevated risk for developing HeH ALL (Hasle, 2001; Benson and Horwitz, 2006). This constitutional chromosome disorder is associated with an overall 20-fold higher risk for developing ALL, but the proportion of HeH ALL is lower in this patient population compared to non-Down syndrome pediatric patients with ALL. However, the total risk of developing HeH ALL is still higher with, than without, a constitutional trisomy 21 (Forestier *et al.*, 2008b).

Concerning individual genes, the immune response gene *HLA-DBP1\*0201* has been demonstrated to occur more frequently in pediatric patients with HeH ALL compared to healthy controls (Taylor *et al.*, 2002; Taylor *et al.*, 2008; Taylor *et al.*, 2009). This could indicate that it is associated with an increased risk; however, no follow-up studies from other groups have as yet confirmed these results. In two recent independent GWA studies, three SNPs in the gene *ARID5B* on 10q21.2 were associated with increased risk for HeH ALL compared to healthy controls (Papaemmanuil *et al.*, 2009; Treviño *et al.*, 2009). Two of these SNP were demonstrated to be linked with methotrexate polyglutamate accumulation in bone marrow (Treviño *et al.*,

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2009), a clinical feature clearly linked to HeH ALL (Synold *et al.*, 1994; Kaspers *et al.*, 1995; Zhang *et al.*, 1998; Belkov *et al.*, 1999; Kager *et al.*, 2005; Whitehead *et al.*, 2005). These germline variations in the *ARID5B* locus might affect the gene's function in B-lineage development and create a susceptibility to HeH ALL, but the mechanisms remain unknown. As regard genetic risk factors, it should however be noted that studies of siblings to HeH patients have shown that they do not have an increased risk of developing ALL, indicating that constitutional genetics has a limited impact on the etiology of pediatric HeH ALL (Schmiegelow and Hjalgrim, 2006).

Little is known about environmental risk factors for HeH ALL. It has been reported that pediatric HeH ALL is less common in children exposed to paternal smoking, something that the authors speculated could be due to that hyperdiploid cells might be sensitive to toxic smoking-associated metabolites (Wiemels *et al.*, 2005). Elevated birth weight and low maternal folate consumption during pregnancy have also been associated with increased general risk of pediatric ALL (Thompson *et al.*, 2001; Hjalgrim *et al.*, 2003b). That birth weight is as a risk factor for HeH ALL has been shown (Hjalgrim *et al.*, 2004), whereas the risk-association with low folate intake did not pinpoint specific genetic subgroups. Interestingly, folate deficiency has been shown to induce aneuploidy of chromosomes 17 and 21 in cultured lymphocytes, suggesting a link between hyperdiploidy and folic acid (Wang *et al.*, 2004). In addition, specific polymorphisms of the *MTHFR* gene, involved in folate metabolism, have been demonstrated to be underrepresented in HeH ALL (Wiemels *et al.*, 2001), further supporting the involvement of folic acid in pediatric HeH ALL etiology.

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## Origin of HeH

How does a cell become HeH? To answer this biologically, and perhaps clinically, important question, four different mechanisms have been suggested: (1) development of a near-haploid cell followed by chromosome-doubling, (2) tetraploidization of the cell followed by chromosome losses, (3) sequential gain of individual chromosomes through several consecutive independent cell divisions and (4) simultaneous gain of chromosomes in one single abnormal mitosis (Onodera *et al.*, 1992). By demonstrating a 2:2 allelic ratio and specific tetraploid pattern of whole chromosome uniparental isodisomies (UPDs), or no pattern of UPDs at all, in virtually all cases investigated, HeH seems to arise by the simultaneous gain mechanism in about 70% of the cases and by the tetraploid pathway in the remaining 30% of the cases (Paulsson *et al.*, 2003; Paulsson *et al.*, 2005; Paulsson and Johansson, 2009).

The accepted theory that hematologic malignancies are initiated and maintained by leukemic stem cells have been supported by the detection of gene fusions, such as [*ETV6/RUNX1*] caused by the t(12;21), in specific precursor lineages (Castor *et al.*, 2005; Hong *et al.*, 2008; Bernt and Armstrong, 2009). However, few studies have addressed this issue in HeH ALL and the results have also been ambiguous. Studies by Larramendy *et al.* (1995) and Quijano *et al.* (1997) concluded that pediatric HeH ALL might well be a disease originating from a pluripotent stem cell since hyperdiploidy were found in CD34<sup>+</sup>CD33<sup>-</sup>CD38<sup>-</sup>CD19<sup>-</sup> cells deemed to belong to primitive compartments of the bone marrow, whereas Kasprzyk *et al.* (1999) detected trisomies only in committed B cell progenitors; however, they could not exclude a stem cell involvement. Le Viseur *et al.* (2008) could by engraftment of flow sorted blast derived from two HeH ALL patients demonstrate that at

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least some cases of HeH have leukemia-initiating activity in populations consistent with later mid-stage development. So, in conclusion, this issue still remains unresolved.

### **Leukemogenesis**

HeH is very likely of utmost importance for leukemogenesis; however, there is ample evidence that it in itself it is not sufficient for leukemic transformation. The strongest support in favor of this has come from studies of neonatal blood spots identifying preleukemic cells carrying clonotypic *IGH@* rearrangements identical to those later detected in the neoplastic HeH cells (Yagi *et al.*, 2000; Panzer-Grümayer *et al.*, 2002; Taub *et al.*, 2002; Maia *et al.*, 2004). In addition, HeH cells with clonotypic *IGH@* rearrangements have been detected in a pair of monozygotic twins with concordant pediatric HeH ALL, where it most likely reflects blood cell chimerism resulting from shared placental vasculature, *i.e.*, the leukemia starts in one twin in utero and spreads to the other via the placenta (Maia *et al.*, 2003). Thus, it could be argued that hyperdiploidy most likely occurs prenatally but secondary events are needed for overt disease. This is further strengthened by the typical age peak of diagnosis around 2-4 years of age shared with the t(12;21) leukemia, where identical *ETV6/RUNX1* genomic fusions have been demonstrated to occur both in preleukemic cells derived from neonatal blood spots and in later diagnostic samples (Greaves, 2005). The latency of disease both for t(12;21)-positive and HeH ALL could, if it is of prenatal origin, reflect the time span needed to acquire additional aberrations necessary for overt disease. Indeed, accompanying genetic lesions are found in a majority of HeH cases (Mitelman *et al.*, 2009). However, the temporal order of these additional aberrations

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remains to be ascertained since it has not yet been clearly demonstrated that they indeed occur after the HeH pattern.

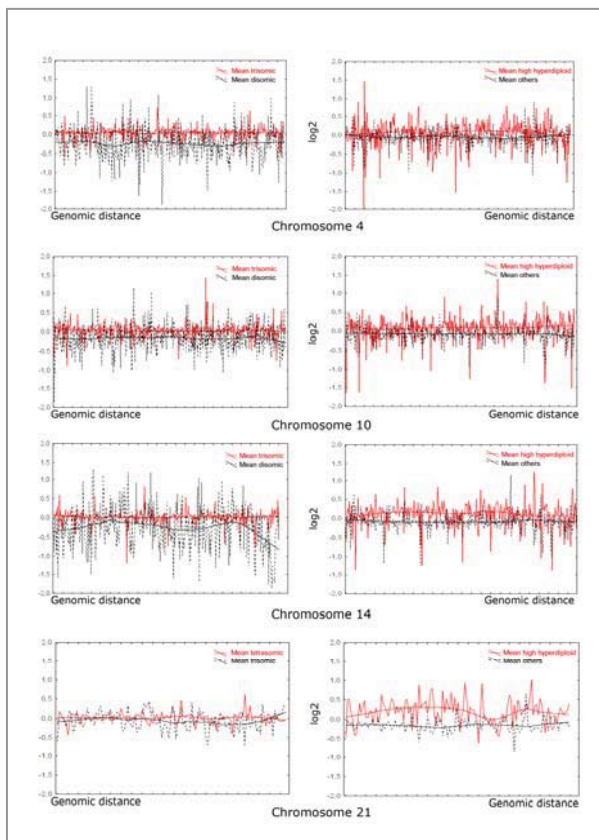
### **Gene dosage**

What is the functional outcome of HeH? Since tetrasomies have been demonstrated to display equal duplication of parental homologues (2:2 allelic ratio) in the majority of cases, neither imprinting defects nor selective duplication of mutated genes seems to be a valid explanation for the pathogenetic mechanism associated with HeH and neoplastic transformation (Paulsson *et al.*, 2003; Paulsson *et al.*, 2005; Paulsson and Johansson, 2009). Expression dysregulation due to gene dosage effects stemming from the massive but nonrandom aneuploidy is a more attractive hypothesis. Indeed, it has been demonstrated that pediatric HeH ALL has an expression signature that is distinctly separate from other pediatric ALL subtypes and that the great majority of genes on the gained chromosomes in HeH display upregulation compared to other ALL subgroups without tri- or tetrasomies (Andersson *et al.*, 2005). In addition, we recently demonstrated global overexpression of genes on the additional chromosomes also when comparing disomic and trisomic/tetrasomic chromosomes within the HeH group itself (*Article II*) (**Figure 3**).

### **Relapse**

Despite the generally very good prognosis of pediatric HeH ALL, approximately 20% of the cases relapse and about 10% of all patients succumb to the disease. The reasons for relapse remain unknown. One possibility could be that the genetic features at the time of diagnosis differ

between cases that subsequently relapse and those who remain in long-term remission. In this respect it has been suggested that HeH with structural genetic changes, in particular i(17q), are associated with a poorer outcome (Pui *et al.*, 1988; Pui *et al.*, 1989); however, this has not been confirmed in later studies (Raimondi *et al.*, 1996; Forestier *et al.*, 2000b; Moorman *et al.*, 2003). Furthermore, some investigators have reported a superior outcome for cases harboring certain trisomies, that is +4, +10, +17 and +18, findings, at least as regards +4, +10 and +17, that are now used for risk stratification by the Children's Oncology Group (COG) (Schultz *et al.*, 2007).



**Figure 3. Expression patterns of HeH ALL.** Lowess curves of mean-centered expression ratios of disomies, trisomies and tetrasomies for chromosomes 4, 10, 14 and 21 among the HeH cases (left) and between HeH cases and all other pediatric B-lineage ALLs (right) (*Article II*).

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Whether the patterns of micro-deletions or mutated genes that recently have been detected in pediatric HeH ALL (Paulsson *et al.*, 2006; Kuiper *et al.*, 2007; Mullighan *et al.*, 2007; Paulsson *et al.*, 2008) have a prognostic impact is currently unknown. One approach to identify changes possibly associated with relapse is to investigate paired diagnostic and relapsed samples. This has to date only been performed in two SNP array studies of a total of eight cases of HeH ALL (Mullighan *et al.*, 2008; Yang *et al.*, 2008). These studies report contradictory findings as regards genetic heterogeneity/homogeneity between pediatric HeH ALL at diagnosis and relapse: the two patients analyzed by Yang *et al.* (2008) displayed identical numbers of aberrations at diagnosis and relapse, whereas Mullighan *et al.* (2008) observed differences in copy number alterations, involving both whole chromosomes and/or micro-aberrations, in diagnostic and relapse samples in five of the six cases analyzed. Clearly further studies are needed to clarify this important issue. To address this, we performed high resolution SNP array and mutation analyses of 11 paired samples from pediatric HeH ALL (*Article III*).





## THE PRESENT STUDY

This section includes the aims of thesis, a brief description of the material and methods used, as well as summary of the main results of each individual article. At the end, a general discussion is given together with the major conclusions of this thesis, and finally some remarks on future directions. For a more thorough description of the individual studies, the reader is referred to the original articles (*Articles I-III*).

### **Aims**

The general aim of this thesis has been to broadly characterize pediatric HeH ALL using a wide spectrum of genomic and epigenomic techniques in order to better understand the pathogenetic mechanisms underlying leukemogenesis, disease progression and potential relapse of this leukemic subtype. More specifically the aims were:

- to perform a genomic and epigenetic investigation of the most common structural chromosome change in pediatric HeH ALL and Burkitt lymphoma (BL), namely dup(1q) (*Article I*).
- to perform genome-wide methylation profiling coupled to global gene expression and copy-number arrays on pediatric HeH ALL, using t(12;21)-positive ALL as a comparison, in order to investigate if

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aberrant methylation patterns play a significant leukemogenic role (*Article II*).

- to identify genetic changes associated with relapse and to gain information about the temporal order of genetic abnormalities and clonality by investigating paired diagnostic and relapse samples from pediatric HeH ALL patients, using a genome-wide screening method as well as mutation analyses of RTK-RAS genes (*Article III*).

## **Patients and samples**

### *Article I*

This study comprised six and four cases of dup(1q)-positive HeH ALL and BL, respectively. All HeH ALL patients had been diagnosed and treated according to the NOPHO-ALL 1992 or 2000 protocols at the Departments of Pediatric Oncology and Hematology, Lund University Hospital, Lund, and Linköping University Hospital, Linköping, Sweden. Bone marrow and/or peripheral blood were collected at diagnosis. The breakpoints of the dup(1q) were investigated in all cases. In addition satellite II methylation was investigated in seven of the ten cases, as well as in four and three non-dup(1q) HeH ALLs and BLs, respectively, using normal peripheral blood and ICF B-lymphocytes as controls in each assay. Finally, gene expression data from three of the ten cases were retrieved from a previously reported dataset (Andersson *et al.*, 2005)

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### *Article II*

Samples from 20 children with B-cell precursor ALL, comprising ten with HeH and ten with t(12;21)(p13;q22), were included in the study. The patients had been diagnosed and treated according to the NOPHO-ALL 1992 or 2000 protocols at the Departments of Pediatric Oncology and Hematology, Lund University Hospital, Lund, and Linköping University Hospital, Linköping, Sweden. Bone marrow (n=19) and peripheral blood (n=1) samples were collected at diagnosis and the samples were cytogenetically characterized by conventional chromosome banding analysis. The presence of the cytogenetically cryptic [*ETV6/RUNX1*] was identified by RT-PCR and verified by FISH, as part of routine diagnostic analyses. The additional chromosomes identified by G-banding in the HeH cases were all confirmed by array CGH.

### *Article III*

Diagnostic and relapse samples from 11 children with pediatric HeH ALL were included. The patients had been diagnosed and treated according to the NOPHO-ALL 1992 or 2000 protocols at the Departments of Pediatric Oncology and Hematology, Lund University Hospital, Lund; Karolinska Institute, Stockholm; Linköping University Hospital, Linköping; Umeå University Hospital, Umeå; Uppsala University Hospital, Uppsala, Sweden; and Rigshospitalet, Copenhagen, Denmark. Cytogenetics of bone marrow samples obtained at diagnosis and relapse were performed using conventional methods. None of the cases had the well-known ALL-associated translocations t(1;19)(q23;p13), t(9;22)(q34;q11), 11q23/*MLL* rearrangements or t(12;21)(p13;q22).

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## Methods

### *Article I*

The proximal as well as the distal breakpoints of the dup(1q) were analyzed using tiling resolution array CGH, which is a method where test and reference DNA are differentially labeled with fluorochromes and subsequently hybridized to slides containing defined genomic sequences. This enables detection of genomic copy-number loss or gain (Pinkel and Albertson, 2005). The 32 k slides used in our study contained 32 433 tiling BAC clones, covering at least 98% of the human genome, and were produced at the SCIBLU DNA microarray resource center at Lund University, Sweden. Labeling of DNA, slide preparation and hybridization were performed as described previously (Jönsson *et al.*, 2007). Initial analyses of the microarray images were performed using the GenePix Pro 4.0 software (Axon Instruments, Foster City, CA). All additional analyses were performed in the Bioarray software environment (BASE) database (Saal *et al.*, 2002) and data normalization was performed for each array subgrid using a lowess curve fitting (Yang *et al.*, 2002). Classification as gain or loss was based on identification as such by the BASE CGH plotter and also by visual inspection of the log<sub>2</sub> ratios.

Satellite II domain methylation status was investigated by Southern blot analysis as previously described (Wong *et al.*, 2001). In short, test DNA was digested using a CpG methyl sensitive restriction enzyme and then blotted to a membrane where it was probed with a radiolabeled oligonucleotide. The amount of fractionated DNA reflects the methylation status of the region investigated (Wong *et al.*, 2001). DNA samples were digested using *Bst*BI (New England BioLabs, Ipswich, MA), and the satellite II probes used were an 18 mer single-stranded oligonucleotide and a cloned Chr1-specific insert

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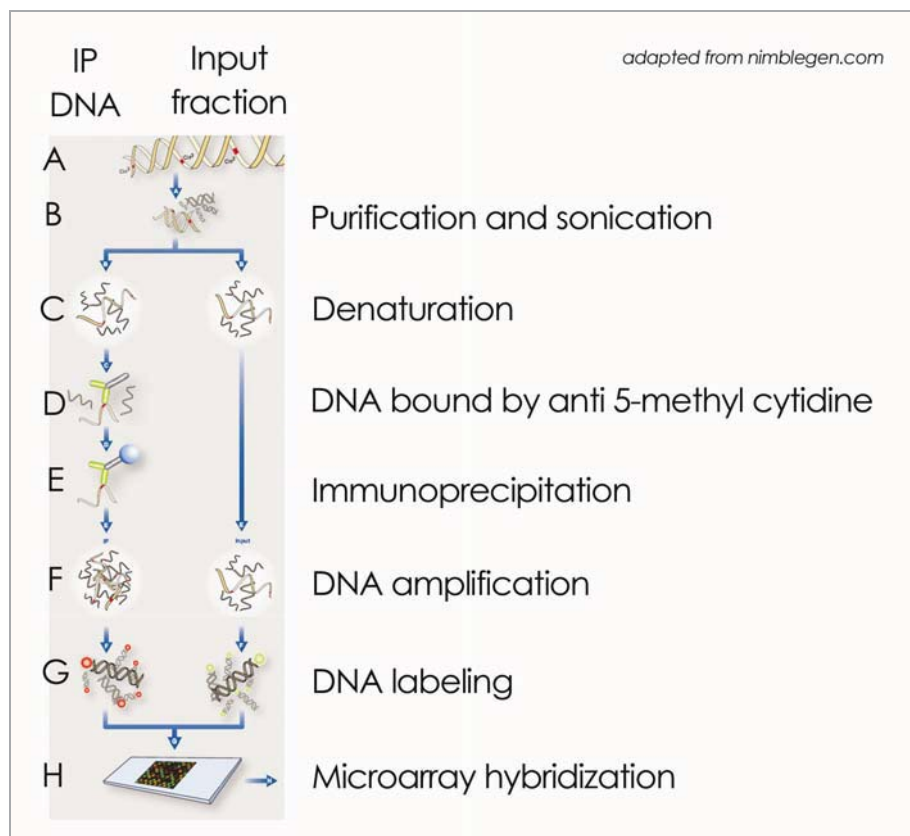
excised from the recombinant plasmid pUC1.77 (Cooke and Hindley, 1979). In each Southern blot analysis, normal peripheral blood and ICF B-lymphocyte (Corriell Cell Repositories, Camden, NJ) (Jeanpierre *et al.*, 1993) DNA was included as methylated and hypomethylated controls, respectively. Using a FLA 3000 phosphoimager (Fujifilm Corporation, Tokyo, Japan), the approximate amount of methylation was assessed by comparing the intensities of the hybridized fragments.

The cDNA microarray analyses had previously been reported (Andersson *et al.*, 2005). The methodology is similar to that of array CGH, but instead of quantifying gene copy-numbers, the hybridization reflects RNA levels and hence gene expression. Samples were hybridized to 27 k microarray slides containing 25 648 cDNA clones (SCIBLU DNA Microarray Resource Center) representing 13 737 Unigene clusters and 11 592 Entrez gene entries, according to the Unigene build 195. RNA extraction, amplification, labeling, hybridization, scanning, post-hybridization washing and feature analysis were performed as described by Andersson *et al.* (2005). The data analyses were all performed in BASE. To identify differentially expressed genes in the dup(1q)-positive ALLs, a t-test comparing HeH ALLs with and without gain of 1q was performed on the expression patterns of genes in the minimally gained 1q region.

### *Article II*

The global methylation levels were measured using methylated DNA immunoprecipitation (MeDIP) (Weber *et al.*, 2005) followed by hybridization to two different microarray systems (**Figure 4**). For genome-wide profiling we used tiling BAC arrays in order to obtain large-scale methylation profiles of

each chromosome and for gene promoter-specific analyses we used a microarray platform (Roche NimbleGen, Madison, WI) comprising a total of 28,226 CpG islands.



**Figure 4. MeDIP.** A) Selection of test DNA. B) High grade purification and fragmentation by sonication. C) DNA denaturation and input fraction (IF) collection. The IF serves as reference in the subsequent microarray hybridizations. D) DNA is treated with a monoclonal antibody against 5-methylcytidine. E) Immunoprecipitation using Protein A agarose beads and subsequent DNA recovery. F) Immunoprecipitated DNA (IP DNA) and IF is amplified. G) IP DNA and IF are differentially labeled using fluorochromes. H) Mixing and hybridization to a suitable microarray slide.

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Unsupervised clustering and principal component analyses were used to investigate methylation differences/similarities between the BAC array large-scale methylation profiles derived from t(12;21)-positive and HeH cases. The two groups were also analyzed in relation to methylation and gene density on each BAC clone to detect crude methylation differences between individual chromosomes in each subtype. The detected highly methylated genes in the promoter and CpG island array were analyzed in three different ways: First, all genes with significantly high methylation levels were grouped according to the genetic ALL subtype in order to identify those that were uniquely hypermethylated in either t(12;21)-positive or HeH cases. Second, an edge preserving smoother analysis determining a sequence with as few jumps as possible was performed. This segmentation analysis used the Potts filter with a penalty parameter in order to identify highly methylated genes throughout the genome (Lingjaerde *et al.*, 2005). Third, we identified all genes that displayed a high level of methylation in  $\geq 30\%$  of cases in either genetic ALL subgroup or that were present in a dataset comprising genes known to be hypermethylated in malignant disorders, involved in B-cell development and mutated or rearranged in B-lineage ALL. Each gene was then functionally annotated using the EASE software (Hosack *et al.*, 2003) and correlated with the gene expression data described below.

Identification of methylation hotspots, *i.e.*, genomic sites harboring a significantly higher number of highly methylated genes compared with the rest of the genome, was performed by plotting the spatial and number distribution of promoters enriched for methylation using a density estimator (Lingjaerde *et al.*, 2005). In order to designate a chromosomal segment as a methylation hotspot four probes had to be present within the segment analyzed.

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To test if methylation hotspots were associated with imprinted genes we used the mapping information provided by Luedi *et al.* (2007) on all known imprinted human genes. We ascertained the number of imprinted genes per chromosome band. Then, we calculated how often bands with a specific number of imprinted genes harbored methylation hotspots.

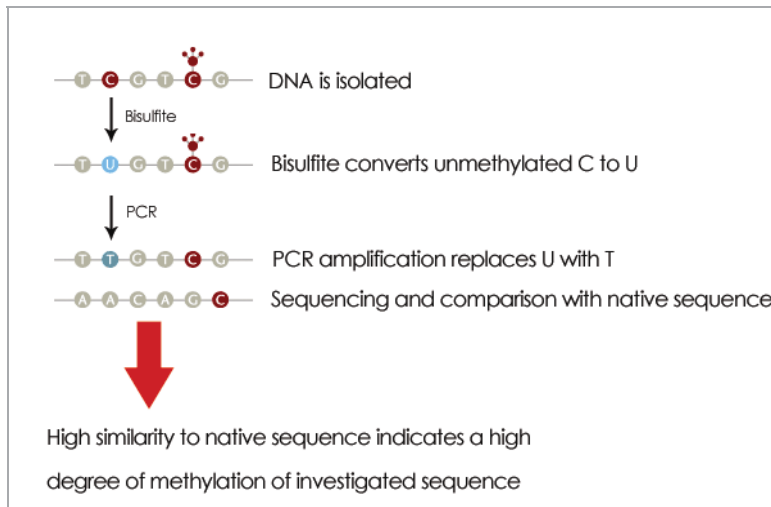
To validate the reliability of the promoter array, eight hypermethylated genes were selected for bisulfite sequencing (**Figure 5**). The bisulfite-treated DNA was used as template in a standard PCR amplification utilizing primers directed towards the promoter regions harboring the hypermethylated CpG islands, as indicated by the array. The primers were designed using the MethPrimer software (Li and Dahiya, 2002), the PCR products sub-cloned using the TOPO-TA system (Invitrogen, Carlsbad, CA) and sequenced using standard methods. Finally, the sequences were analyzed using the BiQ Analyzer software (Bock *et al.*, 2005).

Gene expression data based on cDNA microarrays (Andersson *et al.*, 2005) were used to investigate correlations between hypermethylated target genes and their expression levels. Furthermore, array CGH (Jönsson *et al.*, 2007) was performed on each case to detect genomic imbalances that might affect gene expression and/or be associated with large scale methylation changes.

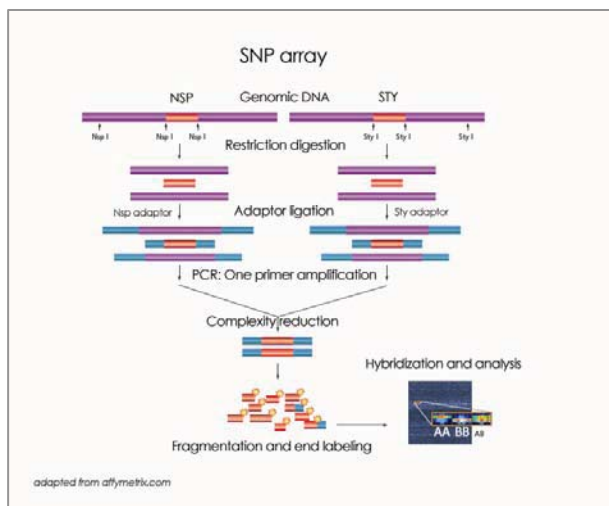
### *Article III*

SNP array analyses were performed using the Affymetrix GeneChip Human 250k *Nsp*, 250k *Sty*, and 10k 2.0 array systems (**Figure 6**) in order to identify genomic copy-number alterations (Heinrichs and Look, 2007). These chips cover approximately 510,000 SNPs, with a median physical distance between





**Figure 5. Bisulfite sequencing.** DNA is treated with sodium bisulfite which converts cytosine CpG motif residues into uracil, unless a methyl group is bound to the carbon 5 of the cytosine. Using PCR amplification, uracils are converted to thymines in the DNA segment, which subsequently can be sequenced and compared with the native sequence. Conserved cytosines indicate methylation whereas replacement by thymine means that the base is unmethylated.



**Figure 6. Affymetrix GeneChip.** DNA is digested with *Nsp* and *Sty* and ligated to adaptors. An adaptor-specific PCR assay is used to amplify fragments. Products from each restriction digest are combined. The amplified DNA is then fragmented, labeled and hybridized to a SNP array.

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the SNPs of <2.5 kb. Hybridization and washes were performed as described previously (Paulsson *et al.*, 2008). The Affymetrix GTYPE software was used for analysis of signal intensity and for genotype calling. As an initial step to identify putative genomic imbalances, log<sub>2</sub> ratios were segmented with the CBS algorithm (Venkatraman and Olshen, 2007) using the DNA copy package in R (<http://www.bioconductor.org>). For further copy number and UPD analyses, the in-house Genome Orientated Laboratory File (GOLF) and the dChip (Lin *et al.*, 2004) software were used. Classification as a copy number alteration was based on visual inspection of the inferred log<sub>2</sub> ratio versus the pooled signal intensity of 10 control samples.

For analyses of RTK-RAS genes, codons 835 and 836 in the second tyrosine kinase domain and of internal tandem duplication of exons 14 and 15 in *FLT3*, codons 12, 13 and 61 in *NRAS* and *KRAS* and of exons 3 and 13 in *PTPN11* were sequenced, as described previously (Paulsson *et al.*, 2007). The PCR products sense and the antisense strands were directly sequenced and analyzed using the Seqscape software (PE Applied Biosystems, Foster City, CA).

## Results

### *Article I*

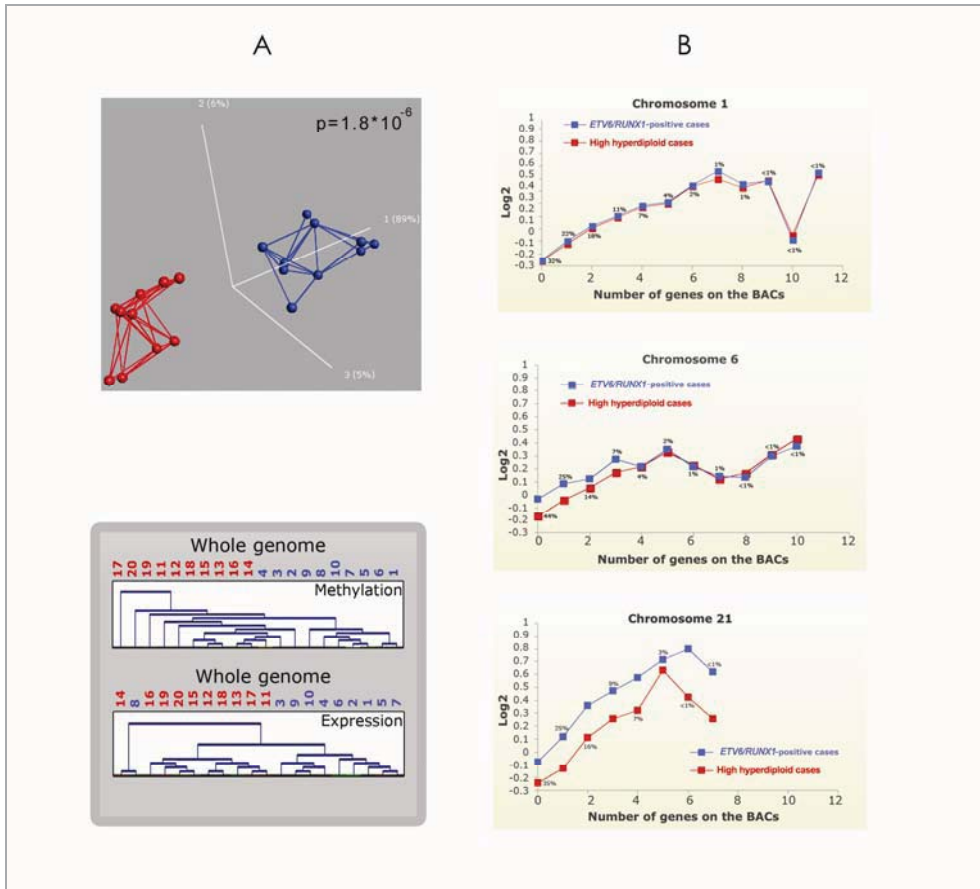
The proximal breakpoints in the ten dup(1q)-positive BLs and HeH ALLs were all near-centromeric, with eight of them clustering within a 1.4 Mb segment in 1q12-21.1. The distal 1q breakpoints were more heterogeneous, being more distal in the HeH ALLs than in the BLs. The minimally gained 1q segments in the ALLs and BLs were 57.4 Mb [dup(1)(q22q32.3)] and 35 Mb [dup(1)(q12q25.2)], respectively. Satellite II DNA on 1q was not

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hypomethylated, as ascertained by Southern blot analyses of 15 BLs/ALLs with and without gain of 1q, indicating that aberrant methylation was not involved in the origin of dup(1q). Global gene expression analyses revealed that 5 genes in the minimally Mb gained region – *B4GALT3*, *DAP3*, *RGS16*, *TMEM183A* and *UCK2* – were significantly overexpressed in dup(1q)-positive ALLs compared to non-dup(1q) HeH ALL. The *DAP3* and *UCK2* genes were among the most overexpressed genes in the sole dup(1q)-positive BL case investigated.

### *Article II*

Unsupervised clustering and principal component analyses of the BAC array chromosome-wide data successfully subgrouped the t(12;21)-positive and HeH cases, indicating that they are characterized by different methylomes (**Figure 7A**). When comparing the mean methylation levels in the two ALL types, differences between the HeH and the t(12;21)-positive ALLs as regards chromosome-wide methylation on chromosomes 6, 10, 14, 16, 17, 18, 19 and 21 were clearly seen, with the former cases generally displaying a lower methylation of these chromosomes (**Figure 7B**). The majority of these chromosomes are the ones commonly gained in HeH ALL. Furthermore, when comparing the mean methylation levels within HeH ALL, they were generally lower in cases with trisomy/tetrasomy than in cases with disomies of the same chromosomes. Thus, gains in HeH cases are associated with decreased methylation of additional chromosomes. This was not seen in t(12;21)-positive ALL.



**Figure 7. BAC array chromosome-wide methylation profiling** A) Principal component and unsupervised clustering analyses of the data could successfully subgroup the majority of t(12;21)-positive (red) and HeH ALLs (blue). B) Mean  $\log_2$  ratios of the HeH (red) and t(12;21)-positive (blue) ALLs plotted against genomic positions on chromosomes 1, 6 and 21. Similar methylation levels are seen for chromosome 1, which is disomic in both subtypes. Lower methylation of gained chromosomes is seen for both chromosomes 6 and 21 in HeH, in which they were gained.

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A total of 8,662 genes with significant methylation scores were identified by the CpG promoter array. The HeH ALLs harbored more hypermethylated genes (n=7650) than the t(12;21)-positive cases (n=3983) and the genes were distributed on all chromosomes, without any clear association with chromosomes frequently gained or lost in either subgroup. Segmentation analysis identified 138 genes that were highly enriched for methylation. It was apparent that enrichment for methylation not necessarily resulted in decreased expression. However, the majority of hypermethylated (60%) genes did show a significantly lowered expression. In addition, a total of 167 different recurring genes and/or genes involved in B-cell development or mutated or rearranged in B-lineage ALL were found to be targets for hypermethylation; 34% of these genes showed a significant decrease in expression.

A total of 58 different methylation hotspots were identified, with 30 of these being common to both t(12;21)-positive and HeH ALLs. The t(12;21)-positive ALLs harbored eight unique methylation hotspots, whereas the HeH ALLs had 20 unique methylation hotspots. In addition, we detected a correlation between the presence of imprinted genes and methylation hotspots, with the frequencies of methylation hotspots increasing with the number of imprinted genes.

In the validation assay of the promoter CpG island methylation profiling, the bisulfite sequencing results corresponded to the array results in all cases, *i.e.*, low or moderate methylation was found when the investigated regions were situated outside the peak and hypermethylation was detected when the analyzed segments were situated within the peak region.

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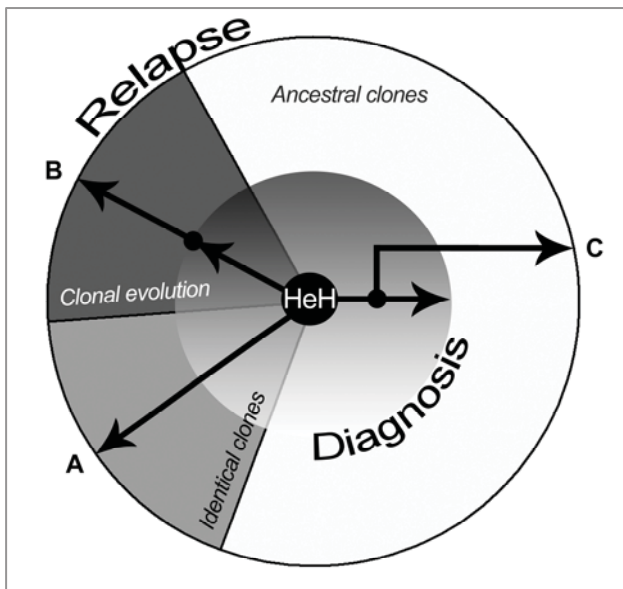
### *Article III*

When analyzing and comparing the 11 investigated paired diagnostic and relapse samples in HeH ALL, the following gains, in decreasing frequency order, were detected: +6 (100%), +21 (100%), +4 (91%), +17 (91%), +18 (91%), +X (86%), +10 (82%), +8 (77%), +14 (45%), +5 (23%), +2 (9%), +7 (9%), +9 (9%), +15 (9%) and +16 (5%). Only one sample harbored a monosomy (-19), and this was not present at relapse. In seven patients, the gains were identical at diagnosis and relapse, whereas they differed by 1-5 trisomies/tetrasomies in four cases. Simultaneous trisomies of chromosomes 4, 10, and 17 – the “triple trisomies” used for stratification of patients into lower-risk by the COG (Schultz *et al.*, 2007) – were found in seven (64%) of the 11 patients.

The number of structural changes varied between 0 and 6 among the 22 samples with a total of 34 different hemizygous deletions, two homozygous deletions and five copy-number gains being identified. Among all 41 structural changes detected, nine were >10 Mb in size; the remaining 32 imbalances may be considered cytogenetically cryptic. None of the five copy-number gains were recurrent. Among the 36 hemi- and homozygous deletions identified, four were recurrent: 7p12.2/*IKZF1*, 9p13.2/*PAX5*, 9p21.3/*CDKN2A* and 9p24.1/*AK3*. None of the homozygous deletions or copy-number gains was shared in paired diagnostic and relapse samples and apart from a homozygous 9p deletion at diagnosis in case 2, these changes were only found at relapse. Among the 34 hemizygous deletions, nine were seen both at diagnosis and relapse in five patients. Of the remaining 25 hemizygous deletions 17 were found at relapse and 8 at diagnosis. Taken together, the mean frequency of structural changes at diagnosis was 1.6, whereas the corresponding frequency

at relapse was 2.9, demonstrating a significantly ( $p < 0.05$ ) higher frequency of such aberrations at relapse. UPDs were only detected in two of the 11 paired samples, comprising a UPD8 at diagnosis and relapse. Five of the 11 paired samples harbored a total of six mutated RTK-RAS genes: one *FLT3* mutation, two *KRAS* mutations and three *PTPN11* mutations. Two different mutations, one in *FLT3* and one in *PTPN11*, were found in one single case. Among all mutations, only one was detected both at diagnosis and relapse. Of the remaining five mutations, one was found at diagnosis and four at relapse.

Based on the clonal relationship between the diagnostic and relapse samples three distinct groups were identified: 1) identical genetic changes at diagnosis and relapse (18%), 2) clonal evolution with all changes at diagnosis being present at relapse (18%), and 3) ancestral clones with some changes present at diagnosis but not at relapse (64%), suggesting the presence of an preleukemic clone (Mullighan *et al.*, 2008) (**Figure 8**).



**Figure 8. The clonal relationship of HeH ALL were divided into three groups. A) Identical clones at diagnosis and relapse. B) Clonal evolution with all changes at diagnosis being present at relapse. C) Ancestral clones, i.e. some changes were conserved and others lost or gained in the diagnostic and relapse samples, respectively.**

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## Discussion

### *Article I*

The results in *Article I* demonstrated near-centromeric proximal 1q breakpoints in all BLs and HeH ALLs investigated. Together with data, derived from other high resolution array studies (Paulsson *et al.*, 2006; Mullighan *et al.*, 2007), this strongly indicates the presence of a breakprone region near the centromeric region of 1q. Why this region is prone to break and to form duplications is unknown; however, it has been hypothesized that hypomethylation of pericentric heterochromatin could be associated with decondensation and instability of satellite sequences, resulting in such rearrangements (Sawyer *et al.*, 1998). However, we found no hypomethylation of the 1q satellite II domain in our series. It is thus highly unlikely that aberrant methylation of the satellite II region should play an important role in the formation of dup(1q), at least not in BLs and HeH ALLs. A more likely explanation could be that the genomic architecture as such, in particular segmental duplications, could be important for this phenomenon. The presence of low-copy repeats has been implicated in the formation of both constitutional and neoplasia-associated chromosomal abnormalities (Ji *et al.*, 2000). Interestingly, such duplications can be found immediately distal to the highly repetitive satellite DNA motifs, such as satellite II, located close to the centromere (Horvath *et al.*, 2001). In addition, small segmental duplications have been mapped to the region on 1q in which the proximal breakpoints occurred in our cases (Sharp *et al.*, 2006). The frequent occurrence of dup(1q) could therefore be explained as a consequence of these rearrangement hotspots.



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Does dup(1q) play any role in leukemogenesis? Murine models inoculated with human leukemic B-cell clones carrying dup(1)(q11q32) developed tumors that were more tumorigenic, grew faster and resulted in more metastases than did those occurring in mice inoculated with clones harboring other chromosomal abnormalities (Ghose *et al.*, 1990). This indicates that dup(1)(q11q32) provides a proliferative advantage. Furthermore, it is noteworthy that the minimally gained regions for both the BLs and the HeH ALLs in our study are included in the abovementioned segment. Hence, one or several genes mapping to the minimally gained region could be pathogenetically important.

The global gene expression analyses revealed five significantly up-regulated genes, namely *B4GALT3*, *DAP3*, *RGS16*, *TMEM183A* and *UCK2*. Two of them (*DAP3* and *UCK2*) were among the most overexpressed genes in the single BL case with gain of 1q investigated with expression array. The *DAP3* has been reported to be highly expressed in invasive glioblastoma multiforme cells (Mariani *et al.*, 2001), although it is normally proapoptotic. The *UCK2* protein has been correlated with sensitivity to anticancer drugs, more specifically certain inhibitors of RNA polymerases (Shimamoto *et al.*, 2002). However, the impact of the up regulation of these genes in dup(1q)-positive high hyperdiploid ALLs and BLs is currently unknown and remains to be further investigated.

### *Article II*

In *Article II*, an intriguing and unexpected finding was the differences between the HeH and the t(12;21)-positive ALLs as regards the chromosome-wide methylation patterns, with the majority of the commonly gained chromosomes

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in the former subtype being less methylated than their disomic counterparts (**Figure 7B**). Only one previous study has reported similar findings (Weber *et al.*, 2005), identifying an overall lowered methylation in gene-poor regions of the trisomic chromosomes 7 and 14 in a colon cancer cell line. However, in the present study the decreased methylation was equally distributed, independent of gene content, which agrees well with previous data showing no clear bias for decreased methylation only of gene-poor regions in the inactive and globally hypomethylated X chromosome in females (Hellman and Chess, 2007). The inactive X chromosome was long believed to be hypermethylated, but was, quite unexpectedly, by the MeDIP approach demonstrated to be globally hypomethylated (Weber *et al.*, 2005).

It has previously been demonstrated that pediatric HeH ALL has a separate expression signature compared with other pediatric ALL subtypes (Andersson *et al.*, 2005) and that the majority of the genes on the gained chromosomes are upregulated compared to other ALL subgroups with no tri- or tetrasomies (**Figure 3**). In *Article II* we demonstrate a global overexpression of genes on the additional chromosomes also when comparing disomic and trisomic/tetrasomic chromosomes within the HeH group. Yeast experimental systems that promote aneuploidy also demonstrate expression of the majority of genes on the extra chromosomes (Torres *et al.*, 2007). However, even if the transcript levels are increased, the amount of detected proteins often is not. Thus, gene overexpression on additional chromosomes does not always translate directly into high amounts of protein. Hence, the role of the decreased methylation that we detected on the gained chromosomes in HeH could be unrelated to gene expression. Instead, these methylation differences could theoretically be involved in the compartmentalization of the tri-

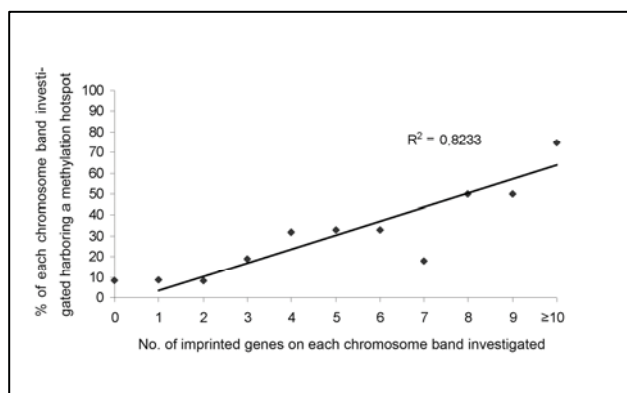
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/tetrasomic chromosomes during interphase, similar to Barr body formation of the inactive X chromosome. Another possibility is that the lowered methylation could contribute to the abrogation of the reduced cellular fitness that has been demonstrated to be associated with aneuploidy (Weaver *et al.*, 2007; Williams *et al.*, 2008).

The detection of subtype-specific methylation hotspots indicates that t(12;21)-positive and HeH ALLs have different global methylation patterns. Further support for this was derived from the clustering analyses which successfully could subgroup the t(12;21)-positive and HeH cases (**Figure 7A**). Thus, genome-wide methylation analyses can subdivide morphologically identical, but genetically and clinically distinct, hematologic malignancies.

One large methylation hotspot on 19q common to both subtypes has previously been reported in ALL. Interestingly, this chromosome segment harbors several maternally imprinted genes (Taylor *et al.*, 2007), indicating that other methylation hotspots identified in *Article II* possibly also could be associated with imprinted genes. To test this hypothesis we used available mapping information (Luedi *et al.*, 2007) on all known, or putatively, imprinted human genes, ascertaining the number of imprinted genes per chromosome band and how often bands with a specific number of imprinted genes harbored methylation hotspots. As seen in **Figure 9**, there was a high correlation between the presence of imprinted genes and methylation hotspots, with the frequencies of methylation hotspots increasing with the number of imprinted genes. This strongly suggests that regions rich in imprinted genes are associated with methylation hotspots in t(12;21)-positive and HeH ALLs. A possible explanation for this could be that imprinted genes

are more susceptible to *de novo* methylation of the active allele and hence biallelic silencing. But this remains to be elucidated.



**Figure 9. Association between methylation hotspots and imprinted gene density.** Mapping information on all known, or putatively, imprinted human genes compared with the location of methylation hotspots. As seen, there is a high correlation

between the presence of imprinted genes and hotspots, with the frequencies of hotspots increasing significantly with the number of imprinted genes.

### *Article III*

In *Article III*, we investigated paired diagnostic/relapse samples using SNP array and mutation analysis of RTK-RAS genes. The array revealed higher frequencies of structural chromosome aberrations in relapse samples compared with diagnostic. This is well in line with previous findings, reporting increased numbers of structural abnormalities in HeH ALL at relapse (Secker-Walker *et al.*, 1989; Shikano *et al.*, 1990; Abshire *et al.*, 1992; Heerema *et al.*, 1992; Vora *et al.*, 1994; Chucrallah *et al.*, 1995; Mullighan *et al.*, 2008). We also detected more RTK-RAS mutations at relapse than at diagnosis. Whether this increase of genetic aberrations is caused by the chemotherapy administered to the patients or is a consequence of inherent clonal evolution over time remains unknown.

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No structural aberration or mutated RTK-RAS gene was found in all cases. This indicates that relapse in HeH ALL most probably can not be related to one single genetic lesion, at least not detectable using current methods. Only four abnormalities, namely deletions involving *AK3*, *CDKN2A/B*, *IKZF1* or *PAX5*, were recurrent in the 11 investigated cases. Submicroscopic deletions in regions harboring these genes have previously been identified in HeH ALL at both diagnosis and relapse (Mullighan *et al.*, 2007; Mullighan *et al.*, 2008; Paulsson and Johansson, 2009). However, the prognostic impact of these aberrations is currently quite unclear (Den Boer *et al.*, 2009; Sulong *et al.*, 2009).

*IKZF1* deletions have been shown to be associated with poor outcome in high-risk pediatric B-cell progenitor ALL (Mullighan *et al.*, 2009). In addition, specific SNPs in the gene have recently been associated with a increased risk of childhood ALL in two GWA studies (Papaemmanuil *et al.*, 2009; Treviño *et al.*, 2009). One of these *IKZF1* SNPs was demonstrated to generate a low *IKZF1* expression in Epstein-Barr virus-transformed lymphocytes (Papaemmanuil *et al.*, 2009), possibly mimicking a downregulation caused by a deletion. However, among the 11 patients investigated in the present series and the six paired samples previously reported by Mullighan *et al.* (2008), only 18% (n=3) harbored *IKZF1* deletions, with the losses being present both at diagnosis and relapse in one case, only at diagnosis in one and only at relapse in one. *IKZF1* deletions thus do not seem to play any significant prognostic role in the HeH genetic subgroup of pediatric ALL.

The simultaneous occurrence of +4, +10, and +17, called the “triple trisomies”, is presently used to stratify low-risk childhood ALL by the COG

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(Schultz *et al.*, 2007). It is hence noteworthy that we found these triple trisomies in the majority (64%) of the investigated paired diagnostic and relapse samples. The incidence of these triple trisomies in the close to 60 diagnostic HeH cases presently analyzed by high resolution arrays (Paulsson *et al.*, 2006; *Article I*; Mullighan *et al.*, 2007; Strefford *et al.*, 2007) is 50%, a frequency slightly lower than the one we observed in the paired samples. Furthermore, these trisomies were also observed in two of the eight previously reported paired diagnostic and relapse samples (Mullighan *et al.*, 2008; Yang *et al.*, 2008). It is thus quite clear that these “favorable” trisomies can be found in a substantial proportion of relapses in HeH, regardless of treatment protocols, thus questioning their favorable impact.

HeH ALL was genetically characterized only by the nonrandom tri- and tetrasomies and a handful of well-defined structural chromosome abnormalities before the advent of array based techniques (Paulsson and Johansson, 2009). However, the genetic patterns have, during recent years, been demonstrated to be more complex, consisting of several submicroscopic aberrations beside HeH and established cytogenetic abnormalities (Paulsson *et al.*, 2006; Mullighan *et al.*, 2007; Strefford *et al.*, 2007; Mullighan *et al.*, 2008). The complexity increases even further when paired diagnostic and relapse samples are compared. Our study (*Article III*) together with the one by Mullighan *et al.* (2008) demonstrates that the genetic relationship between diagnostic and relapse samples can be divided into different groups.

The most common group – the one displaying a complex genetic evolution pattern with some changes conserved and others lost or gained in the diagnostic and relapse samples, respectively – suggests the presence of an ancestral preleukemic clone from which both the diagnostic and relapse clones

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are derived. Although it is not possible to directly characterize this clone genetically, its genotype can be backtracked by comparing diagnostic and relapsed samples from the same patient. When doing this it becomes clear that both structural chromosome changes and mutated genes are secondary to the HeH pattern, occurring later in the temporal order of the evolution of the neoplastic clone. Thus, HeH is the primary genetic abnormality in this ALL subtype.

## **Conclusions**

### *Article I*

- Proximal breakpoints of dup(1q)-positive BL and pediatric HeH ALL cluster close to the centromere in both disorders, suggesting this region to be breakprone.
- Distal breakpoints are more heterogeneous.
- The minimally gained region is much larger in the HeH ALLs than in the BLs.
- The satellite II domain is not hypomethylated in either disorder, ruling out that decondensation of pericentric heterochromatin, due to hypomethylation of satellite II sequences, is the underlying mechanism behind dup(1q) in BL and pediatric HeH ALL.
- Comparing HeH ALL dup(1q)-positive cases with HeH ALL without gain of 1q, five genes on 1q are significantly overexpressed and thus potentially pathogenetically important.

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## *Article II*

- Unsupervised cluster and principal component analyses of BAC array chromosome-wide methylome profiles can accurately subgroup the t(12;21) and HeH ALL subtypes, demonstrating that they are characterized by different methylomes.
- Analysis of the promoter-specific CpG island array demonstrates that several B-cell- and neoplasia-associated genes are hypermethylated and simultaneously underexpressed.
- Methylation hotspots are associated with chromosome bands predicted to harbor imprinted genes, suggesting that regions rich in imprinted genes are associated with aberrant methylation in t(12;21)-positive and HeH ALLs.
- The tri-/tetrasomic chromosomes in the HeH ALLs are less methylated than their disomic counterparts.

## *Article III*

- Structural changes are significantly more common in relapse samples than in diagnostic ones.
- No single, recurrent aberration can be directly linked to relapse.
- The triple trisomies +4, +10, and +17, associated with low-risk ALL, are detected in two-thirds of cases that subsequently relapse.
- Genetic differences between diagnostic and relapse samples suggest the presence of an ancestral HeH clone in the vast majority of patients.
- Structural changes and mutations are secondary to the HeH pattern.



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### *Concluding remarks*

The major aim of this thesis was to analyze the genetic and epigenetic characteristics of pediatric HeH ALL using a spectrum of established, as well as novel techniques. Has this aim been fulfilled? The three original articles in this thesis have all used high-throughput genome-wide screening methods to address three different questions regarding HeH ALL: what are the genetic characteristics of the most common structural abnormality – dup(1q)? Are aberrant methylation patterns involved in leukemogenesis? How does diagnostic and relapse samples relate to each other genetically? In this fashion, a global genetic and epigenetic perspective has been obtained for these three relevant questions regarding HeH ALL pathogenesis.

Has any new significant insight regarding HeH leukemogenesis been gained? The fact that dup(1q) in HeH ALL probably is due to a breakprone region shared with other neoplasms harboring this abnormality indicates that this aberration is not specific for HeH but most likely contributes to neoplastic transformation in general. In the second study it was demonstrated that aberrant methylation resulting in downregulation of expression and that lowered methylation on the gained chromosomes could contribute to pathogenesis. The genetic differences between diagnostic and relapse samples in the third article suggested the presence of a preleukemic ancestral HeH clone giving rise to both diagnosis and relapse clones in the majority of cases. So, in essence, some important data contributing to the understanding of pediatric HeH ALL formation have been obtained. Of course, much work remains to be done in HeH ALL. For example, further investigation of the significance and frequency of different secondary genetic events contributing to overt disease needs to be undertaken in larger series using high-resolution

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and genome-wide techniques. Validating a large proportion of the aberrantly methylated genes detected, as well as testing the functional outcome of their hypermethylation in experimental systems should definitely be performed. Investigating subclonality using SNP arrays, estimating allele frequencies of both diagnostic and relapsed samples, could give more clues to delineating the exact genetic patterns of ancestral clones. Last but definitely not least, the big question of the functional outcome of high hyperdiploidy as such still lingers. This is to a large extent unsolved and presently no experimental technique allows a direct investigation of these massive chromosomal imbalances. In resolving this lies the key to understanding the pathogenetics of pediatric HeH ALL.



## SVENSK SAMMANFATTNING

(SUMMARY IN SWEDISH)

Hematologiska maligniteter är namnet på alla de kliniskt, morfologiskt och immunofenotypiskt heterogena cancersjukdomar som involverar benmärgen och blodet. De akuta leukemierna, vilka är de vanligaste formerna av hematologiska maligniteter hos barn och vuxna, delas in i de myeloiska och de lymfatiska, beroende på vilken blodcellslinje de har uppstått ifrån. Hos barn dominerar akut lymfatisk leukemi (ALL) och hos vuxna akut myeloisk leukemi. Båda karakteriseras av en ackumulering av maligna omogna vita blodkroppar i benmärg och perifert blod. Detta leder i sin tur till ökad infektionsbenägenhet och risk för blodbrist och blödning hos patienten.

Cancer är en sjukdom som karakteriseras av förvärvade genetiska förändringar i en cell, vilka ändrar dess egenskaper vad gäller tillväxt, utmognad och överlevnad. Vad gäller ALL hos barn, har förekomsten en karakteristisk topp mellan 2-5 år. Studier har visat att de första genetiska förändringarna ofta uppstår redan under fosterstadiet och att sedan ytterligare genetiska avvikelser ackumuleras i cellen innan leukemin kan bryta ut. Genetiska förändringar synbara på kromosomnivå detekteras i ungefär hälften av alla akuta leukemier och ger viktig klinisk information med betydelse för diagnostik och behandling. En vanlig strukturell genetisk avvikelse är en så kallad translokation, vilket innebär omflyttning av genetiskt material så att

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cellens kontrollgener påverkas. Det är också vanligt att cellen får ett tillskott av kromosomer. Detta är dock inte lika välstuderat som translokationer.

Syftet med den här avhandlingen var att genetiskt karakterisera den vanligaste formen av barnleukemi: höghyperdiploid barn-ALL. Denna leukemityp karakteriseras främst av att cancercellerna innehåller ett stort antal extra kopior av vissa speciella kromosomer – så kallad hyperdiploidi. Idag vet vi väldigt lite vad detta kromosommönster har för betydelse och funktion i cancercellerna. Tidigare studier har visat att själva hyperdiploidin uppstår i fosterstadiet, genom en enda felaktig celledning, och att ytterligare genetiska och epigenetiska (modifiering av genuttryck som inte beror på ändring av själva DNA sekvensen) förändringar sedan krävs för att en akut leukemi ska uppträda hos patienten. Höghyperdiploid barn-ALL fungerar således som en typisk modell för hur man idag tror naturförloppet hos en barnleukemi är. Målet med de tre studierna som ingår i avhandlingen har varit att identifiera och karakterisera sekundära avvikelser till hyperdiploidin, avvikelser vilka kan ha betydelse för leukemiuppkomsten, men också för återinsjuknande.

I den första studien undersöktes tio stycken höghyperdiploida ALL och Burkitt lymfom, vilka bar på den vanligast förekommande strukturella cytogenetiska avvikelserna, nämligen överskott av kromosommaterial på den långa armen på kromosom 1, d.v.s. dup(1q). Detta gjordes med ”array CGH”, vilken är en teknik som detekterar väldigt små förluster eller tillkomster av genetiskt material. Vi kunde visa att en av de två brottspunkterna på duplikationerna var i samma region, nära kromosomens centrum, i samtliga fall. Det har spekulerats att dup(1q) kan bero på undermetylering av repetitiva DNA-sekvenser på denna kromosomarm. För att undersöka detta användes en metyleringskänslig Southern blot analys. Vi detekterade dock enbart normal

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metylering av DNA-sekvenser på 1q, vilket indikerar att låg metyleringsgrad inte är mekanismen bakom dup(1q).

I studie II så undersökte vi metyleringsmönster över samtliga kromosomer och gener i 20 fall av höghyperdiploid och ALL med en translokation mellan kromosom 12 och 21, d.v.s. t(12;21). Med en antikropp fångade vi metylerade DNA-sekvenser och hybridiserade sedan dessa till en arrayplattform. Detta möjliggör undersökning av vilka gener och regioner som är högmetylerade, vilket ofta innebär att gener inte uttrycks. Följaktligen hittade vi också hypermetylering av ett stort antal gener i båda typerna av leukemi av vilka majoriteten också var nedreglerade uttrycksmässigt. Metylering är således ytterligare ett sätt att påverka gener i leukemiceller. Intressant nog kunde vi också visa en lägre metyleringsgrad i de extra kromosomkopiorna i höghyperdiploid jämfört med t(12;21)-positiv ALL.

I studie III undersökte vi genetiska skillnader mellan diagnos och recidiv hos 11 fall av höghyperdiploid barn-ALL. Metoderna var mutationsanalys och ”SNP array”, vilken liksom array CGH detekterar genetiska obalanser på en hög resolutionsnivå. I linje med vad föregående undersökningar visat så hittade vi mer strukturella avvikelser i recidiv- gentemot diagnosproven. Dessutom fann vi att +4,+10 och +17, vilka har hävdats vara associerade med god prognos, i två tredjedelar av de undersökta fallen. Den genetiska jämförelsen visade också att den vanligaste utvecklingsvägen var från en gemensam höghyperdiploid preleukemisk klon, till vilken för diagnos och recidiv skilda strukturella avvikelser och mutation tillkommer. Sammanfattningsvis så har undersökningarna i denna avhandling lett till ökade insikter om det komplexa mönster av genetiska och epigenetiska förändringar som är associerade med utvecklingen av höghyperdiploid ALL.



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# Tiling resolution array comparative genomic hybridization, expression and methylation analyses of dup(1q) in Burkitt lymphomas and pediatric high hyperdiploid acute lymphoblastic leukemias reveal clustered near-centromeric breakpoints and overexpression of genes in 1q22-32.3

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Although gain of 1q occurs in 25% of Burkitt lymphomas (BLs) and 10% of pediatric high hyperdiploid acute lymphoblastic leukemias (ALLs), little is known about the origin, molecular genetic characteristics and functional outcome of dup(1q) in these disorders. Ten dup(1q)-positive BLs/ALLs were investigated by tiling resolution (32k) array CGH analysis, which revealed that the proximal breakpoints in all cases were near-centromeric, in eight of them clustering within a 1.4 Mb segment in 1q12-21.1. The 1q distal breakpoints were heterogeneous, being more distal in the ALLs than in the BLs. The minimally gained segments in the ALLs and BLs were 57.4 Mb [dup(1)(q22q32.3)] and 35 Mb [dup(1)(q12q25.2)], respectively. Satellite II DNA on 1q was not hypomethylated, as ascertained by Southern blot analyses of 15 BLs/ALLs with and without gain of 1q, indicating that aberrant methylation was not involved in the origin of dup(1q), as previously suggested for other neoplasms with 1q rearrangements. Global gene expression analyses revealed that five genes in the minimally 57.4 Mb gained region—*B4GALT3*, *DAP3*, *RGS16*, *TMEM183A* and *UCK2*—were significantly overexpressed in dup(1q)-positive ALLs compared with high hyperdiploid ALLs without dup(1q). The *DAP3* and *UCK2* genes were among the most overexpressed genes in the BL case with gain of 1q investigated. The *DAP3* protein has been reported to be highly expressed in invasive glioblastoma multiforme cells, whereas expression of the *UCK2* protein has been correlated with sensitivity to anticancer drugs. However, involvement of these genes in dup(1q)-positive ALLs and BLs has previously not been reported.

## INTRODUCTION

Gain of 1q, through duplication, isochromosome formation or unbalanced translocations, is one of the most frequent

acquired cytogenetic abnormalities in human neoplasia (1). In solid tumors, dup(1q) is particularly common in hepatoblastoma (2), melanoma (3), Wilms' tumor (4) and carcinomas of the breast (5), liver (6), lung (7) and pancreas (8). As regards

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hematological malignancies, dup(1q) has been reported in all major diagnostic subgroups (1), although mainly in B-lineage acute lymphoblastic leukemia (ALL) (9), multiple myeloma (MM) (10) and B-cell non-Hodgkin lymphoma (NHL) (11). In these disorders, the incidence of dup(1q) varies significantly among the different cytogenetic subgroups, being quite strongly associated with t(11;14)(q13;q32) in MM, t(3;14)(q27;q32), t(8;14)(q24;q32) and t(14;18)(q32;q21) in NHL and with high hyperdiploidy (>50 chromosomes) in pediatric ALL (1).

Despite the high prevalence of 1q gain in neoplasia, next to nothing is known about its origin, molecular genetic characteristics and functional outcome. However, methylation studies of Wilms' tumors and carcinomas of the breast, liver and ovary have revealed an association between hypomethylation of the heterochromatic 1q region and the presence of dup(1q), and it has been suggested that this undermethylation is the cause of the 1q rearrangements (12–15). Whether this holds true in hematological malignancies has not been investigated. In MM, a number of studies, using chromosome banding, fluorescence *in situ* hybridization (FISH), comparative genomic hybridization (CGH), single nucleotide polymorphism (SNP) and expression analyses, have revealed that the proximal breakpoints (PBs) often are near-centromeric and that several genes on 1q are overexpressed (16–20). The breakpoints in NHL, mainly follicular and diffuse large B-cell lymphomas, have also been shown to localize close to the centromere, often involving the satellite II (sat II) domain (17,21,22).

Surprisingly few studies have focused on dup(1q) in Burkitt lymphomas (BLs) and high hyperdiploid pediatric ALLs, which harbor 1q gains in approximately 25 and 10% of the cases, respectively (1,9,23–27). As in MM and NHL, the PBs in the relatively small number of dup(1q)-positive BLs analyzed have been shown to involve either the sat II domain or to be almost centromeric (17,21). To the best of our knowledge, no ALLs have been investigated in detail in this respect; however, a recent FISH mapping of two high hyperdiploid ALLs with 1q gain revealed near-centromeric PBs (28). In the present study, the PBs as well as the distal breakpoints (DBs) were analyzed in dup(1q)-positive high hyperdiploid ALLs and BLs, using tiling resolution (32k) array CGH. In addition, the methylation pattern of sat II was investigated in ALLs and BLs with and without dup(1q) in order to ascertain whether undermethylation of the heterochromatic region is associated with 1q rearrangements in these disorders. Finally, gene expression analyses, using 27k cDNA microarrays, were performed to elucidate the functional outcome of 1q gains in BLs and ALLs.

## RESULTS

### Cytogenetic characterization of 1q gains

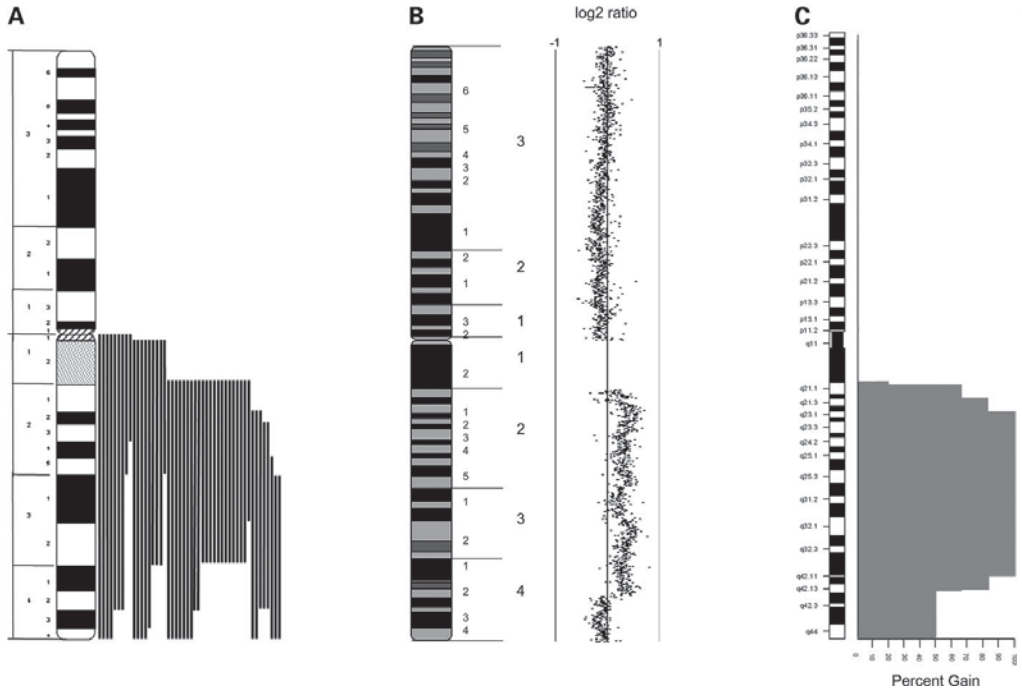
The literature review revealed that the vast majority of the 1q PBs in dup(1q)-positive BLs and pediatric high hyperdiploid ALLs map cytogenetically close to the centromere, in 1q11–21, whereas the DBs are more heterogeneous. Although the duplications are quite large, often involving most of chromosome arm 1q, a minimally gained segment (1q23–25) could be delineated in both the disorders (Figs 1 and 2).

### Array CGH findings

The array CGH analyses confirmed, refined and, in some cases, revised the aberrations identified by chromosome banding (Table 1). In addition, a total of 54 previously undetected abnormalities were revealed by the array CGH in the 10 cases investigated; many of the changes were submicroscopic, whereas a few larger ones most likely had escaped detection because of poor chromosome morphology. Of the 54 additional aberrations, 20 changes were potentially pathogenetic, whereas 25 involved regions known to harbor copy number polymorphisms (CNPs) and nine involved the *IGK@* and *IGH@* loci (Table 1). Excluding numerical aberrations, CNPs and the *IGK@* and *IGH@* rearrangements, 23 different genomic imbalances were found by array CGH (Table 2). All 54 imbalances in the 10 cases are summarized as a heat map in Figure 3.

The mapped 1q breakpoints and hence the sizes of the duplicated segments differed quite extensively between the chromosome banding and array CGH results (Table 1). Because of the often poor chromosome morphology in BLs/ALLs and the difficulty in delineating breakpoints cytogenetically, the findings from the array CGH analyses are most likely more accurate. As seen in Table 2, the 1q PBs in all 10 cases analyzed were near-centromeric, clustering within a 1.4 Mb segment at 1q12–21.1 (between chr 1:141.4 Mb and chr 1:142.8 Mb) in eight of the cases. The 1q DBs were more heterogeneous, generally being more distal in the ALLs than in the BLs. Although no clear-cut clustering was observed, three of the ALLs harbored DBs close to the telomere, at chr 1:245 Mb, whereas all four BLs had breakpoints between chr 1:176 Mb and chr 1:205 Mb (Table 2). The duplicated regions were hence larger in the ALLs (68.1–103.7 Mb) than in the BLs (35.4–63.6 Mb); the minimally gained segment in the ALLs was 57.4 Mb (chr 1:153.5–210.9 Mb), corresponding to dup(1)(q22q32.3), and 35 Mb (chr 1:141.8–176.8 Mb) in BLs, corresponding to dup(1)(q12q25.2) (Table 2; Figs 1 and 2).

Excluding CNPs and immunoglobulin rearrangements, additional cryptic, partial genomic imbalances were detected in two of the six ALLs, namely dup(4)(p16.2p16.3) and del(7)(q34) in case 1 and del(9)(q22.3q22.3) and del(12)(p12.1p13.2) in case 2 (Tables 1 and 2). In addition, the unbalanced 1;5 translocation in case 5 could be revised to der(5)t(1;5)(q22;q21.3) since the array CGH analysis revealed that the terminal deletion of 5q started in sub-band 5q21.3. Further FISH mapping of the 5q breakpoint, using the bacterial artificial chromosome probes RP11-541H04, RP11-505D04 and RP11-345I03 (BACPAC Resources, Oakland, CA, USA) revealed the presence of the first probe, located upstream of the *EFNA5* gene, weak signal of the second probe, covering *EFNA5* and loss of signal of the third probe, located downstream of the gene, strongly suggesting rearrangement of the *EFNA5* gene (data not shown). Despite several attempts to amplify a putative *EFNA5* fusion transcript using 5'-rapid amplification of cDNA ends (Clontech, Mountain View, CA, USA) no such chimera was obtained. The 7p breakpoint in the idic (7)(p11.2) in case 6, previously mapped in detail by locus-specific FISH (29), was confirmed by array CGH. Among the four BLs, cryptic imbalances were identified in two of them—dup(13)(q31.1q32.3) in case 8 and dup(X)



**Figure 1.** Gain of 1q in high hyperdiploid pediatric ALLs. (A) Cytogenetic imbalance map based on chromosome banding analyses of 48 cases reported in the literature (1) and unpublished cases from our department. (B) Representative example of array CGH 1q findings in ALL (case 1). (C) Frequency plot based on array CGH analysis of the 1q gains in cases 1–6.

(q28q28), dup(8)(q24.2q24.3), dup(14)(q32.3q32.3) and dup(18)(p11.2p11.3) in case 10. The presence of dup(8) and dup(14), involving regions in which *MYC* and some of the *IGH* genes map, in the latter case revealed that the cytogenetically balanced t(8;14) was unbalanced on the molecular genetic level. The add(17)(p13) in case 10 most likely represented a der(17)t(17;18)(p13.2;p11.2), considering the observed imbalances of these two chromosome arms. Unfortunately, lack of material precluded FISH confirmation.

#### Methylation status of sat II DNA

The methylation pattern of sat II DNA was investigated in a total of eight ALLs, at diagnosis and during remission; four of these ALLs harbored gain of 1q. Similarly, seven BLs, four of which with dup(1q), were also analyzed at diagnosis and during remission. Compared with a positive control for sat II hypomethylation and to normal peripheral blood DNA, the diagnostic and remission samples from all 15 cases studied showed a sat II methylation status similar to that observed in normal blood, and there were no clear-cut differences in intensities between the diagnostic and remission samples (Fig. 4). Hence, there was no evidence for sat

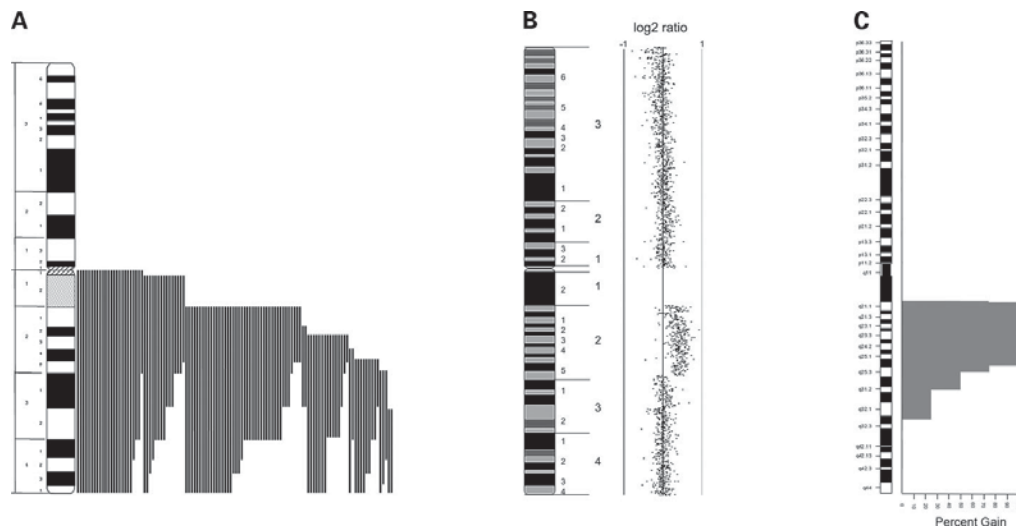
II undermethylation in ALLs and BLs with or without 1q gains.

#### Expression patterns of genes in dup(1q)

The cDNA microarray analyses of 29 high hyperdiploid ALLs revealed that a large number of genes, mapping in the minimally gained 1q region identified herein (1q22–32.3), were highly expressed, irrespective of the genomic 1q status. In order to find genes in this chromosomal segment that were overexpressed in the dup(1q)-positive ALLs only, a *t*-test was performed, identifying six genes to be differentially expressed (*B4GALT3*, *CRB1*, *DAP3*, *RGS16*, *TMEM183A* and *UCK2*). All but one (*CRB1*) was highly expressed in the ALLs with 1q gain. Two of the genes were among the top 15 most highly expressed genes in the single dup(1q)-positive BL case analyzed, namely *DAP3* (rank 12) and *UCK2* (rank 3). The expression findings are summarized in Figure 5.

#### DISCUSSION

As expected, the array CGH analyses identified several imbalances in regions known to harbor CNPs (30) as well as



**Figure 2.** Gain of 1q in BLs. (A) Cytogenetic imbalance map based on G-banding analyses of 116 cases reported in the literature (1) and unpublished cases from our department. (B) Representative example of array CGH 1q findings in BL (case 8). (C) Frequency plot based on array CGH analysis of the 1q gains in cases 7–10.

**Table 1.** Clinical and cytogenetic features of the six high hyperdiploid ALLs and the four BLs with gain of 1q

Case	Diagnosis	Sex/age	Karyotypes based on chromosome banding (top row) and array CGH (lower row) analyses <sup>a</sup>
1 <sup>b,c</sup>	ALL	M/7	50-54, XY, +4, +6, +10, +14, +17, +18, +20, +20, +mar 55, XY, +X, dup(1)(q21.1q42.1), +4, dup(4)(p16.2p16.3), +6, del(7)(q34), +10, <b>del(15)(q11.2q11.2)</b> , +17, +18, +18, <b>del(19)(p13.2p13.2)</b> , +21, +21
2 <sup>b</sup>	ALL	M/11	57, XY, der(1)t(1;1)(p36;q21), +4, +6, +14, +17, +21, +21, inc 57, XY, +Y, dup(1)(q21.2q44), del(2)(p11.2p11.2), +4, +4, <b>del(4)(p11p11)</b> , +6, +8, del(9)(q22.3q22.3), +10, <b>del(11)(p14.1p14.1)</b> , <b>del(11)(q22.1q22.1)</b> , del(12)(p12.1p13.2), +14, +17, +18, +21, +21, <b>del(22)(q11.2q11.2)</b>
3 <sup>b</sup>	ALL	F/3	56, XX, +X, dup(1)(q?), +4, +6, +8, +10, +14, +14, +18, +21, +21 56, XX, +X, dup(1)(q21.1q32.3), del(2)(p11.2p11.2), +4, +6, <b>dup(7)(q22.1q22.1)</b> , +8, +10, +14, +14, +18, +21, +21
4 <sup>b</sup>	ALL	M/3	52, XY, +X, dup(1)(q22q42), +6, +10, +11, +21, +21 52, XY, +X, dup(1)(q21.1q42.1), +6, <b>del(8)(p23.2p23.2)</b> , +10, +11, <b>dup(11)(p15.5p15.5)</b> , <b>dup(20)(q13.3q13.3)</b> , +21, +21
5 <sup>b</sup>	ALL	M/2	61-63, XXY, +Y, -1, -2, -3, der(5)t(1;5)(p?;q?), -7, -13, +14, -15, -16, -19, -20, +21 61-63, XXY, +Y, -1, dup(1)(q22q44), -2, -3, del(5)(q21.3), -7, -13, +14, -15, <b>del(15)(q11.2q11.2)</b> , -16, <b>del(16)(p13.3)</b> , -19, -20, +21
6 <sup>b,c</sup>	ALL	M/3	54-55, XY, +X, +4, +6, idic(7)(p11), +8, +10, +14, +17, +21, inc 54, XY, +X, dup(1)(q12q44), <b>dup(2)(p11.2p11.2)</b> , +4, +5, +6, del(7)(p11.2), dup(7)(q11.2q36.3), +8, +10, +14, <b>del(14)(q11.1q11.2)</b> , del(14)(q32.3q32.3), +17, +21
7	BL	M/7	46, XY, dup(1)(q25q42), t(8;14)(q24;q32)/46, XY, ?dup(1)(q21q11), t(8;14) 46, XY, dup(1)(q12q31.2), <b>dup(2)(p11.2p11.2)</b>
8	BL	F/39	46, XX, t(8;14)(q24;q32), der(13)t(1;13)(q21;q32)/46, idem, der(1)dup(1)(q?)add(1)(q42) 46, XX, dup(1)(q12q25.3), del(2)(p11.2p11.2), <b>del(4)(p16.3)</b> , <b>del(5)(q31.3q31.3)</b> , <b>del(8)(q21.2q21.2)</b> , <b>del(10)(q26.3)</b> , <b>del(11)(p15.5)</b> , dup(13)(q31.1q32.3), del(13)(q32.3), del(14)(q32.3), <b>dup(15)(q11.1q11.2)</b> , <b>del(22)(q11.2q11.2)</b>
9	BL	F/38	46, XX, dup(1)(q31q44), der(14)t(8;14)(q24;q32) 46, XX, dup(1)(q12q32.1), del(2)(p11.2p11.2), <b>dup(7)(q22.1q22.1)</b> , dup(8)(q24.2q24.3), del(14)(q32.3), <b>del(15)(q11.1q11.2)</b>
10	BL	M/45	46, XY, dup(1)(q25q31), t(8;14)(q24;q32), add(17)(p13) 46, dup(X)(q28q28), Y, dup(1)(q12q25.2), del(2)(p11.2p11.2), dup(8)(q24.2q24.3), dup(14)(q32.3q32.3), del(14)(q32.3), del(17)(p13.2), dup(18)(p11.2p11.3), <b>del(22)(q11.2q11.2)</b>

<sup>a</sup>Abnormalities in bold are suspected CNPs.

<sup>b</sup>Original karyotype previously published in (29,60–62).

<sup>c</sup>The gain of 1q in these cases was identified by SNP array analyses (unpublished data).

Table 2. Imbalances detected by array CGH in the 10 analyzed cases

Chromosome band <sup>a</sup>	Position (Mb)	Size (Mb)	Gain/Loss	Case(s)	Genes <sup>b</sup>	Clone <sup>c</sup>
1q12-q25.2	141.4-176.8	35.4	G	10		
1q12-q31.2	141.4-190.0	48.6	G	7		
1q12-q32.1	141.4-205.0	63.6	G	9		
1q12-q44	141.4-245.1	103.7	G	6		
1q12-q25.3	141.8-179.7	37.9	G	8		
1q21.1-q32.3	142.8-210.9	68.1	G	3		
1q21.1-q32.1	142.8-225.9	83.1	G	1,4		
1q21.2-q42.1	149.3-245.0	95.7	G	2		
1q22-q44	153.5-245.2	91.7	G	5		
2p11.2	87.2-88.6	1.4	G	6,7	<i>PLGLB1, PLGLB2, RGPDI, RGPD2, KRCC1, SMYDI, FABP1</i>	RP11-532020
2p11.2	88.8-88.9	0.1	L	2, 3, 8-10	<i>IGK@</i>	RP11-1517
4p16.3	0.0-1.0	1.0	L	8	<i>ZNF595, ZNF718, ZNF141, PIGG, PDE6B, ATP5I, MTL5, MFSDF7, PCGF3, CPLX1, GAK, DGKQ, IDUA, FGFRL1</i>	RP11-46704
4p16.2-p16.3	0.0-4.8	4.8	G	1	No known genes	RP11-77M9
4p11	48.9-49.5	0.6	L	2		RP11-445N8
5q21.3-q35.3	108.6-180.7	72.1	L	5		
5q31.3	140.0-140.4	0.4	L	8	<i>TMC06, NDUFA42, JK, WDR55, DND1, HARS, HARS1, ZMAT2, PCDHAI1-PCDHAI3, PCDHAC1, PCDHAC2</i>	RP11-171H22
7p11.2-p22.3	0.0-56.2	56.2	L	6		
7q11.2-q36.3	61.8-158.6	96.8	G	6		
7q22.1	101.7-101.9	0.2	G	3,9	<i>CUTL1, PRKRI1, ALKBH4</i>	RP13-771B7
7q34-q36.3	141.5-158.5	17.0	L	1		
8q23.2	3.7-5.2	1.5	L	4	<i>CSMD1</i>	RP11-626K14
8q21.2	86.6-87.0	0.4	L	8	<i>REXO1L1</i>	RP11-574H12
8q24.2-q24.3	128.8-146.2	17.4	G	9, 10	<i>BAX1, PTPDCC1, ZNF169, HIATL1, FBP2, FBP1, FANCC, PTCHI, HSD17B3, SLC35D2, ZNF367</i>	RP11-351M19
9q22.3	95.6-98.2	2.6	L	2		
10q26.3	133.6-135.4	1.8	L	8	<i>PPP2R2D, BNIP3, DPYSL4, STK32C, LRRRC27, PWWP2, INPP4, GPR123, KNDCl, UTF1, FENIX, ADAM8, TUBGCP2, ZNF511, DRD1P, PRAP1, ECHS1, PAOX, MTGI, SPRN, CYP2E1, SYCE1, DUX4, FRG2</i>	RP11-692I8
11p15.5	0.0-2.7	2.7	G/L	4,8	<i>SCGB1C1, ODF3, BET1L, RC8A, SIRT3, PSMD13, NALP6, ATH1L1, IFITM1-IFITM3, IFITM5, B4GALNT4, PKP3, SIGIRR, TMEM161, PTDSS2, RNH1, HRAS, LRRRC56, RASSF7, IRF7, MUCDHL, SCT, DRD4, DEAF1, TMEM480, EPRS92, TALDO1, PDDC1, RM88, LRDD, CEND1, LRDD, RPLP2, PNPLA2, EFCAB4A, CD151, POLR2L, TSPAN4, CHD1, AP2A2, MUC2, TOLLIP, BRK2, HCC42, DUSP8, FAM994, CTSD, SYR8, TNM2, LSP1, TNNT3, MRPL23, H19, IGF2, IGF2AS, INS, TH, ASCL2, TSPAN32, CD81, TSSC4, TRPM5, KCNQ1</i>	RP11-721I2
11p14.1	29.1-30.1	1.0	L	2	<i>KCNV4</i>	RP11-106M13
11q22.1	98.2-99.0	0.8	L	2	<i>CNTN5</i>	RP11-784P17
12p12.1-p13.2	10.4-22.8	12.4	L	2		
13q31.1-q32.3	85.2-100.3	15.1	G	8		
13q32.3-q34	100.3-114.1	13.8	L	8		
14q11.1-q11.2	18.1-19.4	1.3	L	6	<i>POTE14, OR11H2, OR4Q3, OR4Q3, OR4M1, OR4N2</i>	RP11-558C21
14q32.3	102.5-105.3	2.8	G	10	<i>CDCA2BP8, TNFAIP2, EEP3, MARK3, CBR, BAG3, KNS2, XRCC3, ZFYFE21, TDRD9, ADSS1, SIVA, AKTI, PLD4, CDCA44, GPR132, JAG2, IGHA1, IGHI</i>	RP11-68H21
14q32.3	105.3-106.3	1.0	L	6,8-10	<i>IGHG2, IGHA1, IGHI, IGH@</i>	RP11-761D24

Continued



Table 2. Continued

Chromosome band <sup>a</sup>	Position (Mb)	Size (Mb)	Gain/Loss	Case(s)	Genes <sup>b</sup>	Clone <sup>c</sup>
<b>15q11.1-q11.2</b>	18.3–22.9	4.6	G/L	1, 5, 8, 9		RP13-609E6
<b>16p13.3</b>	0.1–4.7	4.6	L	5		RP11-598P9
<b>17p13.2-p13.3</b>	0.0–4.9	4.9	L	10		RP11-229E7
<b>18p11.2-p11.3</b>	0.0–12.8	12.8	G	10		RP11-680A23
<b>19p13.2</b>	8.7–8.8	0.1	L	1		RP11-1021O15
<b>20q13.3</b>	61.2–62.4	1.2	G	4	OR2Z1, ZNF558, YTHDF1, BIRC7, ARFGAP1, COL20A1, CHRNA4, KCNQ2, EEF1A2, PTK6, SRMS, PRIC2A5, GAMEB2, STAM3, RTEL1, TNFRSF6B, ARFRP1, ZGPAT, LIME1, RTBD4, ZBTB46, TPD52L2, DNVA1C3, UCKL1, SAND10, PRPF6, PRP17, SOX18, TCEA2, RGS19, OPRLL1, NPBW2, MYT1, PCM2T2, GGT2, HIC2, MAPK1, PPM1F, TOP3B, IGLC1, PPREB1, SUSH2, PRAME, GGT4	
<b>22q11.2</b>	19.8–21.6	1.8	L	2, 8, 10	LCAP, AVPR2, ARHGAP4, ARD1A, RENBP, HCFCT, IRAK1, MECP2, OPN1LW, OPN1MW2, OPN1MH, TKTL1, FLNA, EMD, RPL10, DNASE1L1, TAZ, ATP6A1, GDI1, FAM30A, PLXNA3, LAGE3, UBL4A, FAM3A	RP11-757E24
Xq28	152.8–153.4	0.6	G	10		RP11-333O6

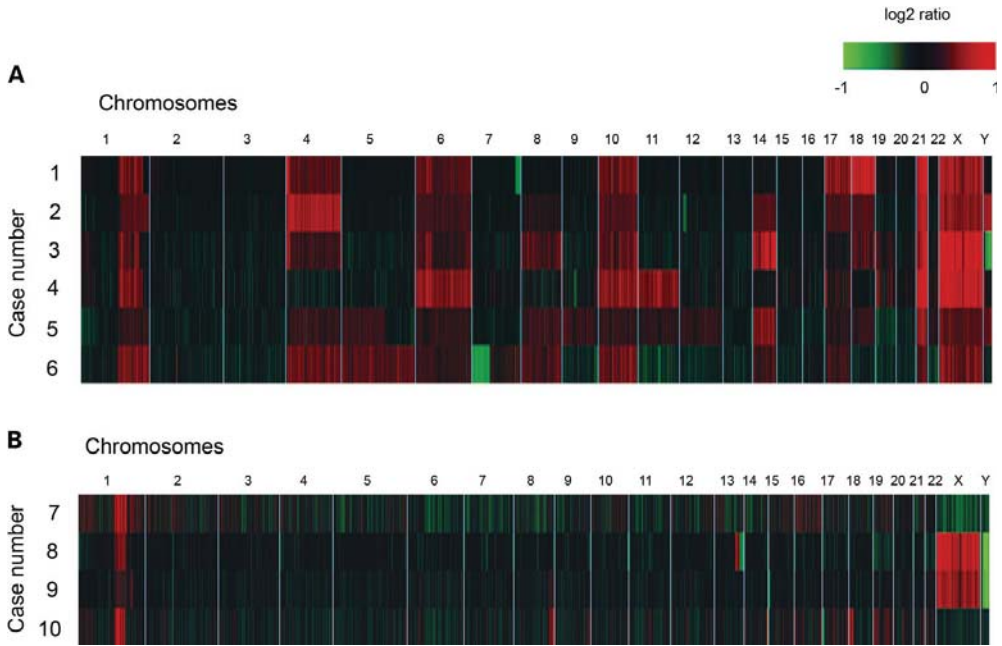
<sup>a</sup>Chromosome bands in which CNPs have been described in two or more studies reported in the Database of Genomic Variants (<http://projects.tcag.ca/variation/>) are denoted in bold. CNPs were only considered for segments  $\leq 3$  Mb.

<sup>b</sup>Genes present in the region according to the USCS Genome Browser (<http://genome.ucsc.edu/>). Genes were only listed for segments  $\leq 3$  Mb.

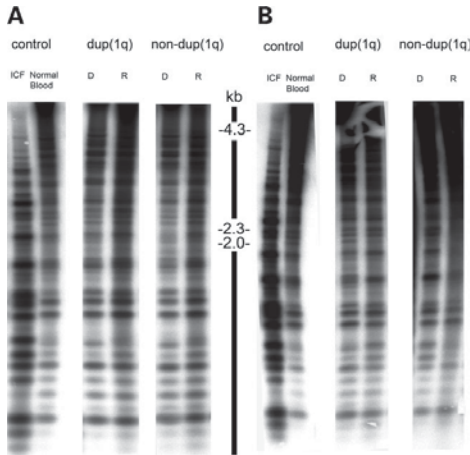
<sup>c</sup>A representative clone is only given for segments  $\leq 5$  Mb.

deletions involving the *IGK@* and *IGH@* genes, the latter probably representing somatic immunoglobulin rearrangements clonotypic for the malignant lymphoid cells (31); these changes most likely did not contribute to the pathogenesis of the malignant disorders. On the other hand, submicroscopic, putative pathogenic aberrations were found in two of four BLs and two of six pediatric ALLs. Similar high frequencies of additional changes, using array CGH, have previously been reported in high hyperdiploid ALLs (27,32) and in BLs (33,34). As regards BLs, none of the cryptic changes identified so far have been recurrent and their molecular genetic consequences remain to be elucidated, as do their clinical impact. Including the present series, array CGH findings in 15 high hyperdiploid ALLs, 12 of which analyzed with the 32k array set, have now been reported (27,32). Among these, a total of 14 additional genomic imbalances have been observed in seven cases. To date, only one change has been recurrent, namely a deletion of 12p including, among other genes, *ETV6*. Interestingly, very recently an SNP array analysis of 39 high hyperdiploid ALLs revealed *ETV6* deletions in two of the cases (35). Thus, cryptic hemizygous loss of this gene occurs in 5–10% of pediatric high hyperdiploid ALLs, suggesting that haploinsufficiency or loss of tumor suppressor function of *ETV6* may play a role in this common childhood leukemia.

The 1q PBs mapped close to the centromere in all BLs and ALLs investigated (Table 2 and Figs 1–3), a finding similar to what has been reported for other tumor types with 1q duplications (1). In the recent SNP array analysis of pediatric ALLs (35), approximately 30% of the high hyperdiploid cases were reported to harbor 1q gains. By using the publicly available dataset (<http://ftp.stjude.org/pub/data/ALL-SNP1/>) and the dchip software (36), the 1q PBs were extracted and shown to localize in the same region as the PBs in the present ALLs (data not shown). However, it should be noted that due to the repetitive nature of DNA sequences in this segment, the SNP density is lower than elsewhere in the genome, making accurate mapping difficult. This notwithstanding, the available data strongly indicate that the near-centromeric region of 1q is breakpoint. The reason(s) for this is presently unknown, although it has been hypothesized that demethylation of the pericentric heterochromatin is associated with decondensation and subsequent instability of satellite sequences, resulting in 1q rearrangements (16), with several studies having reported undermethylation of sat II DNA in different neoplastic disorders (12–15). However, we found no evidence for hypomethylation of sat II in the present series of dup(1q)-positive BLs and ALLs (Fig. 4). Thus, we deem it highly unlikely that aberrant methylation of this region is the underlying mechanism behind dup(1q), at least not in BLs and ALLs. Instead, the genomic architecture as such, in particular segmental duplications, could be responsible. In fact, the presence of low-copy repeats has been implicated in the formation of both constitutional and neoplasia-associated chromosomal abnormalities (37–39). Interestingly, such duplications are very common immediately distal to highly repetitive satellite DNA close to the centromere, such as sat II (40), and recently (41–43), segmental duplications have been mapped to the 1q region in which the PBs occurred in our cases (Table 2). It is hence tempting



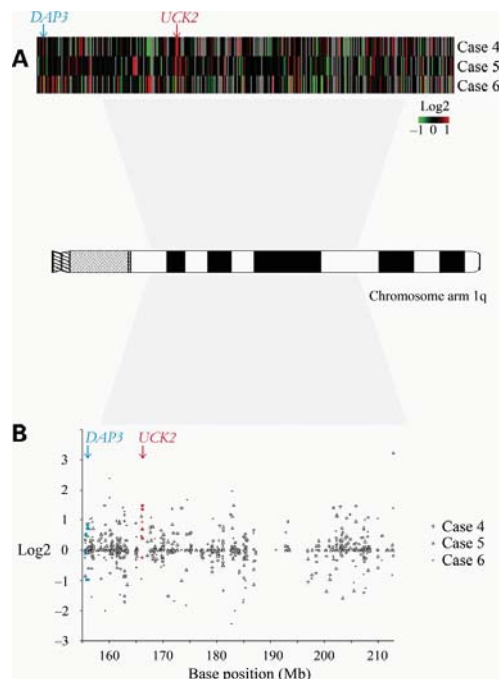
**Figure 3.** Heat map of genomic imbalances detected by array CGH analyses of six high hyperdiploid ALLs (cases 1–6) and four BLs (cases 7–10) with dup(1q).



**Figure 4.** Representative Southern blot analyses of sat II DNA methylation with *Bst*BI, using DNA from ICF B-cells as a positive control for hypomethylation and DNA from normal peripheral blood as a reference and the sat II oligonucleotide probe. (A) Examples of high hyperdiploid ALLs, one with and one without dup(1q), at diagnosis (D) and at remission (R). (B) Similar examples of BLs.

to speculate that the dup(1q) in BLs and ALLs arises as a consequence of these rearrangement hotspots.

The 1q DBs were clearly more variable than the proximal ones, strongly indicating that there is not a common break-prone segment on distal 1q; in fact, none of the DBs (Table 2) are located in regions known to harbor such hotspots (42). Furthermore, the DBs also differed between the BLs and the ALLs, generally being more distal in the latter group (Table 2 and Figs 1–3). Thus, the minimally gained segment was larger in the ALLs (57.4 Mb) than in the BLs (35 Mb), corresponding to dup(1)(q22q32.3) and dup(1)(q12q25.2), respectively. Interestingly, in a previous array CGH analysis of high hyperdiploid ALLs (27) one case, without a cytogenetically identified dup(1q), harbored a submicroscopic 0.6 Mb gain of 1q22, close to the minimally gained segment identified herein. It has been reported that tumors arising in nude mice inoculated with a human leukemic B-cell clone carrying dup(1)(q11q32) were more tumorigenic, grew faster and resulted in more metastases than did those occurring in mice inoculated with clones harboring other chromosomal abnormalities, suggesting that gain of this 1q segment provides a proliferative advantage (44). It is noteworthy that the minimally gained regions in both the BLs and the ALLs are included in that segment. Hence, one or several genes mapping to the minimally gained region are potentially pathogenetically important.



**Figure 5.** Expression profile of cDNA clones in the minimally gained 1q region in dup(1q)-positive high hyperdiploid ALLs. (A) Heat map of expressed cDNA clones in cases 4–6, with the location of the *DAP3* and *UCK2* genes, overexpressed also in a dup(1q)-positive BL, indicated. (B) Plot of cDNA clones in relation to Log<sub>2</sub> ratios and genomic position on 1q. The *DAP3* and *UCK2* harboring clones are indicated in blue and red, respectively.

The microarray analyses revealed that six genes, namely *B4GALT3*, *CRB1*, *DAP3*, *RGS16*, *TMEM183A* and *UCK2*, were significantly differentially expressed in the dup(1q)-positive ALLs compared with the other high hyperdiploid cases. Surprisingly, one of these, the *CRB1* gene, was underexpressed. Thus, gain of chromosomal material does not necessarily lead to overexpression of all the duplicated genes, as previously reported also in other tumor types (45,46). As regards the five up-regulated genes, two of them, i.e. *DAP3* and *UCK2* (Fig. 5), were among the most overexpressed genes in the BL case with gain of 1q. Interestingly, the *DAP3* (death associated protein 3) protein, which, if intact, is normally proapoptotic, has been reported to be highly expressed in invasive glioblastoma multiforme cells (47), whereas expression of the *UCK2* protein has been correlated with sensitivity to a few anticancer drugs, i.e. certain inhibitors of RNA polymerases (48). However, the leukemogenic impact of these genes in dup(1q)-positive high hyperdiploid ALLs and BLs remains to be elucidated.

In conclusion, the salient findings in the present study of dup(1q)-positive BLs and pediatric high hyperdiploid ALLs,

using tiling resolution array CGH, methylation and expression analyses, were that the PBs clustered close to the centromere in both these disorders, the DBs were more heterogeneous, the minimally gained region was much larger in the ALLs than in the BLs, the sat II domain was not hypomethylated, several additional changes were observed and that five genes on 1q were significantly overexpressed in the dup(1q)-positive cases compared with ALLs without gain of 1q.

## MATERIALS AND METHODS

### Patients

The array CGH study comprised six cases of B-cell precursor childhood high hyperdiploid ALL (five males, one female; age 2–11 years) and four cases of BL (two males, two females; age 7–45 years) (Table 1), all of which harbored 1q gains in the form of dup(1q) or unbalanced translocations involving 1q. Because of lack of material, FISH characterization of the 1q gains could only be performed in case 5. In the subsequent methylation investigations, diagnostic and remission DNA samples from cases 1–4 and 7–10 as well as from four additional high hyperdiploid ALLs and three BLs, without 1q gains, were analyzed. The gene expression patterns of cases 4–6 and 7 were extracted from a previously reported dataset (49).

### Cytogenetic mapping of 1q gains

The Mitelman Database of Chromosome Aberrations in Cancer (1) was used to retrieve all cytogenetically characterized BLs and pediatric high hyperdiploid ALLs with 1q gains reported in the literature. In addition, unpublished cases from our department were included. In total, 116 BLs and 48 ALLs were identified. The PBs and DBs and the corresponding genomic imbalances were ascertained in all these cases.

### Array CGH and gene expression analyses

DNA was extracted from bone marrow or peripheral blood cells obtained at the time of diagnosis and stored at  $-80^{\circ}\text{C}$ . Male genomic DNA (Promega, Madison, WI, USA) was used as reference in all hybridizations. The 32k slides, containing 32 433 tiling bacterial artificial chromosome clones covering at least 98% of the human genome, were produced at the SWEGENE DNA microarray resource center at Lund University, Sweden. Labeling of DNA, slide preparation and hybridization were performed as described with minor modifications (50). Analyses of the microarray images were performed with the GenePix Pro 4.0 software (Axon Instruments, Foster City, CA, USA). For each spot, the median pixel intensity minus the median local background for both dyes was used to obtain the ratio of test gene copy number to reference gene copy number. Data normalization was performed for each array subgrid using lowess curve fitting with a smoothing factor of 0.33 (51). For all cases, the sex chromosomes and, for the high hyperdiploid ALLs, also the tri- and tetrasomic chromosomes were excluded when calculating the correction factor in the normalization. All analyses were performed in the Bioarray software environment (BASE) database

(52). To identify imbalances, the MATLAB toolbox CGH plotter (53), the SeeGH software (54) and the TM4 software suite (55) were applied using moving mean average over three clones and log<sub>2</sub> limits of >0.2. Classification as gain or loss was based on identification as such by the CGH plotter and also by visual inspection of the log<sub>2</sub> ratios. Ratios >0.5 in five adjacent clones were classified as aberrant, with ratios 0.5–1.0 interpreted as duplications/hemizygous deletions and >1.0 as amplifications/homozygous deletions.

The cDNA microarray analyses have previously been reported (49). In short, samples from 87 pediatric B-lineage ALLs, including 29 high hyperdiploid ALLs and two BLs, were hybridized to 27k microarray slides containing 25 648 cDNA clones (Swegene DNA Microarray Resource Center) representing 13 737 Unigene clusters and 11 592 Entrez gene entries, according to the Unigene build 195. RNA extraction, amplification, labeling, hybridization, scanning, post-hybridization washing and feature analysis were performed as described (49). The data analyses were all performed in BASE and in the CGH-Explorer software, and when investigating chromosome-specific expression patterns, genes were mean-centered and analyzed with a BASE implementation of the MATLAB toolbox CGH-plotter (53,56). Three of the six high hyperdiploid ALLs (cases 4–6) and one of the four BLs (case 7) with dup(1q) in the present study were included in the dataset. To identify differentially expressed genes in the dup(1q)-positive ALLs, a *t*-test comparing high hyperdiploid ALLs with and without gain of 1q was performed on the expression patterns of genes in the minimally gained 1q region. *P*-values <0.05 were considered significant.

### Methylation analysis

Sat II methylation status was investigated by Southern blot analysis as described (12). Two micrograms of DNA were digested with 20 U of the CpG methyl-sensitive restriction enzyme *Bst*BI (New England BioLabs, Ipswich, MA, USA) for 18 h in standard conditions. The digested and fractionated DNA was blotted on a GeneScreen Plus hybridization transfer membrane (Perkin-Elmer, Waltham, MA, USA). The sat II probes used were an 18-mer single-stranded oligonucleotide, with the consensus sequence 5'-TCGAGTCCATTTCGAT GAT-3' (57), and a cloned *Chr1*-specific insert excised from the recombinant plasmid pUC1.77 (58). Hybridization was performed in Rapid-Hyb buffer (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ, USA) at 42°C for the oligonucleotide probe and at 65°C for the cloned DNA probe. The oligonucleotide probe was radiolabeled with 5'-[γ-<sup>32</sup>P]-end dATP and the cloned *Chr1*-specific insert with α-<sup>32</sup>P-dCTP (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ, USA). In each Southern blot analysis, normal peripheral blood and ICF B-lymphocyte (59) (Corriell Cell Repositories, Camden, NJ, USA) DNA was included as methylated and hypomethylated controls, respectively. Using a FLA 3000 phosphorimager (Fujifilm Corporation, Tokyo, Japan), the approximate amount of methylation was assessed by inspection, comparing the intensities of the hybridized fragments.

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*Conflict of Interest statement.* None declared.

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# The DNA methylome of pediatric acute lymphoblastic leukemia

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Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy, with high hyperdiploidy [51–67 chromosomes] and the t(12;21)(p13;q22) [*ETV6/RUNX1* fusion] representing the most frequent abnormalities. Although these arise *in utero*, there is long latency before overt ALL, showing that additional changes are needed. Gene dysregulation through hypermethylation may be such an event; however, this has not previously been investigated in a detailed fashion. We performed genome-wide methylation profiling using bacterial artificial chromosome arrays and promoter-specific analyses of high hyperdiploid and *ETV6/RUNX1*-positive ALLs. In addition, global gene expression analyses were performed to identify associated expression patterns. Unsupervised cluster and principal component analyses of the chromosome-wide methylome profiles could successfully subgroup the two genetic ALL types. Analysis of all currently known promoter-specific CpG islands demonstrated that several B-cell- and neoplasia-associated genes were hypermethylated and underexpressed, indicating that aberrant methylation plays a significant leukemogenic role. Interestingly, methylation hotspots were associated with chromosome bands predicted to harbor imprinted genes and the tri-/tetrasomic chromosomes in the high hyperdiploid ALLs were less methylated than their disomic counterparts. Decreased methylation of gained chromosomes is a previously unknown phenomenon that may have ramifications not only for the pathogenesis of high hyperdiploid ALL but also for other disorders with acquired or constitutional numerical chromosome anomalies.

## INTRODUCTION

The two most common genetic subgroups of pediatric B-cell precursor acute lymphoblastic leukemia (ALL) are the ones represented by t(12;21)(p13;q22), leading to the *ETV6/RUNX1* fusion, or high hyperdiploidy [51–67 chromosomes], characterized by tri-/tetrasomies involving mainly chromosomes X, 4, 6, 8, 10, 14, 17, 18 and 21 (1). Although these genetic aberrations most likely are of utmost importance for leukemogenesis, there is ample evidence that they are not sufficient for leukemic transformation (2). The strongest support in favor of this has come from studies identifying *ETV6/RUNX1* or high hyperdiploidy in neonatal blood spots from children who later developed ALL. Thus, these changes occur prenatally but secondary events are needed for overt disease. Regarding *ETV6/RUNX1*, frequent additional changes include losses of the *EBF1*, *ETV6* and *PAX5* genes and gains of chromosome 21 and chromosome arm Xq,

whereas common additional aberrations in high hyperdiploid ALL include duplication 1q, deletions of *CDKN2A* and *PAX5* and mutations of *FLT3*, *NRAS*, *KRAS* and *PTPN11* (3–8). Many of the secondary changes are genomic imbalances, aberrations that generally have been shown to affect gene dosage and lead to aberrant expression patterns (9). Gene dysregulation through epigenetic abnormalities may hence be an additional mechanism that also plays an important role. However, prior to the present study this has not been investigated in a detailed and genome-wide fashion in these common childhood ALL subtypes.

Hypermethylation of CpG islands situated within, or in close proximity of, promoters is an epigenetic modification frequently resulting in down-regulation or silencing of genes (10). To date, most methylation studies have been performed on individual genes in malignant solid tumors (11) (Supplementary Material, Table S1), and although several genes have been reported to be hypermethylated in B-lineage ALL (Supplementary Material,

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Tables S2 and S3), such as *CDKN2B* and *FHIT*, nothing is known about the complete methylomes in this disorder. During recent years, several platforms assessing global CpG island methylation patterns have been developed (11–13). Until now, however, these methods have mainly been used on cell lines (13–17), and even though such analyses are important as a proof of principle, cell lines may have significantly altered methylomes due to *in vitro* culturing (15,18). As regards primary pediatric B-lineage ALL, most previous studies on aberrant methylation patterns have only focused on a few or even single genes (Supplementary Material, Table S3). In fact, we know of only two studies utilizing array-based methodologies to identify differential methylation of a larger number of genes in B-lineage ALL, one screening close to 60 genes (17) and one investigating ~2300 genes (16); however, these studies included mainly adult patients. Thus, next to nothing is known about the DNA methylome of childhood ALL.

Herein, we investigated primary patient samples from the two most common genetic subgroups of pediatric ALL, namely *ETV6/RUNX1* and high hyperdiploidy (Table 1). We analyzed large-scale global methylation profiles of each chromosome as well as direct gene silencing through hypermethylation by the use of a microarray platform comprising a total of 28 226 CpG islands in order to delineate the DNA methylome and to identify target genes for hypermethylation. In addition, gene expression data based on cDNA microarrays (19) were used to investigate correlations between hypermethylated target genes and their expression levels. Furthermore, tiling resolution array comparative genomic hybridization (array CGH) (20) was performed on each case to detect genomic imbalances that might affect gene expression and/or be associated with large-scale methylation changes.

**RESULTS**

**Hypermethylated genes**

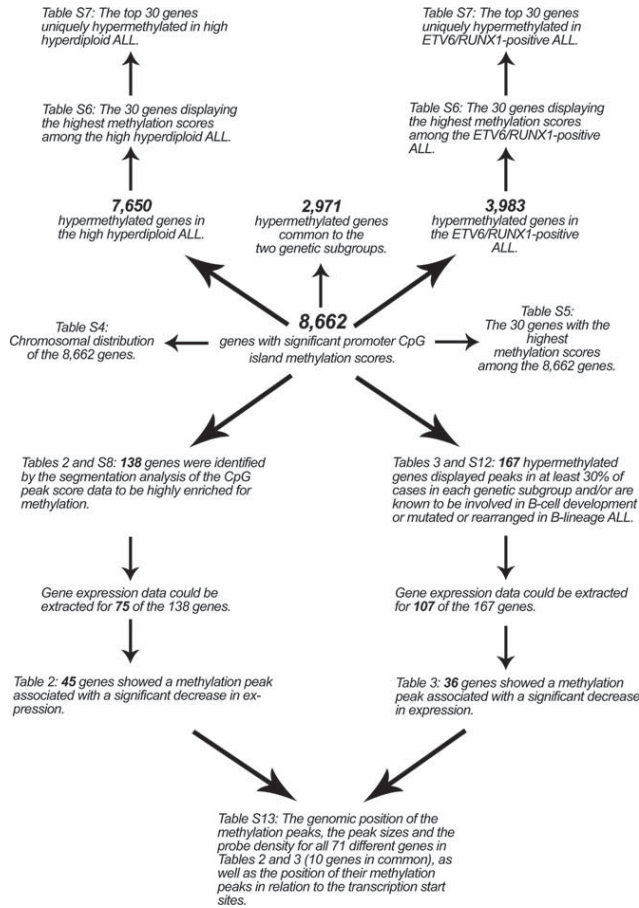
A total of 8662 different genes with significant 5' promoter CpG island methylation scores were identified by the CpG promoter array, constituting ~30% of all genes present on this platform. As seen in the flow chart in Figure 1, the high hyperdiploid ALLs harbored more hypermethylated genes than the *ETV6/RUNX1*-positive cases (7650 versus 3983), with 2971 being common to the two groups. The hypermethylated genes were distributed on all chromosomes, without any clear association with chromosomes frequently gained in *ETV6/RUNX1*-positive or high hyperdiploid ALLs (Supplementary Material, Table S4). The 30 genes displaying the highest methylation scores among all the 8662 hypermethylated genes are listed in Supplementary Material, Table S5 and the top 30 scored genes in the two genetic subtypes are given in Supplementary Material, Table S6 which includes six genes common to both cytogenetic subgroups. Supplementary Material, Table S7 lists only the top genes uniquely hypermethylated in each ALL subtype. As seen in these tables, the largest gene ontology (GO) group was transcription regulation.

Segmentation analysis of the CpG peak score data of the 8662 genes (see above and Fig. 1) identified 138 genes

**Table 1.** Clinical and cytogenetic features of the 20 analyzed pediatric ALLs with *ETV6/RUNX1* or high hyperdiploidy

Case no.	Sex/age (years)	Genetic subgroup	Genomic imbalances based on G-banding and array comparative genomic hybridization
1	M/5	<i>ETV6/RUNX1</i>	dup(X)(q21.31q28),dup(5)(q34q35.3),del(6)(q14.1q27),del(11)(q14.1q25)
2	M/9	<i>ETV6/RUNX1</i>	dup(12)(p13.2p13.33),dup(21)(q11.2q22.12)
3	M/5	<i>ETV6/RUNX1</i>	dup(X)(q25q28)
4	F/4	<i>ETV6/RUNX1</i>	del(6)(q14.1q22.1),del(6)(q24.3q27),dup(8)(q13.3q24.3),dup(12)(p13.2p13.33),del(13)(q13.1q31.1),dup(21)(q11.2q22.12)
5	M/8	<i>ETV6/RUNX1</i>	del(12)(p12.3p12.32),del(19)(q13.32q13.33)
6	M/8	<i>ETV6/RUNX1</i>	dup(10)(p11.1p15.3),+16,+21
7	F/3	<i>ETV6/RUNX1</i>	+X,del(18)(p11.23p11.32),dup(18)(p11.1p11.23),dup(18)(p11.1q23),+21
8	F/7	<i>ETV6/RUNX1</i>	+X,del(1)(p36),+5,del(6)(q21),del(11)(p11),-12,del(21)(q22)
9	M/9	<i>ETV6/RUNX1</i>	dup(X)(q25q28),dup(12)(p13.2p13.33),dup(21)(q11.2q22.12)
10	M/14	<i>ETV6/RUNX1</i>	+5,del(9)(p21.3p21.3),del(9)(p21.3p22.1),dup(12)(p13.2p13.33),del(12)(p12.1p12.12),dup(21)(q11.2q22.12)
11	M/10	High hyperdiploid	+X,+6,del(9)(p11.1p24.3),+10,+14,dup(14)(q11.2q11.2),+17,+18,+21,+21
12	M/3	High hyperdiploid	+X,+4,+6,+7,+10,+14,+17,+18,+21
13	F/3	High hyperdiploid	+X,+4,+6,+14,+17,+18,+21,+21
14	F/1	High hyperdiploid	+X,+6,+8,+14,+17,+18,+21,+21
15	M/1	High hyperdiploid	+X,+4,+6,+14,+17,+18,+21,+21
16	F/16	High hyperdiploid	+X,+4,+5,+6,+7,+8,del(9)(p21.3p21.3)X2,+10,+14,+17,+18,+21,+21
17	F/4	High hyperdiploid	+X,+4,+6,+14,+17,+18,dup(18)(q22q23),+21,+21
18	M/2	High hyperdiploid	+X,+Y,dup(1)(q22q44),+4,dup(5)(p11p15.3),dup(5)(q11q21.2),+6,+8,+9,+10,+11,+12,+14,+14,+17,+18,+21,+21,+22
19	F/4	High hyperdiploid	+X,+4,+8,+10,+18,+21
20	F/6	High hyperdiploid	+X,+4,+6,+14,+17,+21

The original karyotypes of all cases and the array CGH data on cases 1–10, 12 and 18 have previously been reported (5,7,8).



**Figure 1.** Flow chart showing how the 8662 genes with significant promoter CpG island methylation scores were grouped according to genetic ALL subtype, chromosomal distribution, peak scores and effect on gene expression.

that were highly enriched for methylation (Supplementary Material, Fig. S1). These genes could be subgrouped into seven GO groups, with the most common being cell signaling, transcription regulation and apoptosis. Gene expression data, corresponding to log<sub>2</sub> ratios for >50% of cases in each genetic subgroup, could be extracted for 75 of the 138 genes. It was apparent that enrichment for methylation not necessarily resulted in decreased expression. In fact, 11 of the 75 genes displayed a higher mean expression level in cases with methylation peaks than in those without such peaks, whereas 19 did not differ substantially between methylated and non-methylated cases (Supplementary Material, Table S8). However, the majority (45 genes; 60%) showed a peak associated with a significant decrease in expression (Table 2).

A total of 117 genes displayed peaks in ≥30% of cases in each genetic subgroup, and among the 236 different genes listed in Supplementary Material, Tables S1, S2 and S9–S11, 97 displayed CpG island peaks, 47 of which were also included among the 117 methylated genes identified earlier. Thus, a total of 167 different recurring genes and/or genes involved in B-cell development or mutated or rearranged in B-lineage ALL were found to be targets for hypermethylation. Only 18 of these were among the 138 genes found by the segmentation analysis (see above and Fig. 1). The 167 genes could be subgrouped into nine specific GO groups, with the most common being transcription regulation, apoptosis and cell signaling (Supplementary Material, Fig. S2). Gene expression data could be extracted for 107 of the 167 genes. Thirteen of the 107 genes displayed a higher mean expression

**Table 2.** The 45 genes shown by segmental analysis to be highly enriched for methylation and that displayed significantly<sup>a</sup> decreased expression in *ETV6/RUNX1*-positive and/or high hyperdiploid cases

Gene	Location <sup>b</sup>	Function <sup>b</sup>	Genetic subgroup <sup>c</sup>
<i>BRDT</i>	1p22.1	Transcription regulation	<i>ETV6/RUNX1</i>
<i>ETV1</i>	7p21.3	Transcription regulation	<i>ETV6/RUNX1</i>
<i>HLF</i>	17q22	Transcription regulation	High hyperdiploid
<i>PARP14</i>	3q21.1	Transcription regulation	High hyperdiploid
<i>SOX30</i>	5q33	Transcription regulation	<i>ETV6/RUNX1</i> , high hyperdiploid
<i>SSX1</i>	Xp11.22–11.23	Transcription regulation	<i>ETV6/RUNX1</i>
<i>SSX4</i>	Xp11.23	Transcription regulation	High hyperdiploid
<i>TULP4</i>	6q25–26	Transcription regulation	High hyperdiploid
<i>CSF2RA</i>	Xp22.32, Yp11.3	Cell signaling	<i>ETV6/RUNX1</i> , high hyperdiploid
<i>DUSP10</i>	1q41	Cell signaling	High hyperdiploid
<i>HRH1</i>	3p25	Cell signaling	High hyperdiploid
<i>PAQR3</i>	4q21.21	Cell signaling	High hyperdiploid
<i>PRKD3</i>	2p21	Cell signaling	High hyperdiploid
<i>PPF14A</i>	1q32.1	Cell signaling	High hyperdiploid
<i>PTPN3</i>	9q31	Cell signaling	<i>ETV6/RUNX1</i> , high hyperdiploid
<i>RGS12</i>	4p16.3	Cell signaling	High hyperdiploid
<i>BBC3</i>	19q13.3–13.4	Apoptosis	<i>ETV6/RUNX1</i>
<i>BOK</i>	2q37.3	Apoptosis	<i>ETV6/RUNX1</i> , high hyperdiploid
<i>CASP2</i>	7q34–35	Apoptosis	High hyperdiploid
<i>CASP9</i>	1p36.1–36.3	Apoptosis	<i>ETV6/RUNX1</i> , high hyperdiploid
<i>CD36</i>	7q11.2	Apoptosis	High hyperdiploid
<i>DBC1</i>	9q32–33	Apoptosis	<i>ETV6/RUNX1</i>
<i>FOXO3A</i>	6q21	Apoptosis	High hyperdiploid
<i>CD151</i>	11p15.5	Cell adhesion	<i>ETV6/RUNX1</i>
<i>CLDN1</i>	3q28–29	Cell adhesion	High hyperdiploid
<i>DMP1</i>	4q21	Cell adhesion	High hyperdiploid
<i>GNG4</i>	1q42.3	Cell growth	<i>ETV6/RUNX1</i>
<i>IGFBP6</i>	12q13	Cell growth	High hyperdiploid
<i>LTBP1</i>	2p21–22	Cell growth	<i>ETV6/RUNX1</i> , high hyperdiploid
<i>ARHGAP25</i>	2p14	Signal transduction	High hyperdiploid
<i>ARTN</i>	1p32–33	Signal transduction	High hyperdiploid
<i>CD2</i>	1p13	Signal transduction	High hyperdiploid
<i>CDK2AP1</i>	12q24.31	Cell cycle	High hyperdiploid
<i>PDPN</i>	1p36.21	Cell cycle	<i>ETV6/RUNX1</i> , high hyperdiploid
<i>BRC41</i>	17q21	DNA repair	High hyperdiploid
<i>CDC14B</i>	9q22.33	Protein dephosphorylation	<i>ETV6/RUNX1</i>
<i>CES2</i>	16q22.1	Metabolism	High hyperdiploid
<i>CPSF3L</i>	1p36.33	snRNA processing	<i>ETV6/RUNX1</i> , high hyperdiploid
<i>FAT</i>	4q35	Morphogenesis	<i>ETV6/RUNX1</i>
<i>FGFBP1</i>	4p15–16	Cell proliferation	High hyperdiploid
<i>HIST1H1A</i>	6p21.3	Nucleosome assembly	High hyperdiploid
<i>PLGLB1</i>	2p11.2	Unknown	High hyperdiploid
<i>SMURF1</i>	7q22.1	Cell differentiation	High hyperdiploid
<i>TRIM10</i>	6p21.3	Hematopoiesis	High hyperdiploid
<i>UCHL1</i>	4p14	Various	<i>ETV6/RUNX1</i> , high hyperdiploid

<sup>a</sup>Defined as a log<sub>2</sub> difference of at least 0.4 compared with the mean expression in cases without a methylation peak.

<sup>b</sup>Function and location are based on data in NCBI Entrez Gene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>). Only one major function per gene is given.

<sup>c</sup>As identified by the segmentation analysis.

level in cases with methylation peaks than in those without such peaks and 58 did not differ between methylated and non-methylated cases (Supplementary Material, Table S12). The remaining 36 (34%) genes showed a methylation peak associated with a significant decrease in expression (Table 3).

The genomic positions of the methylation peaks, the peak sizes, the probe densities and the positions of the peaks in relation to the transcription start sites of all genes listed in Tables 2 and 3 are given in Supplementary Material, Table S13.

### DNA methylation hotspots throughout the genome

A total of 58 different regions/hotspots with a peak score density higher than four hypermethylated genes per 4 Mb

was identified, with 30 of these being common to both *ETV6/RUNX1*-positive and high hyperdiploid ALLs. The *ETV6/RUNX1*-positive ALLs harbored eight unique methylation hotspots, whereas the high hyperdiploid ALLs had 20 unique methylation hotspots (Fig. 2, Supplementary Material, Fig. S3).

### Clustering analysis of chromosome-wide methylomes and gene expression data

Unsupervised clustering and principal component analyses (PCA) of the bacterial artificial chromosome (BAC) array chromosome-wide methylome profiles could successfully subgroup the majority of *ETV6/RUNX1*-positive and high

**Table 3.** The 36 hypermethylated genes recurrently methylated and/or involved in B-cell development or mutated or rearranged in B-lineage ALL that displayed significantly<sup>a</sup> decreased expression in *ETV6/RUNX1*-positive and/or high hyperdiploid cases

Gene	Location <sup>b</sup>	Function <sup>b</sup>	Genetic subgroup	Inclusion criterion
<i>BBC3</i>	19q13.3–13.4	Apoptosis	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated
<i>BCL10</i>	1p22	Apoptosis	<i>ETV6/RUNX1</i>	Supplementary Material, Table S9
<i>BOK</i>	2q37.3	Apoptosis	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated
<i>CASP9</i>	1p36.1–36.3	Apoptosis	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated
<i>DBC1</i>	9q32–33	Apoptosis	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated+Supplementary Material, Table S2
<i>FOXO3A</i>	6q21	Apoptosis	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated
<i>TP53I3</i>	2p23.3	Apoptosis	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated
<i>AR</i>	Xq11.2–12	Cell signaling	high hyperdiploid	Supplementary Material, Table S1
<i>CSF1R</i>	5q33–35	Cell signaling	high hyperdiploid	Supplementary Material, Table S9
<i>GALNAC4S-6ST</i>	10q26	Cell signaling	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated
<i>NOTCH1</i>	9q34.3	Cell signaling	<i>ETV6/RUNX1</i> , high hyperdiploid	Supplementary Material, Table S9
<i>PLRL</i>	5p13–14	Cell signaling	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated+Supplementary Material, Table S1
<i>SFRP1</i>	8p11.1–12	Cell signaling	high hyperdiploid	Supplementary Material, Tables S1 and S2
<i>TGFBR1</i>	9q22	Cell signaling	high hyperdiploid	Recurrently methylated
<i>CEBPA</i>	19q13.1	Transcription regulation	high hyperdiploid	Supplementary Material, Table S10
<i>HLF</i>	17q22	Transcription regulation	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated+Supplementary Material, Table S10
<i>MSC</i>	8q21	Transcription regulation	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated
<i>NFKB2</i>	10q24	Transcription regulation	<i>ETV6/RUNX1</i>	Supplementary Material, Table S9
<i>FAT</i>	4q35	Morphogenesis	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated+Supplementary Material, Table S1
<i>HCK</i>	20q11–12	Morphogenesis	high hyperdiploid	Supplementary Material, Table S2
<i>NEFL</i>	8p21	Morphogenesis	<i>ETV6/RUNX1</i> , high hyperdiploid	Supplementary Material, Table S1
<i>TPD52</i>	8q21	Morphogenesis	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated
<i>CNND2</i>	12p13	Cell cycle	high hyperdiploid	Supplementary Material, Table S2
<i>GAK</i>	4p16	Cell cycle	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated
<i>PRKCDBP</i>	3p21.3	Cell cycle	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated+Supplementary Material, Table S1
<i>IGF2</i>	11p15.5	Cell proliferation	high hyperdiploid	Supplementary Material, Table S1
<i>LDOC1</i>	Xq27	Cell proliferation	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated
<i>CD44</i>	11p13	Cell adhesion	<i>ETV6/RUNX1</i> , high hyperdiploid	Supplementary Material, Tables S1 and S2
<i>CDH13</i>	16q24.2–24.3	Cell adhesion	high hyperdiploid	Supplementary Material, Tables S1 and S2
<i>BRCA1</i>	17q21	DNA repair	high hyperdiploid	Supplementary Material, Table S1
<i>WRN</i>	8p11.2–12	DNA repair	high hyperdiploid	Recurrently methylated+Supplementary Material, Table S1
<i>CDC14B</i>	9q22.3	Protein phosphorylation	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated
<i>RBPI</i>	3q23	Transport	<i>ETV6/RUNX1</i> , high hyperdiploid	Supplementary Material, Table S1
<i>SDHC</i>	1q23.3	Electron transport	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated
<i>TES</i>	7q31.2	Ion binding	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated+Supplementary Material, Table S1
<i>UCHL1</i>	4p14	Various	<i>ETV6/RUNX1</i> , high hyperdiploid	Recurrently methylated

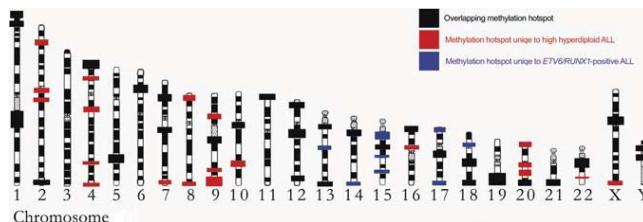
<sup>a</sup>Defined as a log<sub>2</sub> difference of at least 0.4 compared with the mean expression in cases without a methylation peak.

<sup>b</sup>Function and location are based on data in NCBI Entrez Gene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>). Only one major function per gene is given.

hyperdiploid cases (Fig. 3), indicating that they, to a large extent, are characterized by different chromosome-wide methylation patterns and expression profiles. In agreement with previous findings (13), gene rich BAC clones tended to be more methylated than those harboring no or only a few genes (Fig. 4A, Supplementary Material, Fig. S4A). However, it is important to stress that a high methylation level of a BAC clone not necessarily was associated with CpG hypermethylation of individual genes. In fact, a large proportion of the 71 different genes that had methylation peaks and that were underexpressed (Tables 2 and 3) did not map to hypermethylated BAC clones (data not shown).

### Comparison of chromosome-wide methylomes in *ETV6/RUNX1*-positive and high hyperdiploid ALL

When comparing the mean methylation levels in the two ALL types, differences between the high hyperdiploid and the *ETV6/RUNX1*-positive ALL as regards chromosome-wide methylation patterns on chromosomes 6, 10, 14, 16, 17, 18, 19 and 21 are clearly seen, with the former cases generally displaying a lower methylation of these chromosomes (Fig. 4, Supplementary Material, Fig. S4). It is noteworthy that the majority of these chromosomes are the ones that are most commonly gained in ALL with high hyperdiploidy (1).



**Figure 2.** Fifty-eight different methylation hotspots, derived from the promoter CpG island array, were identified in high hyperdiploid and *ETV6/RUNX1*-positive ALLs. The hotspots common to both genetic subtypes are shown as black boxes ( $n = 30$ ), the hotspots unique for the *ETV6/RUNX1*-positive cases are blue ( $n = 8$ ), and the ones unique for high hyperdiploid ALLs are red ( $n = 20$ ).

The only exceptions were chromosome 17, which did not display decreased methylation despite being trisomic in nine of the high hyperdiploid cases, and chromosomes 16 and 19, which were less methylated but only trisomic in one of the *ETV6/RUNX1*-positive cases. Furthermore, when comparing the mean methylation levels per chromosome within the high hyperdiploid group, they were lower in cases with trisomy/tetrasomy for chromosomes 4, 8, 10 and 21 than in cases with disomies of these chromosomes (Supplementary Material, Fig. S5). Thus, gains in high hyperdiploid cases were strongly associated with decreased methylation of the additional chromosomes. The same phenomenon was not seen in *ETV6/RUNX1*-positive ALL. Among the 10 cases analyzed, 16 had either trisomy 21 or gain of a large part of that chromosome (Table 1). However, there were no methylation differences between those with or without such gains (Supplementary Material, Fig. S5).

#### Gene expression profiling of disomic and trisomic/tetrasomic chromosomes

Lowess curves of mean-centered expression ratios of disomies, trisomies and tetrasomies for all chromosomes within the high hyperdiploid subgroup and between high hyperdiploid cases and all other pediatric B-lineage ALL analyzed in the study by Andersson *et al.* (19) were compared. We observed global overexpression of genes on the additional chromosomes in the high hyperdiploid cases, when compared with other ALL subtypes as well as when comparing disomic and trisomic/tetrasomic chromosomes within the high hyperdiploid group (Supplementary Material, Fig. S6).

#### Bisulfite sequencing

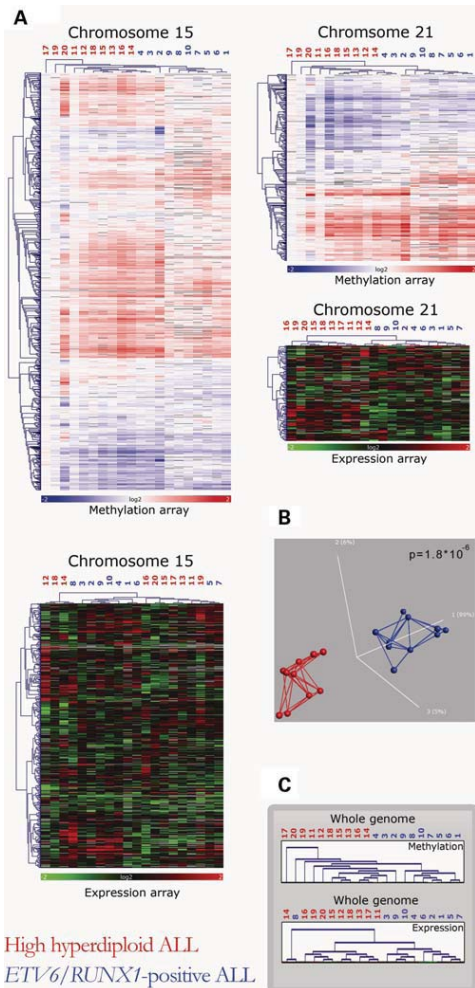
In the validation assay of the promoter CpG island methylation profiling, the array results corresponded to the bisulfite sequencing results of the seven selected genes (*BAD*, *BBC3*, *CAVI*, *CDK2AP1*, *NPM1*, *PRKCDBP* and *THEM4*) in all cases, i.e. low or moderate methylation was found when the investigated regions were situated outside the peak and hypermethylation was detected when the analyzed segments were situated within the peak region (Supplementary Material, Fig. S7).

#### DISCUSSION

During recent years, our knowledge about the global patterns of genomic alterations in childhood ALL has expanded dramatically (4,7,8). In contrast, no studies have focused on the genome-wide epigenetics of pediatric ALL; this is surprising considering that CpG hypermethylation of tumor suppressor genes has been demonstrated to be frequent in various neoplastic disorders (10). For this reason, we performed an in-depth analysis of global DNA methylation changes in the two most common genetic types of pediatric hematologic malignancies, including only primary patient material in order to avoid methylation artifacts arising from *in vitro* culturing.

It is noteworthy that none of the 30 genes displaying the highest methylation peak scores in the two genetic subgroups (Supplementary Material, Tables S5–S7) have previously been implicated as hypermethylated in neoplasia (Supplementary Material, Tables S1 and S2), a finding that emphasizes the value of performing genome-wide methylation analyses to identify novel hypermethylated candidate genes. As seen in Supplementary Material, Tables S5–S7, transcription regulation factors were particularly frequent among the genes with the highest methylation scores. Most likely, some of these genes, in particular those common to both subtypes (Supplementary Material, Table S6), may represent genes that are physiologically hypermethylated in early B-cell precursors and that hence may be associated with normal B-cell development. On the other hand, the genes that were differentially hypermethylated in the two different genetic subgroups (Supplementary Material, Table S7) are probably of leukemogenic importance.

Interestingly, among the genes with significant promoter methylation (Supplementary Material, Figs S1 and S2), only 30–60% were shown to be underexpressed (Tables 2 and 3). This clearly shows that hypermethylation per se does not necessarily lead to decreased gene expression. This has previously been demonstrated in high hyperdiploid ALL for the *FHIT* gene, which displayed no differential expression between methylation-positive and -negative cases (21). In fact, among the 16 hypermethylated genes identified in the study by Paulsson *et al.* (21), six were also found in the present study, namely *CD44*, *CDH13*, *FHIT*, *PAX6*, *RARB* and *WT1*, and expression data were available for five of these, showing downregulation of *CD44* and *CDH13*, no



**Figure 3.** Analysis of the chromosome-wide BAC array methylome profiles could successfully subgroup the majority of *ETV6/RUNX1*-positive and high hyperdiploid ALLs, both on the whole genome level as well as on individual di- or trisomic chromosomes. (A) Unsupervised hierarchical clustering analysis of chromosomes 15 and 21. The former chromosome was disomic in all cases, whereas the latter was gained in all high hyperdiploid ALLs (red) and in the majority of the *ETV6/RUNX1*-positive cases (blue). (B) Principal component analysis of the chromosome wide methylation data. (C) Enhanced dendrograms of unsupervised clustering analysis of whole genome methylation and expression data.

differential expression for *FHIT* and *RARB*, and upregulation of *WT1*, again emphasizing that other factors besides CpG island methylation influence gene expression patterns. As regards the hypermethylated and underexpressed genes

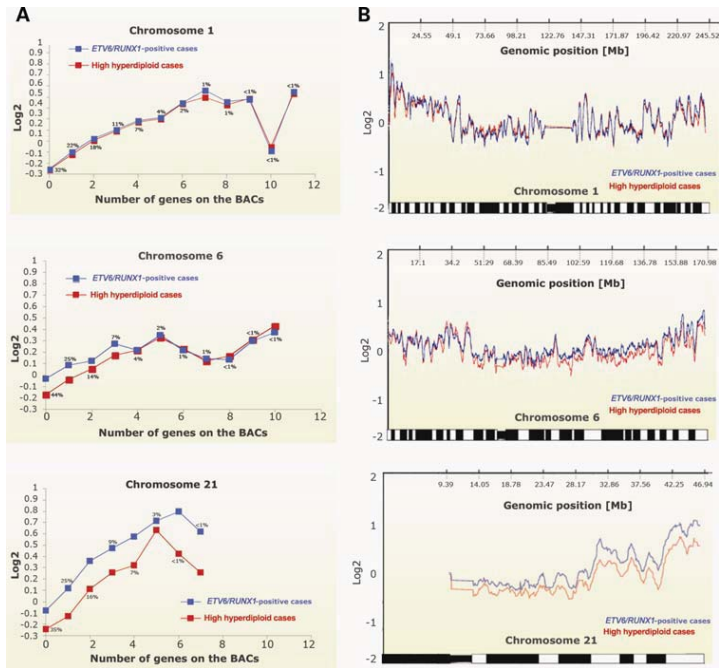
(Tables 2 and 3), the three most common GO groups were (i) transcription regulation, with *BRDT*, *HLF*, *SOX30* and *SSX4* being hypermethylated in the majority of cases, (ii) apoptosis, with *BBC3* and *FOXO3A* frequently being hypermethylated in both high hyperdiploid and *ETV6/RUNX1*-positive ALLs and (iii) cell signaling, with frequent hypermethylation of the *CSF2RA*, *GALNAC4S-6ST* and *PTPN3* genes (Supplementary Material, Figs S1 and S2). These genes could hence be potentially important targets and markers for future treatment trials with demethylating agents.

It has previously been demonstrated that simultaneous occurrence of hypermethylation and deletion causing biallelic gene inactivation is a rare event (22). Our findings agree well with this since none of the deletions identified by the array CGH analysis (Table 1) were associated with hypermethylated BAC clones or promoters in the retained chromosomal regions (Fig. 5 and data not shown). Cooperation between methylation events and copy number alterations is therefore unlikely to play a pathogenetically significant role in pediatric ALL.

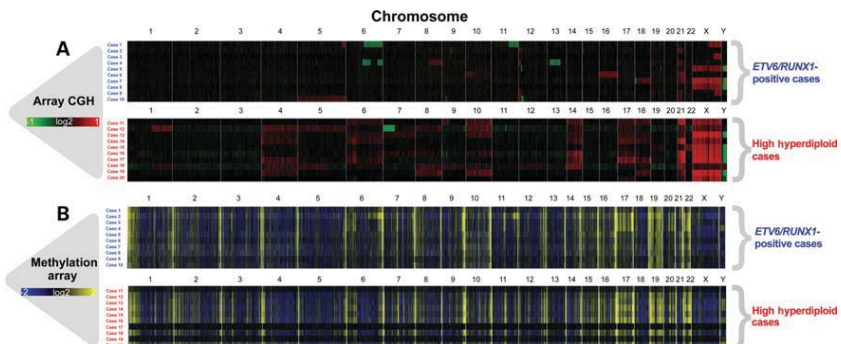
The present genome-wide promoter methylation analysis identified a total of 58 different methylation hotspots, with eight and 20 being unique for the *ETV6/RUNX1*-positive and high hyperdiploid ALLs, respectively (Fig. 2, Supplementary Material, Fig. S3). One large methylation hotspot common to both subtypes was located on chromosome arm 19q (Fig. 2). Interestingly, this methylation hotspot has previously been reported in ALL and harbors several maternally imprinted genes (16). This could suggest that the other methylation hotspots identified herein also include imprinted genes. To test this possibility we used the comprehensive mapping information provided by Luedi *et al.* (23) on all known, or putatively, imprinted human genes. We then ascertained the number of imprinted genes per chromosome band and evaluated how often bands with a specific number of imprinted genes harbored methylation hotspots. As seen in Figure 6, there is a high correlation between the presence of imprinted genes and methylation hotspots, with the frequencies of methylation hotspots increasing with the number of imprinted genes. This strongly suggests that regions rich in imprinted genes are associated with methylation hotspots in *ETV6/RUNX1*-positive and high hyperdiploid ALLs. One may hypothesize that imprinted genes are more susceptible to *de novo* methylation of the active allele and hence biallelic silencing.

The detection of subtype-specific methylation hotspots (Fig. 2) indicates that *ETV6/RUNX1*-positive and high hyperdiploid ALLs are characterized by different global methylation patterns. Further support for this was derived from the clustering analyses which successfully could subgroup the *ETV6/RUNX1*-positive and high hyperdiploid cases (Fig. 3). It has previously been reported that the profiles of CpG island hypermethylation of tumor suppressor genes differ between different tumor entities (10) and that acute myeloid leukemia and ALL can be separated using a 57 gene/249 CpG methylation array (17). The present study is the first to use global methylation patterns to distinguish different genetic subtypes of ALL. Thus, genome-wide methylation analyses can be used to subdivide morphologically identical, but genetically and clinically distinct, hematologic malignancies, a finding that may have ramifications also for other tumor types.

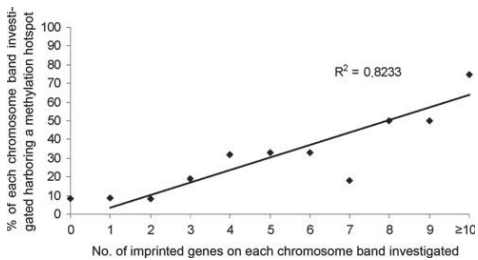




**Figure 4.** (A) Mean log<sub>2</sub> ratios of the high hyperdiploid (red) and *ETV6/RUNX1*-positive (blue) ALLs plotted against the number of genes on the BACs for chromosomes 1, 6 and 21. Chromosome 1 was disomic in all cases, chromosome 6 was trisomic in all but one of the high hyperdiploid cases and disomic in all *ETV6/RUNX1*-positive ALLs and chromosome 21 was gained in the vast majority of all cases. The percentages that the BAC clones with different number of genes comprise of the whole chromosome are given above each BAC group. As seen, the high hyperdiploid cases display a lower methylation of the frequently gained chromosomes 6 and 21 when compared with the *ETV6/RUNX1*-positive cases. (B) Mean log<sub>2</sub> ratios of the high hyperdiploid (red) and *ETV6/RUNX1*-positive (blue) ALLs plotted against genomic positions on chromosomes 1, 6 and 21. Again, the same pattern, i.e. lower methylation of gained chromosomes, is seen for both chromosomes 6 and 21.



**Figure 5.** Genome and DNA methylome profiles of *ETV6/RUNX1*-positive and high hyperdiploid ALLs. (A) Heat maps displaying genomic imbalances detected by array CGH. The gained chromosomes in the high hyperdiploid cases are clearly seen (indicated in red). The imbalance patterns of the *ETV6/RUNX1*-positive cases are quite different, with more losses (indicated in green), for example deletions of 6q. (B) Heat maps displaying chromosome-wide methylation profiles. As seen, none of the genomic imbalances identified by array CGH were associated with hypermethylated BAC clones in the corresponding regions.



**Figure 6.** Mapping information on all known, or putatively, imprinted human genes was retrieved from Luedi *et al.* (23) and compared with the location of methylation hotspots. As seen, there is a high correlation between the presence of imprinted genes and hotspots, with the frequencies of hotspots increasing significantly with the number of imprinted genes.

An intriguing and unexpected finding was the clear-cut differences between the high hyperdiploid and the *ETV6/RUNX1*-positive ALLs as regards the chromosome-wide methylation patterns, with the majority of the commonly gained chromosomes in the former subtype being less methylated than their disomic counterparts (Fig. 4, Supplementary Material, Fig. S4). Since we did not observe the same phenomenon in the *ETV6/RUNX1*-positive ALL cases with or without trisomy 21 (Supplementary Material, Fig. S5) it is apparent that gains as such do not correlate with chromosome-wide differential methylation and that the methylation changes hence depend on other factors, for example chromosome type, number of gains and cell of origin. We know of only one previous study reporting similar findings, namely the one by Weber *et al.* (13) who identified an overall lowered methylation in gene-poor regions of trisomic chromosomes in a colon cancer cell line. However, we observed decreased methylation in primarily gene-poor regions only on chromosomes 6 and 8 in the high hyperdiploid ALLs. In contrast, this was not the case for chromosomes 4, 10, 14, 16, 18, 19 and 21; on these chromosomes the lowered methylation was equally distributed irrespective of the number of genes on the BAC clones (Supplementary Material, Figs S4 and S5). The latter finding agrees well with previous data showing no clear bias for decreased methylation only of gene-poor regions in the inactive and hypomethylated X chromosome in females (24).

It has previously been demonstrated that aneuploidy in yeast is associated with expression of the great majority of the genes on the extra chromosomes. However, even if the amount of transcript is increased, the protein levels are often not increased because of either non-translation or degradation (25). Thus, the overexpression of genes on additional chromosomes in high hyperdiploid cases, when compared with other ALL subtypes (19) or when comparing disomic and trisomic chromosomes within the high hyperdiploid group (Supplementary Material, Fig. S6), does not by necessity translate into protein abundance. Hence, the role of the decreased methylation of the gained chromosomes could be unrelated to gene expression. Instead, the differential methylation may be necessary for proper compartmentalization of the tri-/tetrasomic chromosomes in interphase, akin to Barr body formation of the inactive X chromosome, which, surprisingly, has been

shown to be globally hypomethylated relative to its active counterpart (13).

Recently, it was shown that aneuploidy generally inhibits tumorigenesis in murine experimental systems, being associated with decreased proliferation; however, the opposite effect—oncogenesis—may occur with gains of certain specific chromosomes (26,27). An abnormal chromosome number as such is hence unlikely to be sufficient to drive oncogenesis—other genetic or epigenetic changes are needed in addition. The lowered methylation identified herein may be one such event, perhaps abrogating the reduced cellular fitness associated with aneuploidy, and may have ramifications not only for the pathogenesis of high hyperdiploid ALL but also for other disorders with numerical chromosome anomalies.

## MATERIALS AND METHODS

### Patients and samples

Samples from 20 children with B-cell precursor ALL, comprising 10 with high hyperdiploidy and 10 with the *ETV6/RUNX1* fusion, were included in the study (Table 1). All patients had been diagnosed and treated according to the NOPHO-ALL 1992 or 2000 protocols at the Departments of Pediatric Oncology and Hematology, Lund University Hospital, Lund, and Linköping University Hospital, Linköping, Sweden. The study was reviewed and approved by the regional research ethics boards at Lund and Linköping Universities, and informed consent was obtained from the patients' parents or guardians in accordance with the Declaration of Helsinki. DNA was extracted, at the time of diagnosis, from bone marrow samples in cases 1–19 and from peripheral blood in case 20. All the samples were cytogenetically characterized by conventional chromosome banding analysis. The presence of the cytogenetically cryptic *ETV6/RUNX1* fusion was ascertained by RT-PCR and verified by FISH using locus-specific probes for the *ETV6* and *RUNX1* genes. The additional chromosomes identified by G-banding in the high hyperdiploid cases were, in the present study, all confirmed by array CGH (see in what follows). DNA and RNA were extracted using standard methods, and the DNA was purified further with the DNeasy Blood & Tissue Kit (Qiagen, Solna, Sweden) and quantified using a Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE).

### Array comparative genomic hybridization

Array CGH slides, containing 32 433 tiling BAC clones covering at least 98% of the human genome, were produced at the SCIBLU DNA microarray resource center at Lund University, Sweden. Labeling of DNA, slide preparation and hybridization were performed as described previously (20). Male genomic DNA (Promega, Madison, WI) was used as reference in all hybridizations.

Analyses of the microarray images were performed as described previously (8). To identify imbalances the BASE (28), the CGH Explorer software (29) and the TM4 microarray software suite (30) were applied. Classification as gain or loss was based on the log<sub>2</sub> ratios, where  $> \pm 0.5$  in five adjacent clones were classified as aberrant, with  $\pm 0.5$ –1.0

interpreted as duplications/hemizygous deletions and  $> \pm 1.0$  as amplifications/homozygous deletions.

### Methylated DNA immunoprecipitation

Six micrograms of DNA from each sample was diluted in deionized water, yielding a concentration of 75 ng/ $\mu$ l. The DNA was sonicated with a Sonifier set to 20% amplitude for 0.5 s in 60 cycles to generate fragments ranging from 200 to 1000 bp (verified by gel electrophoresis). The fragmented DNA was diluted to a concentration of 13.4 ng/ $\mu$ l and denatured for 10 min at 95°C, after which an input fraction (~250 ng), serving as a reference in subsequent microarray hybridizations, was collected and stored at -80°C. The remaining DNA was immunoprecipitated overnight with a monoclonal antibody against 5-methylcytidine (Diagenode, Liège, Belgium) in a total of 500  $\mu$ l 1 $\times$  IP buffer at 4°C, as previously described (13). The mixture was then incubated with 80  $\mu$ l of Protein A Agarose beads (Invitrogen, Carlsbad, CA) for 2 h at 4°C and subsequently washed three times with 1 $\times$  IP buffer to remove DNA with no or low level methylation. The beads were then treated overnight with proteinase K at 55°C. After centrifugation, methylated DNA in the supernatant was recovered using standard phenol-chloroform extraction and ethanol precipitation. Both the input fraction and the methylated DNA were then amplified using the WGA2 kit (Sigma-Aldrich, St. Louis, MO), generating sufficient amounts (~6  $\mu$ g) for the various microarray hybridizations, as detailed in what follows.

### CpG island and promoter methylation profiling

Four micrograms of methylated and input fraction DNA were analyzed using the NimbleGen CpG Island-Plus-Promoter Array (Roche NimbleGen, Madison, WI) by the NimbleGen Service Group (Reykjavik, Iceland) who performed the quality control, labeling, hybridization and scanning. This array platform covers all 28 226 UCSC-annotated CpG islands and promoter regions for all RefSeq genes, including 385 000 probes of 50–75 mer length per array, with an upstream and downstream promoter tiling of 800 bp and 200 bp, respectively.

Signal intensity data were extracted from the scanned images. Each feature on the array has a corresponding log<sub>2</sub> ratio, which is the ratio of the input signals for the methylated DNA and the input fraction DNA. The log<sub>2</sub> ratio was scaled in order to center the ratio data around zero by subtracting the bi-weight mean for the log<sub>2</sub> ratio values for all features on the array from each log<sub>2</sub> ratio value. A modified ACME algorithm (31), where a fixed-length window (750 bp) is placed around each consecutive probe, and the one-sided Kolmogorov–Smirnov test were then applied on the scaled log<sub>2</sub> ratio data to determine whether the probes were drawn from a significantly more positive distribution (peak) of intensity log<sub>2</sub> ratios than the other probes in the array. The resulting score for each probe is the  $-\log_{10}$  *P*-value from the windowed Kolmogorov–Smirnov test around that probe.

### Delineation of hypermethylated genes

Peak data were generated by searching for probes above a *P*-value minimum cut-off ( $-\log_{10}$ ) of 2. Peaks within 500 bp

of each other were merged. Each annotated gene was subsequently searched for peaks appearing in a specified promoter region around the transcription start site. The region searched was design-specific, spanning from 5 kb upstream to 1 kb downstream of the transcription start site. Genes that had at least two probes with peaks above the *P*-value minimum cut-off were considered to have significant CpG island methylation scores for hypermethylation. In this fashion only 5' promoter CpG islands were investigated. The detected hypermethylated genes were analyzed in three different ways.

First, all genes with significant CpG island methylation scores were grouped according to ALL subtype in order to identify those that were uniquely hypermethylated in either *ETV6/RUNX1*-positive or high hyperdiploid cases. Furthermore, the chromosomal distribution of all genes was ascertained.

Second, an edge preserving smoother analysis (29), determining a sequence with as few jumps as possible, was performed. We used the Potts filter with the penalty parameter set to 2 and limited the least allowed aberration size to two clones. This segmentation analysis of the peak score data was performed in order to identify peaked genes throughout the genome that were highly enriched for methylation.

Third, we identified all genes that displayed methylation peaks in  $\geq 30\%$  of cases in either genetic ALL subgroup or that were present in Supplementary Material, Tables S1, S2 or S9–S11, which comprise genes known to be hypermethylated in malignant disorders, involved in B-cell development or mutated or rearranged in B-lineage ALL. Each gene was then functionally annotated using the EASE software (32) and correlated with gene expression data.

### Identification of DNA methylation hotspots

Identification of regions with a dense occurrence of hypermethylated genes, i.e. genomic sites harboring a significantly higher number of peak-scored probes compared with the rest of the genome, was performed by plotting the spatial and number distribution of peak scored promoters using an Epanechnikov kernel density estimator in CGH Explorer (29). The size determinant was set to 4 Mb. In order to designate a chromosomal segment as a methylation hotspot at least four peak-scored probes had to be present within the 4 Mb segment analyzed.

### Chromosome-wide methylation profiling

Of the methylated and input fraction DNA, 1.5  $\mu$ g was differentially labeled with Cy3 and Cy5, respectively, and hybridized to 32K BAC array slides. Labeling of DNA, slide preparation, hybridization and analysis were performed as described previously (8,20). Classification as relative enrichment for methylation was based on the log<sub>2</sub> ratio for each of the 32 433 BAC clones smoothed with the factor of 0.33, with a positive ratio indicating enrichment of methylation.

Unsupervised clustering analysis, after using a variance filter with a cut-off value of 0.25 standard deviation to limit the number of probes and a Pearson uncentered test (30) for average linkage clustering, was used to investigate methylation differences/similarities between the *ETV6/RUNX1*-positive and high hyperdiploid cases. In addition, a two

group comparison was performed using PCA in the Qlucore Software (Qlucore AB, Lund, Sweden).

Average log<sub>2</sub> ratios for each BAC clone of the ten high hyperdiploid and the ten *ETV6/RUNX1*-positive ALLs, respectively, were plotted towards the genomic position on each chromosome. The two groups were also analyzed in relation to the gene density on each clone by ascertaining, using a custom-made script, the number of annotated RefSeq genes on each consecutive clone. All clones on each chromosome were grouped in relation to the number of genes they harbored, generally ranging from 0 to 12 genes. The average log<sub>2</sub> ratio for each such group was then plotted against their gene content.

### Global gene expression analysis

RNA extraction, amplification, labeling, hybridization to 27K cDNA microarray slides, scanning, post-hybridization washing and feature analysis have previously been described (19,33). In short, the samples were hybridized to microarray slides containing 25 648 cDNA clones (SCIBLU DNA microarray resource center) and amplified RNA from the Universal Human Reference (Agilent Technologies, Palo Alto, CA) was used as reference. The data analyses were all performed using the BASE (28), TM4 (30) and Statistica (StatSoft Scandinavia AB, Uppsala, Sweden) software. The same type of clustering analysis that was performed on the chromosome-wide methylation profiles (discussed earlier) was used on the expression data.

### Bisulfite sequencing

To validate the reliability of the promoter array, eight genes (*BAD*, *BBC3*, *CAVI*, *CDK2AP1*, *NPM1*, *PRKCDBP*, *TES* and *THEM4*) were selected from the identified 167 recurring target genes (Supplementary Material, Fig. S2; see Results). DNA (2 µg) from eight leukemic samples, comprising four ALL with high hyperdiploidy and four with *ETV6/RUNX1*, was treated with sodium bisulfite using the EpiTect kit (Qiagen, Valencia, CA). The bisulfite-treated DNA was used as a template in standard PCR amplification using primers directed towards the promoter regions harboring the hypermethylated CpG islands, as indicated by peaks in the array (primer sequences available upon request). The primers were designed using the MethPrimer software (34). The PCR products were sub-cloned using the TOPO-TA system (Invitrogen, Carlsbad, CA) and sequenced using standard methods (MCLab, San Francisco, CA). After sequencing 8–12 clones per gene and case, one gene (*TES*) was excluded because it harbored too few informative CpG sites. The sequences were analyzed using the BiQ Analyzer software (35). For a gene to be considered hypermethylated, the following criterion was used: at least 80% of the investigated clones should be methylated in at least 80% of the sequenced CpG sites.

### Array data

The microarray data are deposited in the Array Express database (<http://www.ebi.ac.uk/microarray.as/ae/>).

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

*Conflict of Interest statement:* None declared.

## FUNDING

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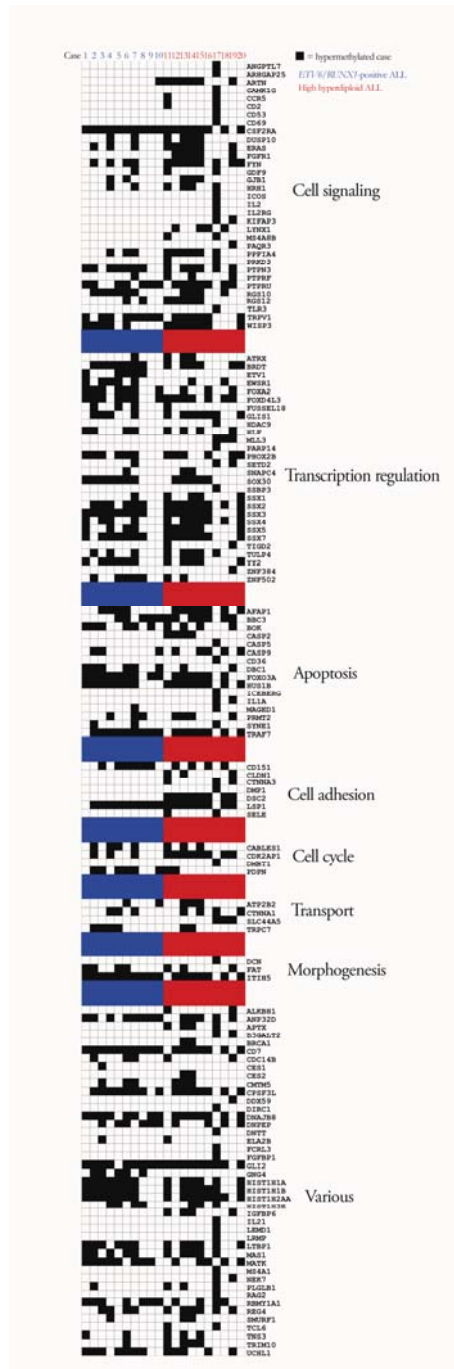
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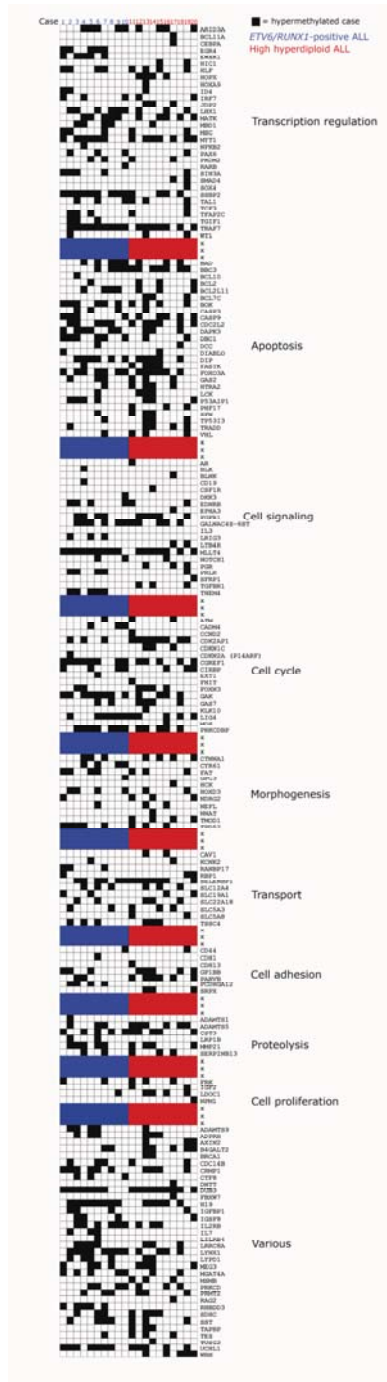
# Supplementary material II





**Figure S1.** A total of 138 genes were identified by the segmentation analysis to be highly enriched for methylation among the *ETV6/RUNX1*-positive and high hyperdiploid ALLs.

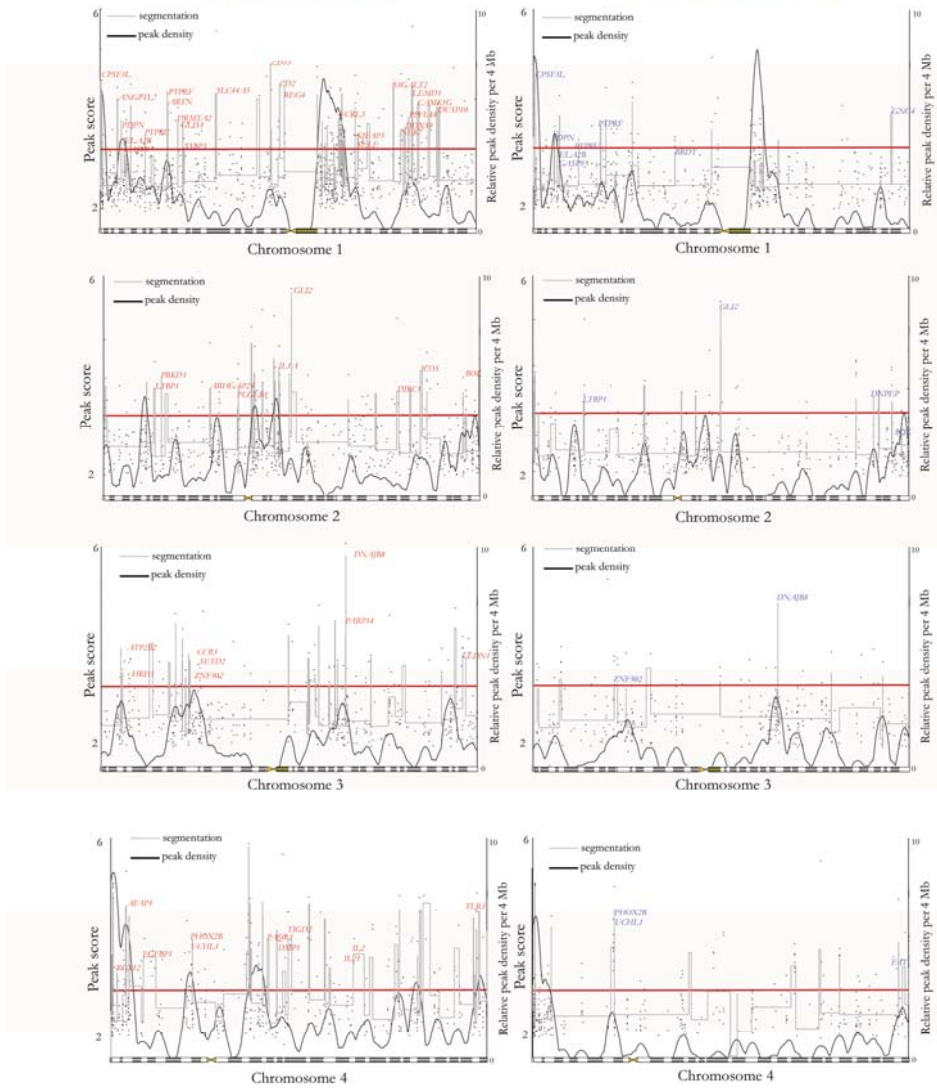




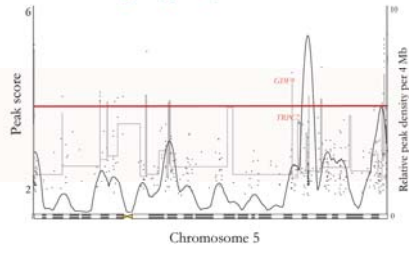
**Figure S2.** A total of 167 genes displaying methylation peaks in at least 30% of the cases in each genetic subgroup or being present in Tables S1, S2 and S9-S11 were identified.

### High hyperdiploid ALL

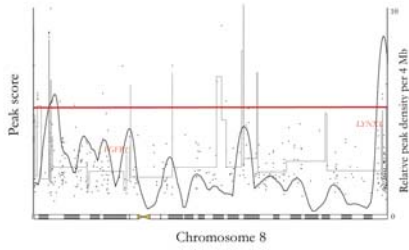
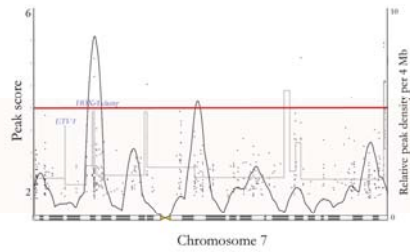
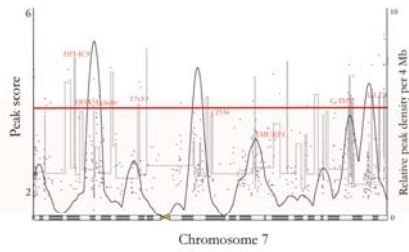
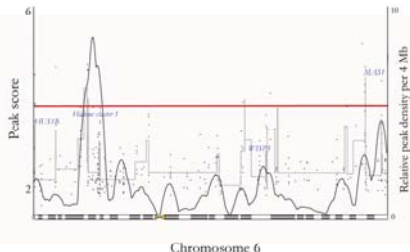
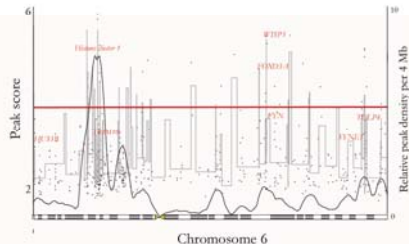
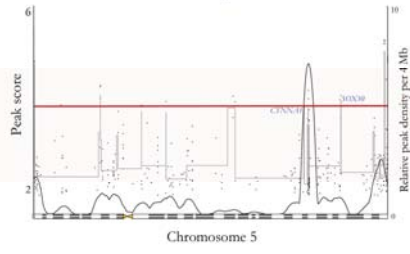
### ETV6/RUN1-positive ALL



### High hyperdiploid ALL

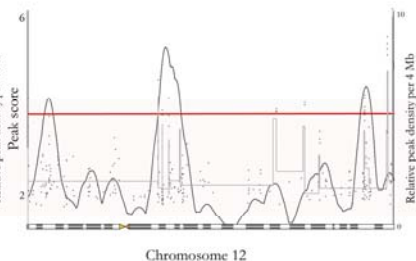
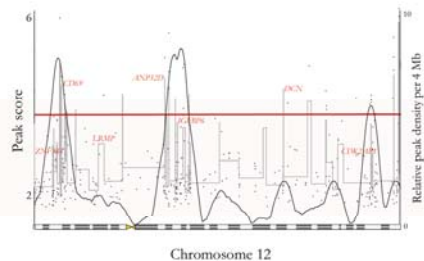
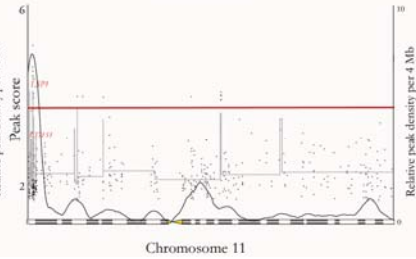
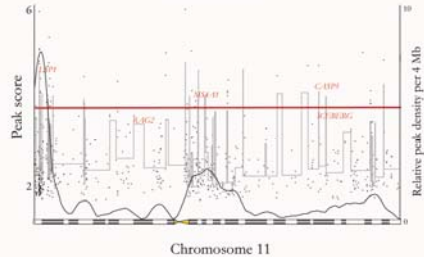
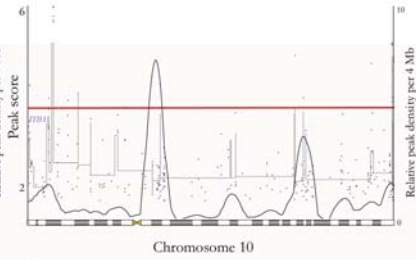
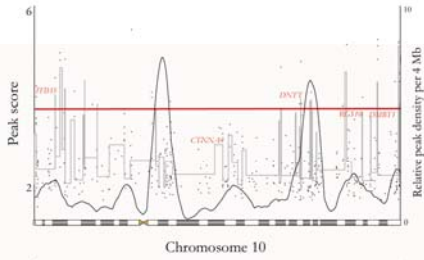
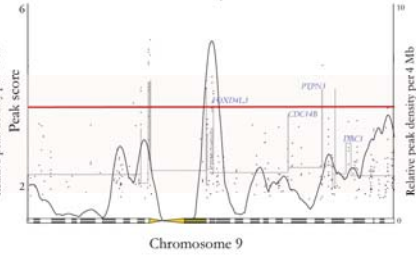
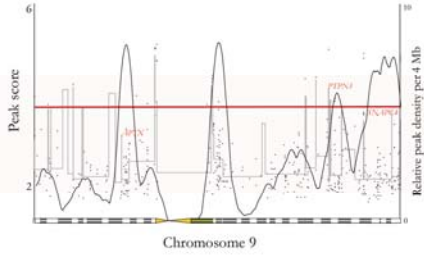


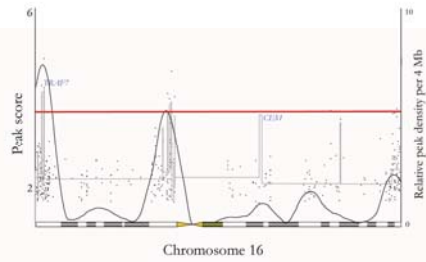
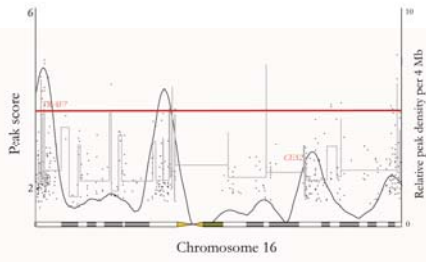
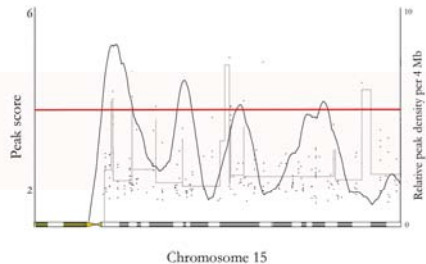
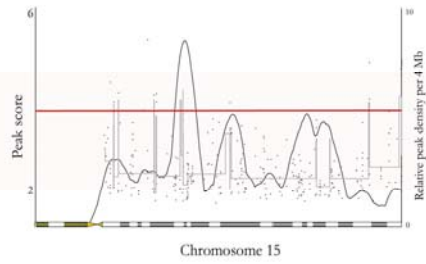
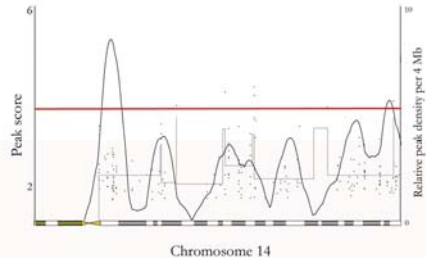
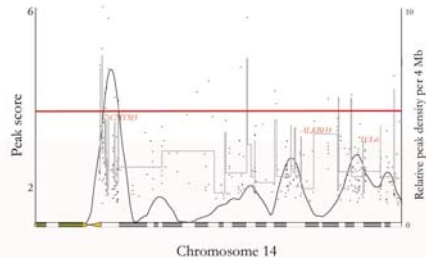
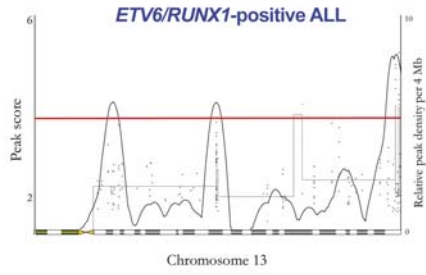
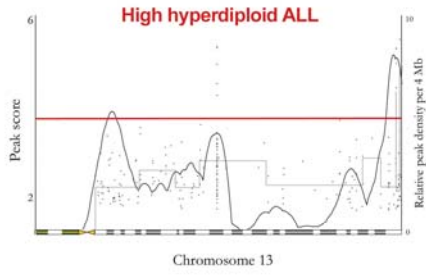
### ETV6/RUNX1-positive ALL



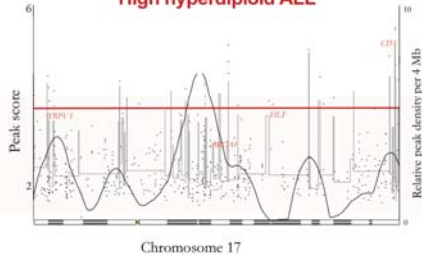
**High hyperdiploid ALL**

**ETV6/RUNX1-positive ALL**

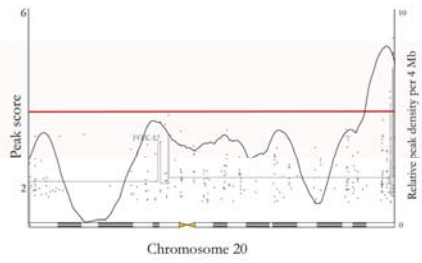
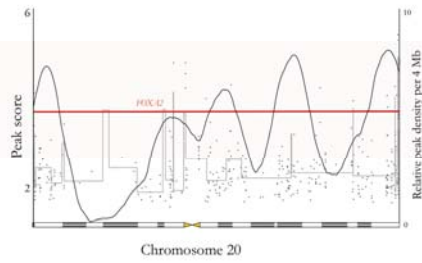
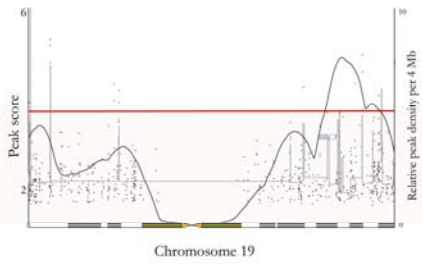
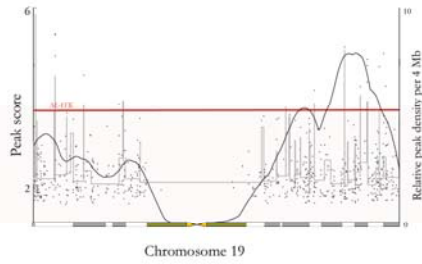
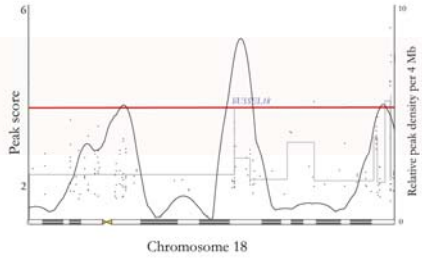
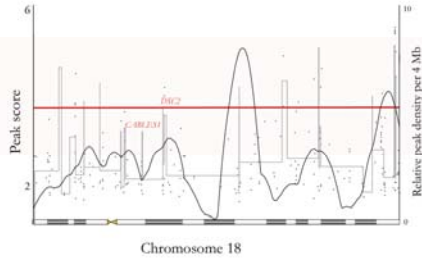
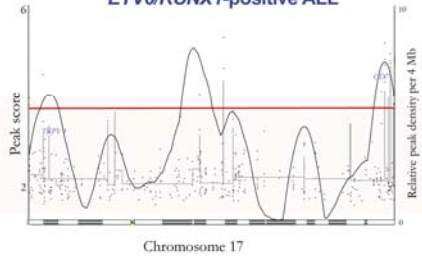


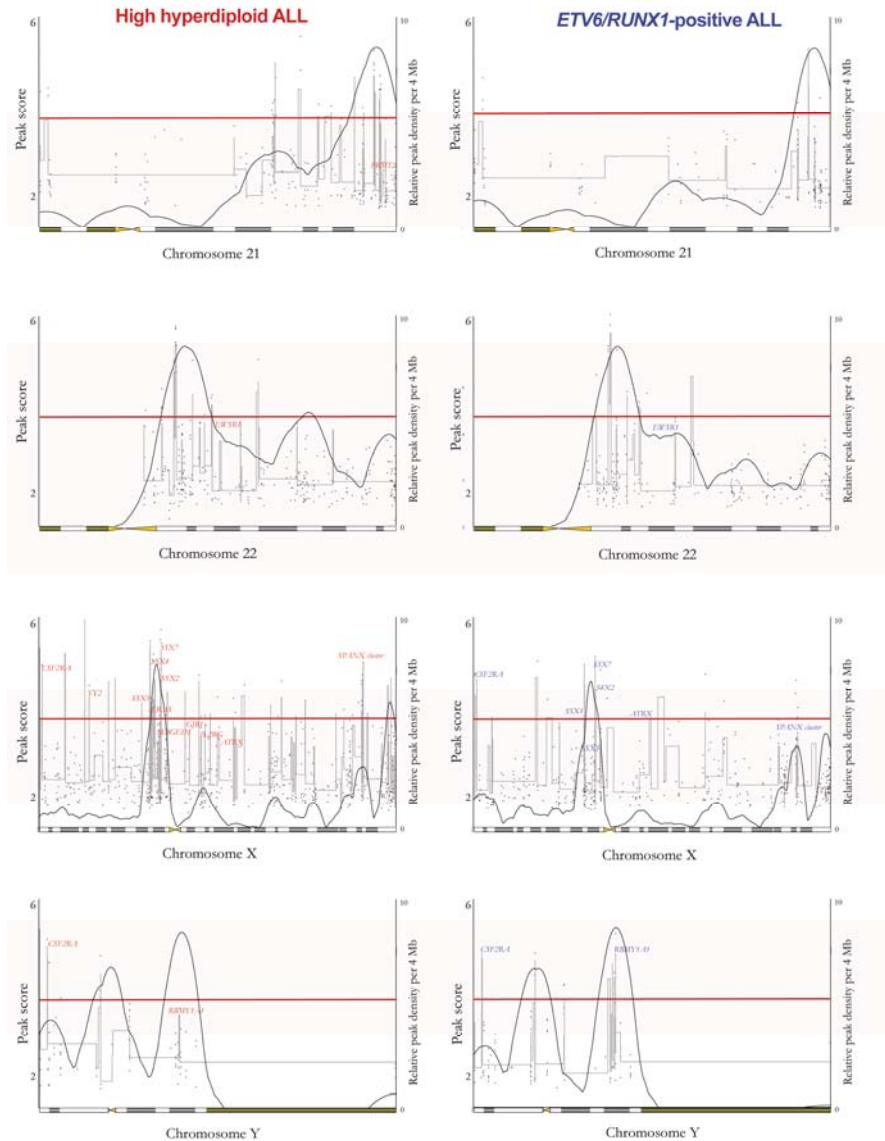


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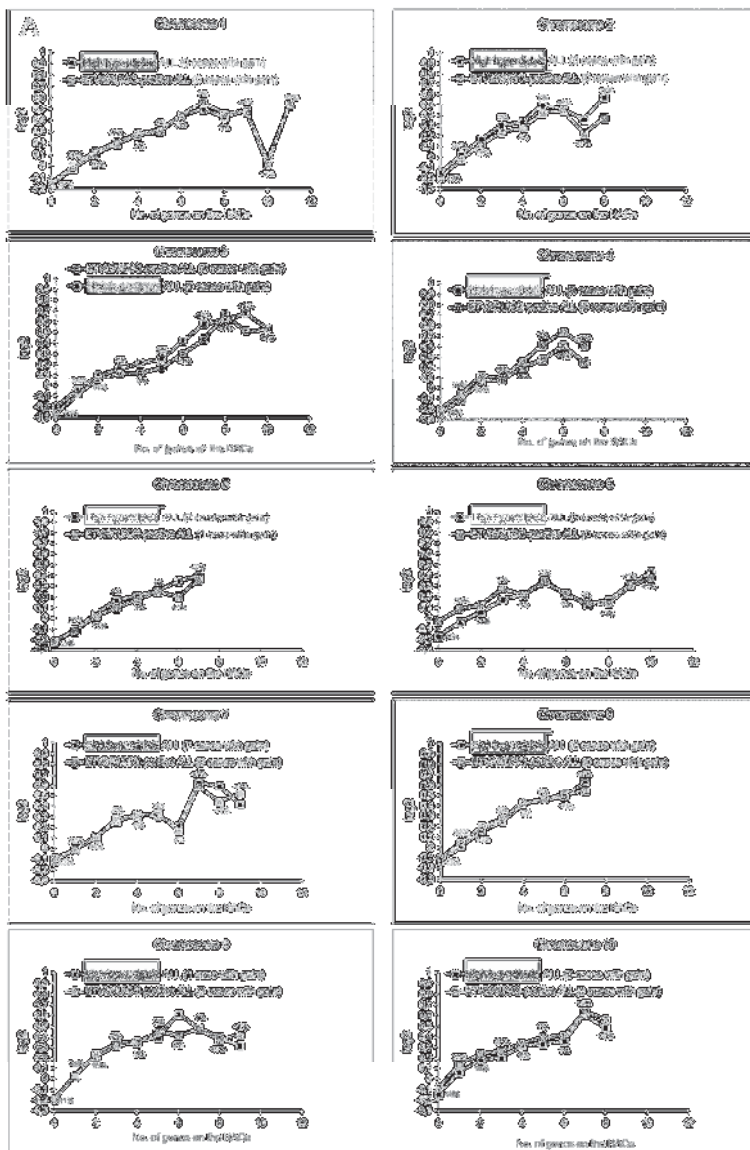


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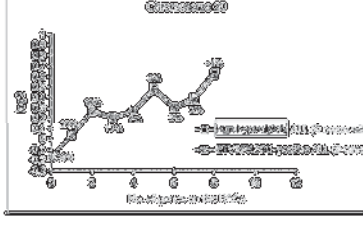
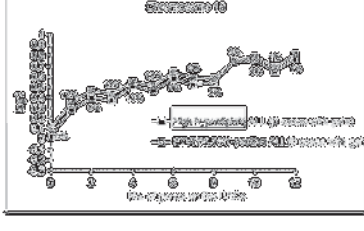
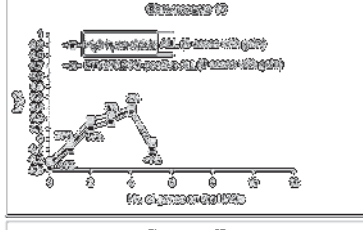
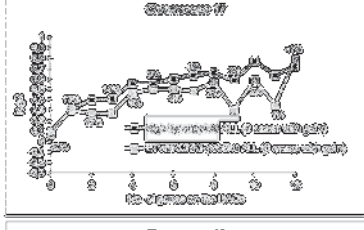
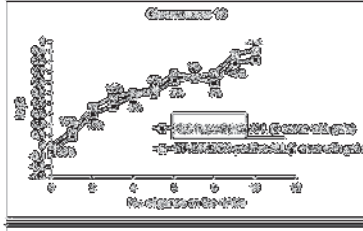
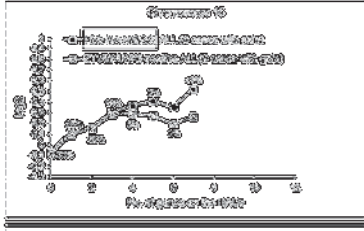
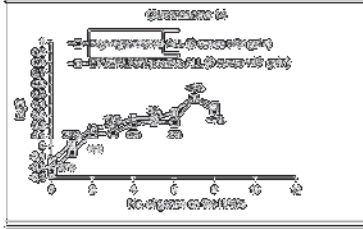
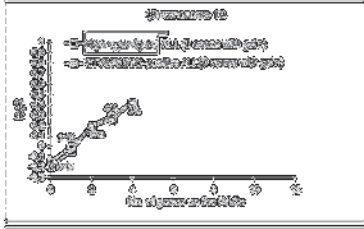
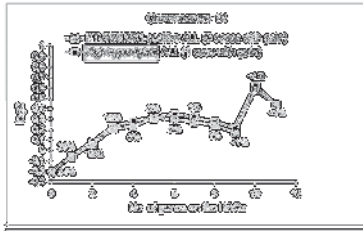
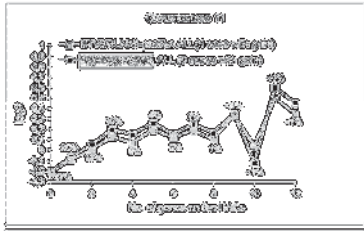


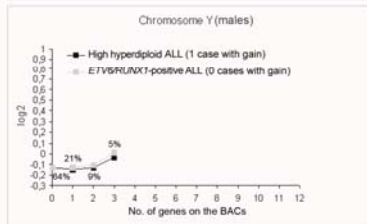
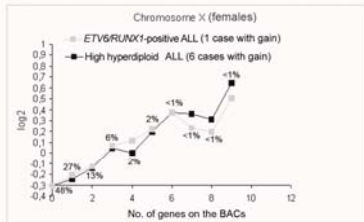
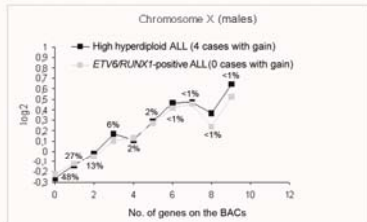
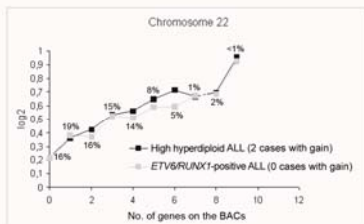
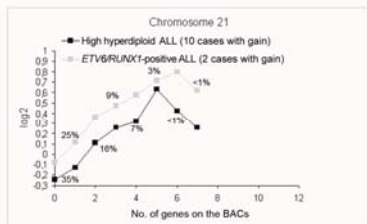


**Figure S3.** The segmentation analysis identified peaked genes that were highly enriched for methylation. These are labeled in red and blue for high hyperdiploid and *ETV6/RUNX1*-positive ALLs, respectively. The spatial and number distribution of peak-scored probes was plotted using a density estimator with the size determinant set to 4 Mb. In order to designate a chromosomal region as a methylation hotspot at least four peak-scored probes had to be present within the 4 Mb segment analyzed. This cutoff is indicated with the red horizontal line. Note that the *ETV6/RUNX1*-positive ALLs had no peaks for chromosome 8 (Supplementary Material, Table S4).

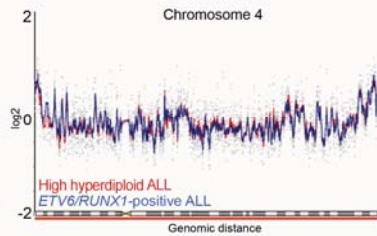
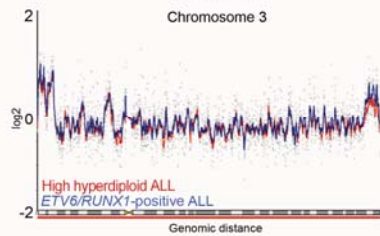
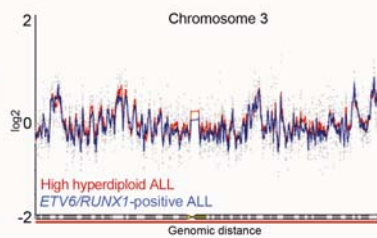
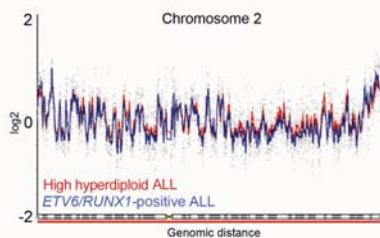
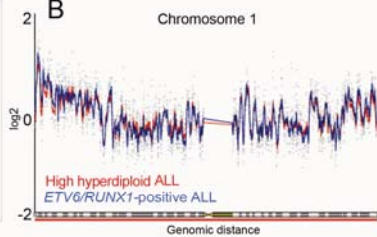


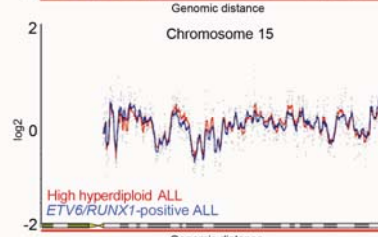
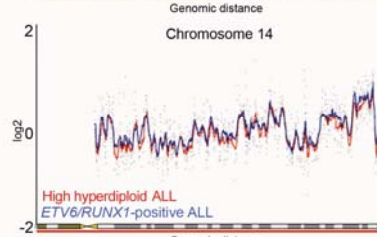
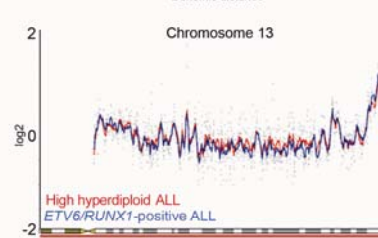
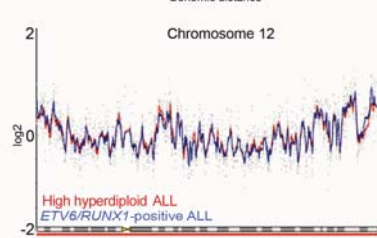
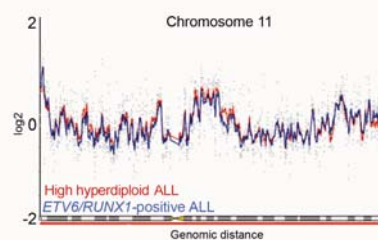
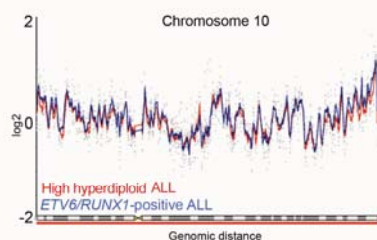
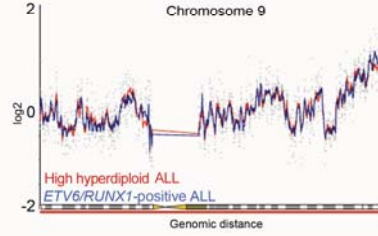
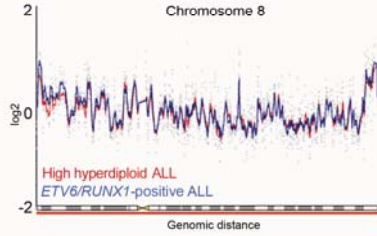
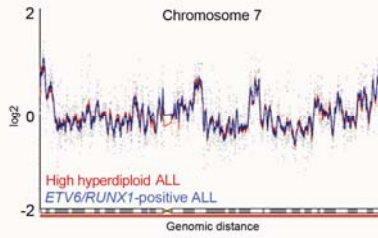
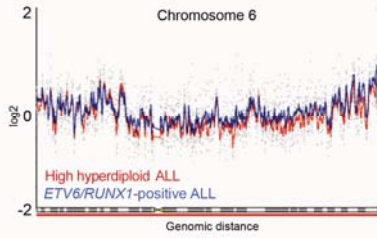


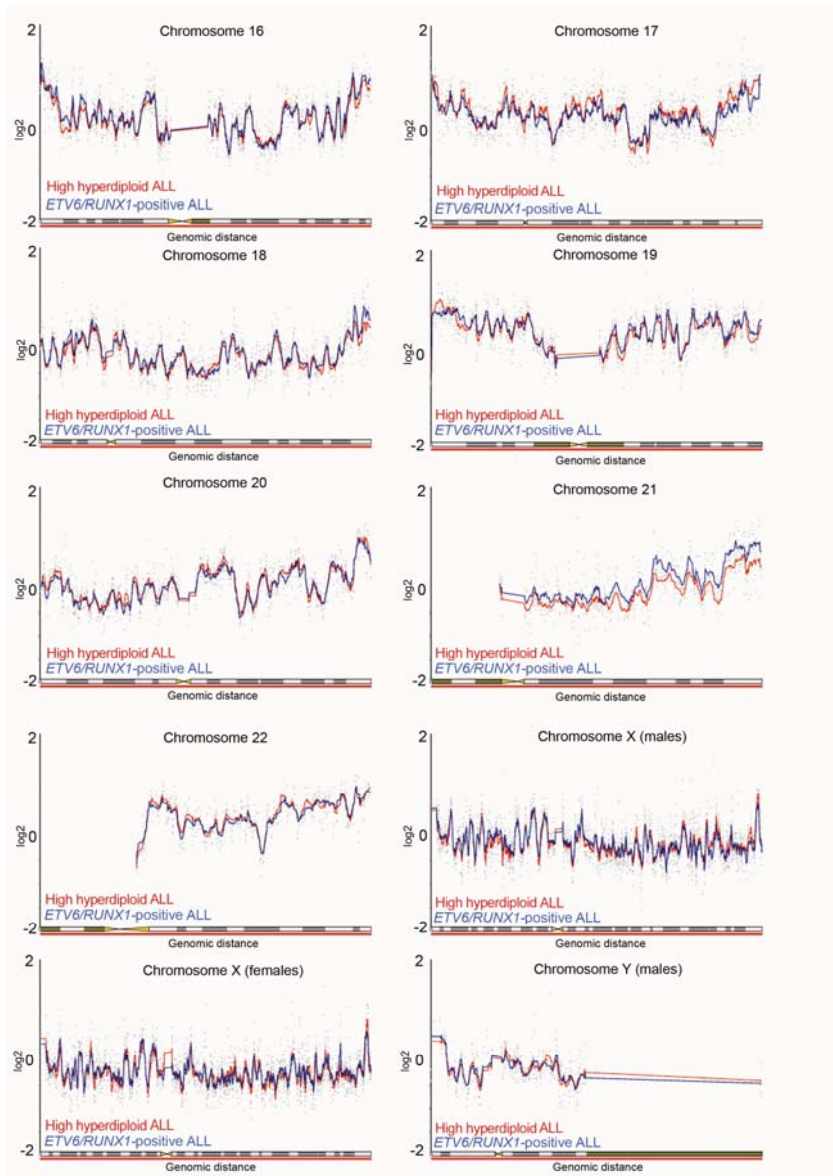




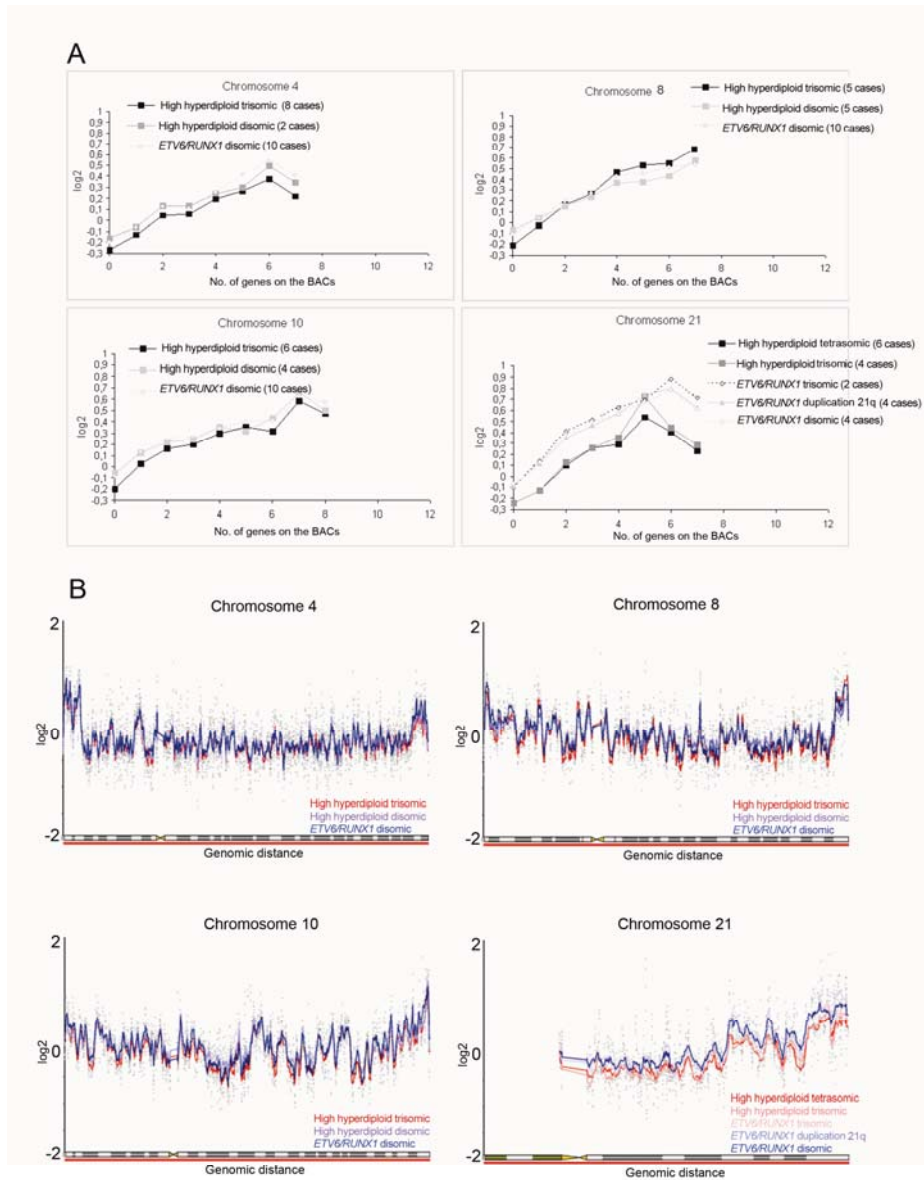
**B**

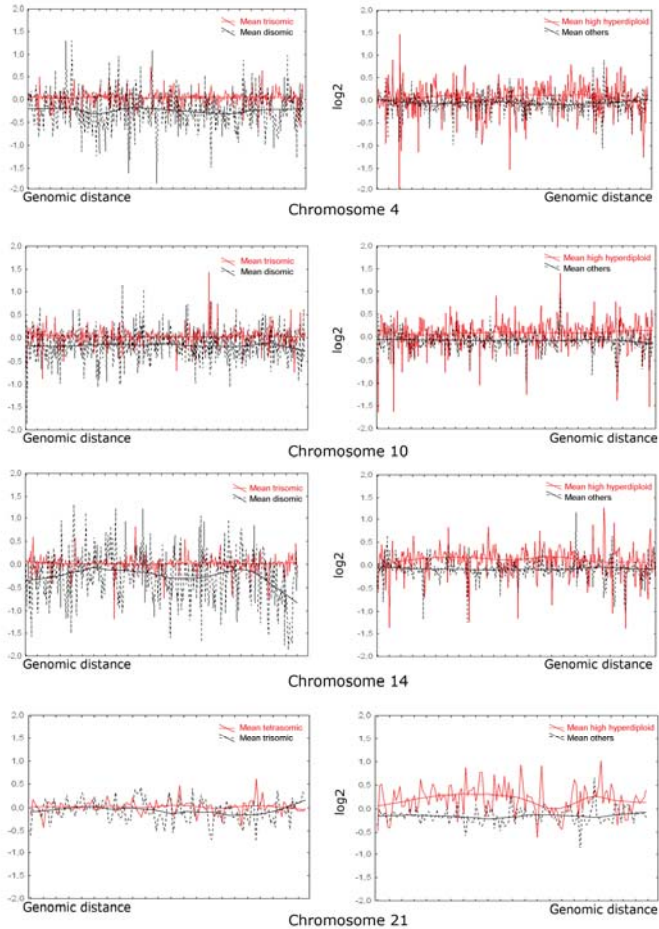






**Figure S4.** (A) Mean  $\log_2$  ratios of the *ETV6/RUNX1*-positive and high hyperdiploid ALLs plotted against the number of genes on the bacterial artificial chromosome clones for all chromosomes. The percentages that the clones with different number of genes comprise of the whole chromosome are given above each bacterial artificial chromosome group. (B) Mean  $\log_2$  ratios of the *ETV6/RUNX1*-positive (blue) and high hyperdiploid (red) ALLs plotted against genomic positions on chromosomes 1-22, X and Y





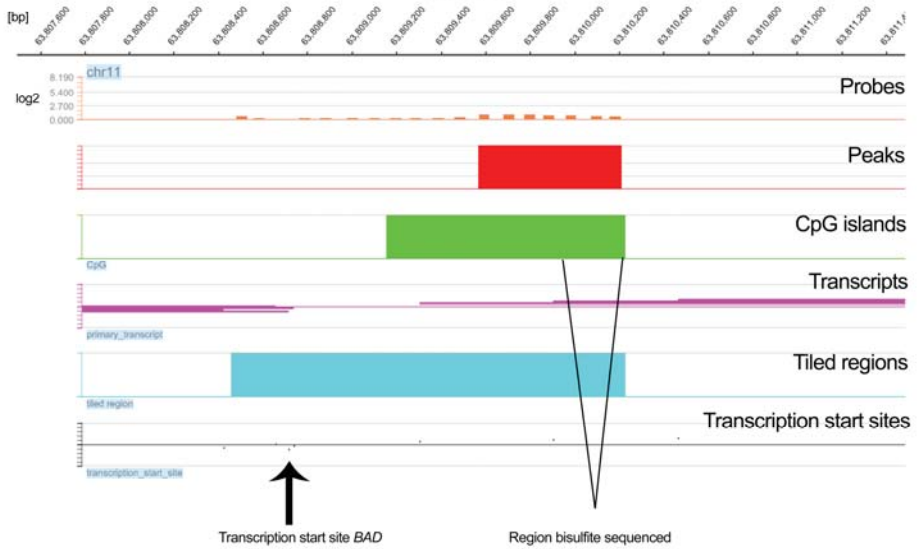
**Figure S6.** Lowess curves of mean-centered expression ratios of disomies, trisomies and tetrasomies for chromosomes 4, 10, 14 and 21 among the high hyperdiploid cases (left) and between high hyperdiploid cases and all other pediatric B-lineage ALLs (right) analyzed in the study by Andersson *et al.* (1).

## Reference

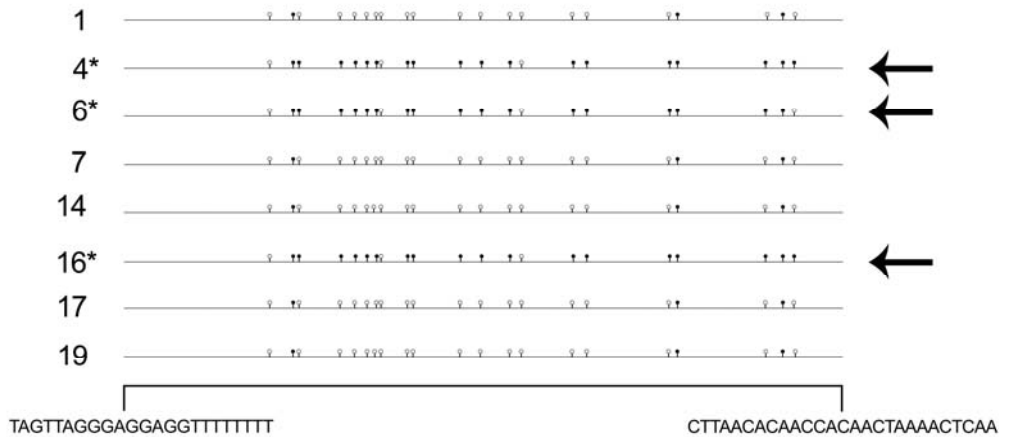
1. Andersson, A., Olofsson, T., Lindgren, D., Nilsson, B., Ritz, C., Edén, P., Lassen, C., Råde, J., Fontes, M., Möirse, H. *et al.* (2005) Molecular signatures in childhood acute leukemia and their correlations to expression patterns in normal hematopoietic subpopulations. *Proc. Natl. Acad. Sci. USA* **102**, 19069-19074.

# BAD

## Case 16

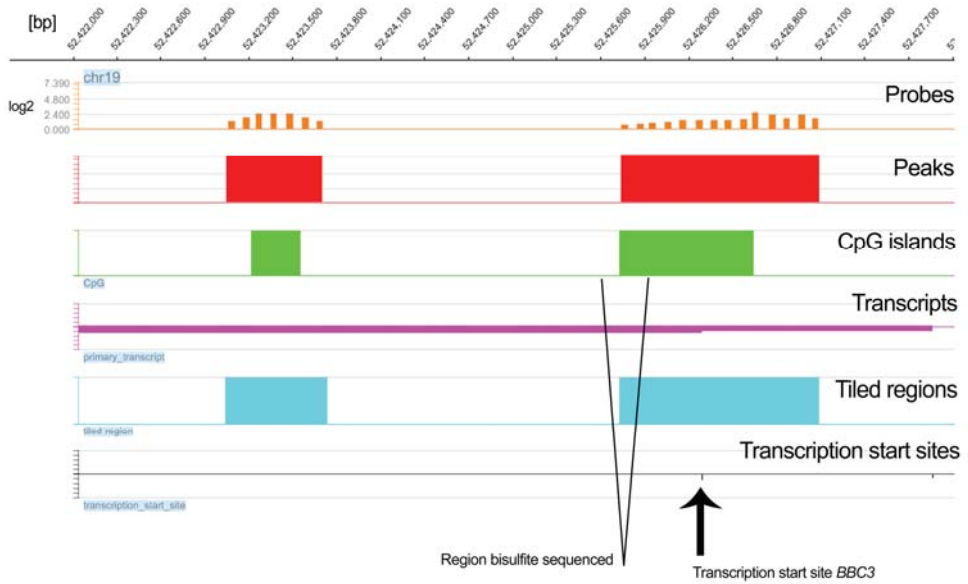


### Case



# BBC3

Case 16



## Case



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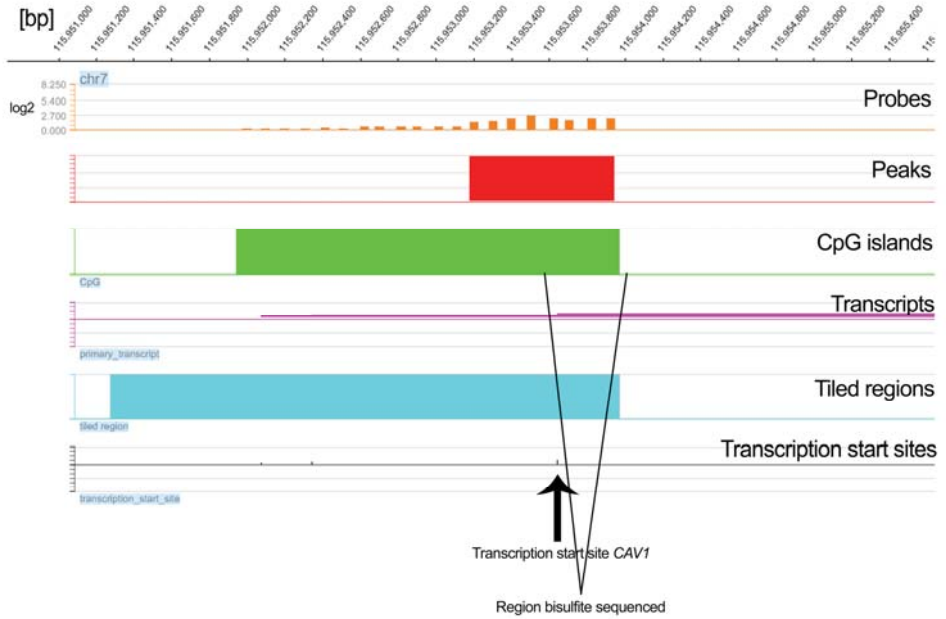
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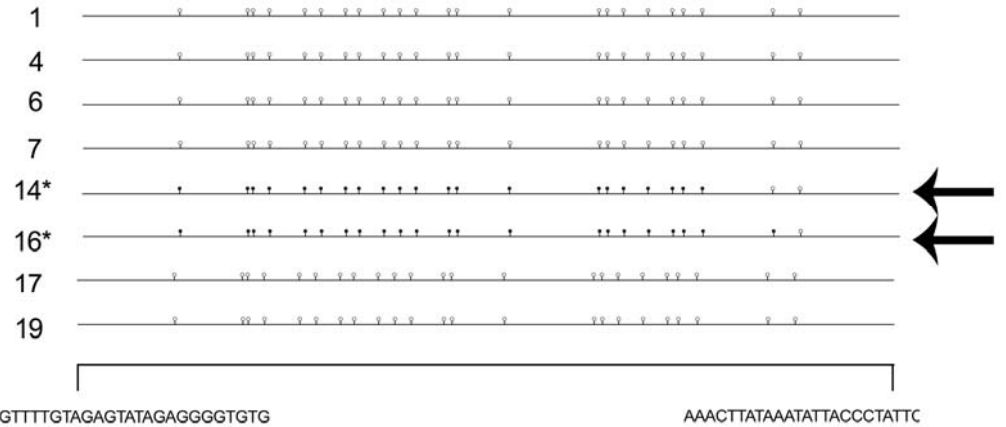
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Case 16

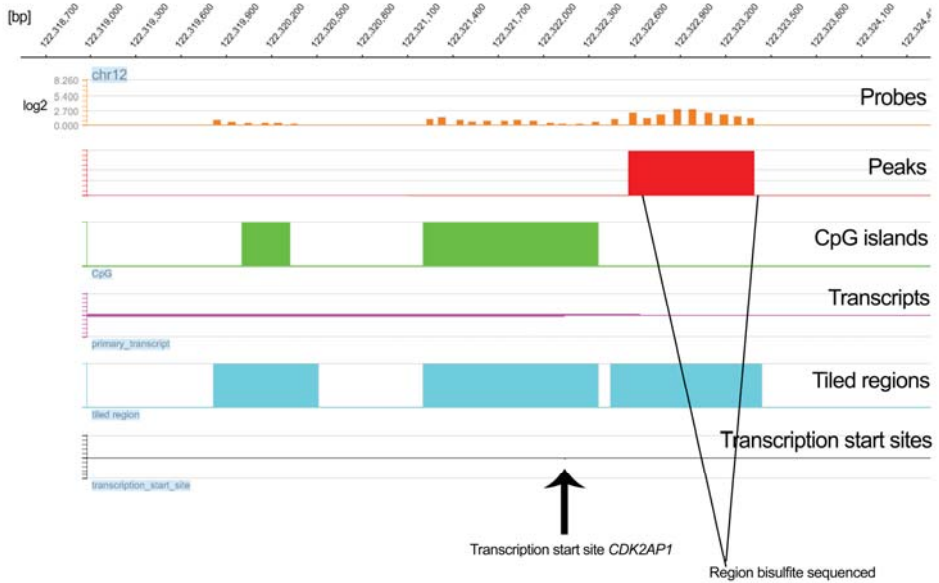


Case

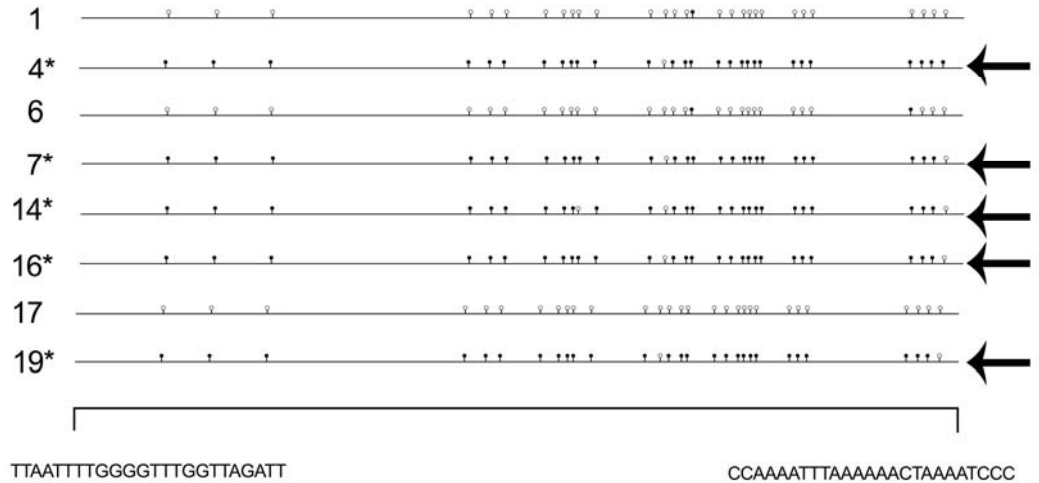


# CDK2AP1

Case 16

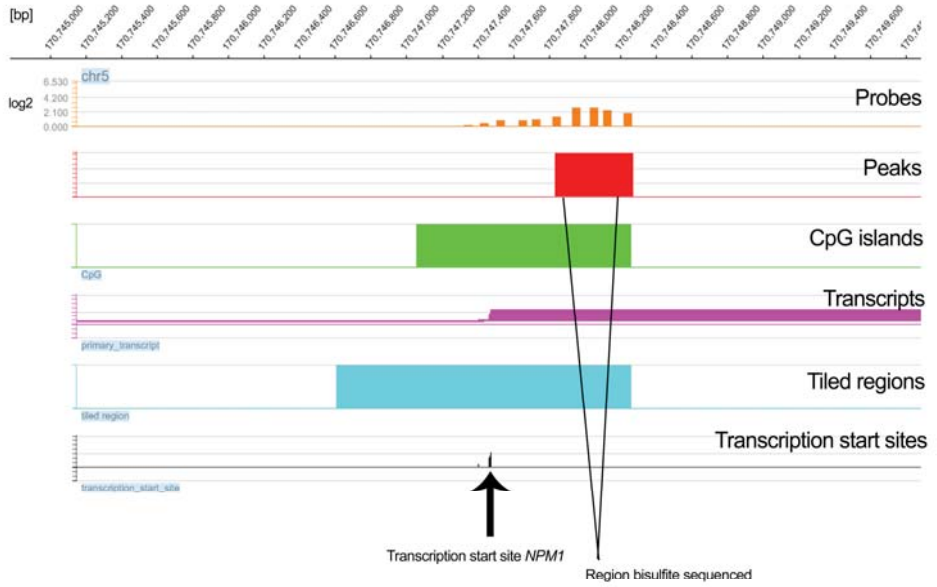


Case

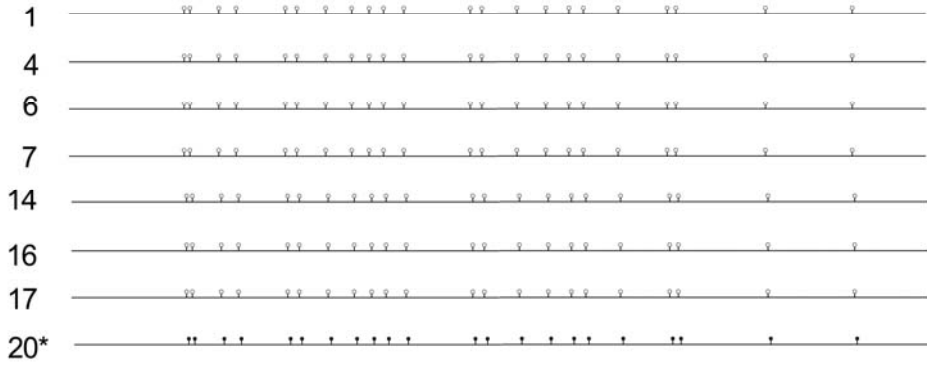


# NPM1

Case 20



## Case



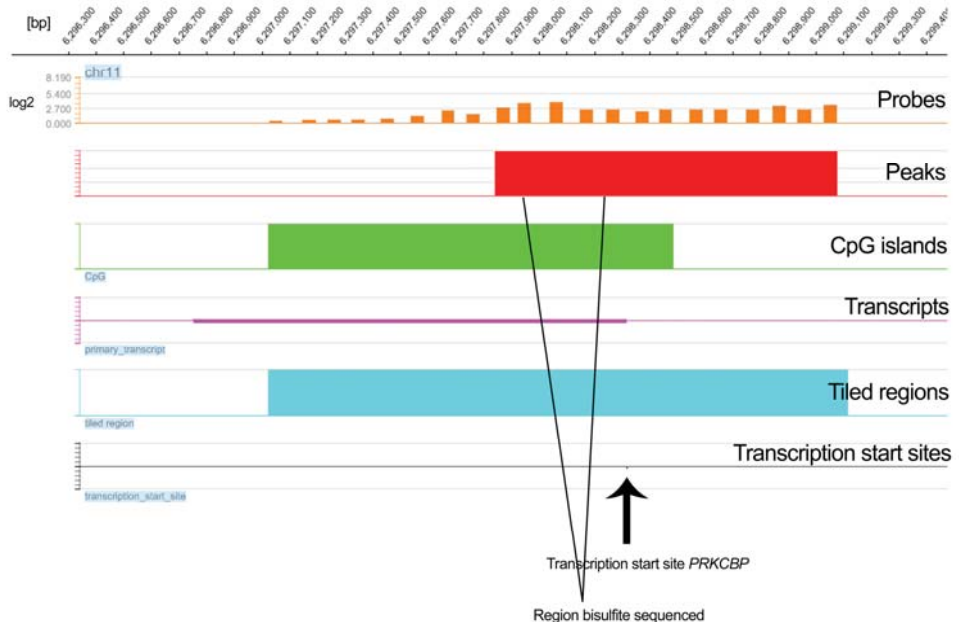
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AACAACAACCCATACTCC

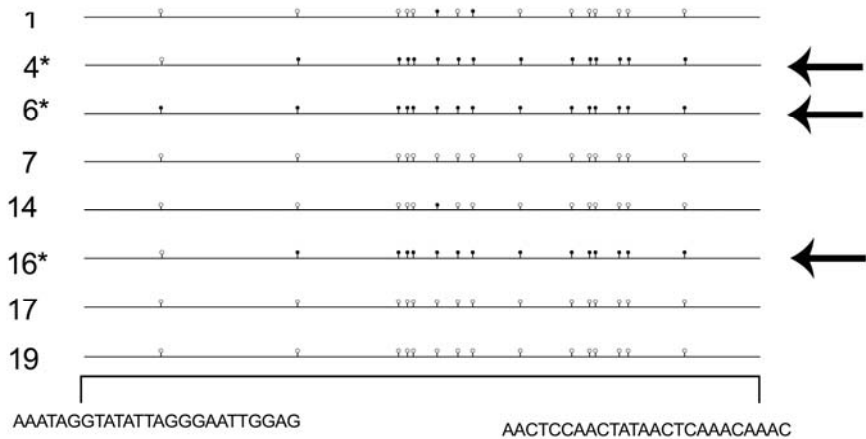


# PRKCBP

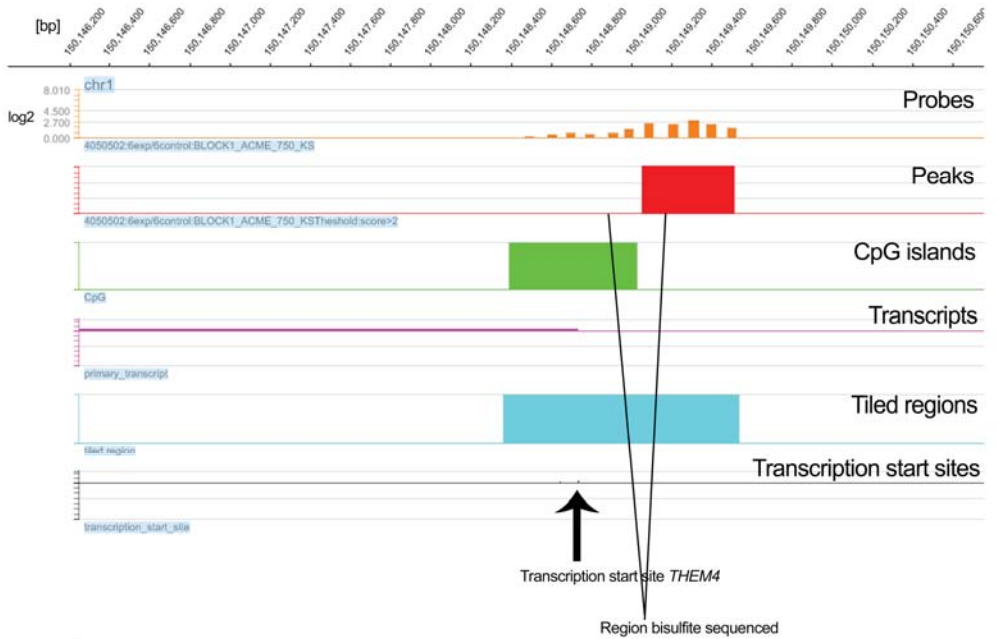
Case 16



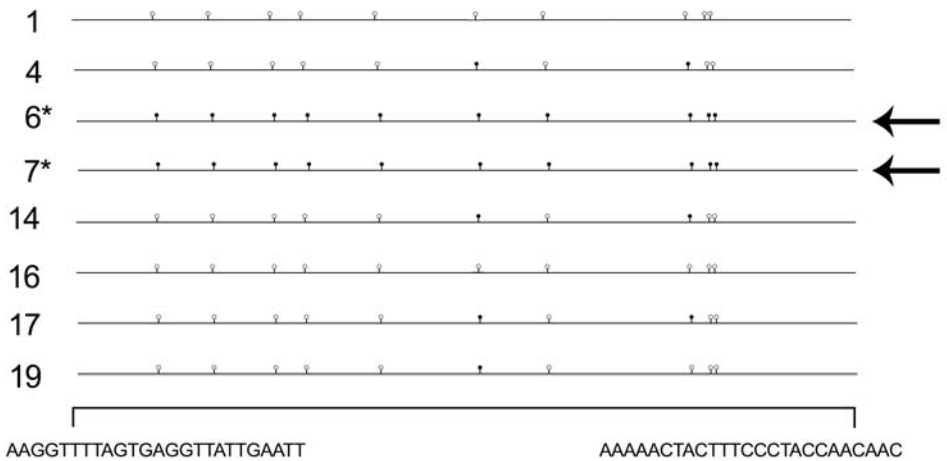
## Case



# THEM4 Case 6



## Case



**Figure S7.** Bisulfite sequencing validating the array results. Cases harboring a methylation peak detected by the promoter array are marked with an asterisk. Sequences demonstrated to be hypermethylated by bisulfite sequencing are indicated with arrows. Primer sequences used are given below each gene.

**Table S1.** Genes that previously have been reported to be frequently hypermethylated in malignant disorders<sup>a</sup>

Gene	Function <sup>b</sup>	Location <sup>b</sup>	Tissue/organ <sup>a</sup>
<i>ABCB1</i>	Drug resistance	7q21.1	Hematopoietic
<i>ABCB4</i>	Drug resistance	7q21.1	Various
<i>APC</i>	Cell cycle	5q21-22	Various
<i>AR</i>	Cell signaling	Xq11.2-12	Prostate
<i>BRCA1</i>	DNA repair	17q21	Breast, ovary
<i>CALCA</i>	Cell signaling	11p15.1-15.2	Various
<i>CASP8</i>	Apoptosis	2q33-34	Neuronal
<i>CAVI</i>	Transport	7q31.1	Breast
<i>CCNA1</i>	Cell cycle	13q12.3-13	Various
<i>CD44</i>	Cell adhesion	11p13	Prostate
<i>CDH1</i>	Cell adhesion	16q22.1	Various
<i>CDH13</i>	Cell adhesion	16q24.2-24.3	Breast, lung
<i>CDKN1B</i>	Cell cycle	12p12-13.1	Various
<i>CDKN1C</i>	Cell cycle	11p15.5	Stomach
<i>CDKN2A</i>	Cell cycle	9p21	Various
<i>CDKN2B</i>	Cell cycle	9p21	Hematopoietic
<i>CFTR</i>	Ion transport	7q31.2	Various
<i>DAPK1</i>	Apoptosis	9q34.1	Various
<i>DKK1</i>	Cell signaling	10q11.2	Colon
<i>EDNRB</i>	Cell signaling	13q22	Prostate
<i>EPHA3</i>	Cell signaling	3p11.2	Hematopoietic
<i>EPO</i>	Cell signaling	7q22	Colon
<i>ESR1</i>	Cell signaling	6q25.1	Breast
<i>EXT1</i>	Cell cycle	8q24.1	Hematopoietic, skin
<i>FABP3</i>	Cell proliferation	1p32-33	Breast, colon
<i>FAT</i>	Cell adhesion	4q35	Colon
<i>FHIT</i>	Cell cycle	3p14.2	Esophagus
<i>GATA4</i>	Transcription regulation	8p22-23.1	Colon, stomach
<i>GATA5</i>	Transcription regulation	20q13.3	Colon, stomach
<i>GJB2</i>	Cell signaling	13q11-12	Breast
<i>GPC3</i>	Morphogenesis	Xq26.1	Ovary
<i>GSTP1</i>	Transferase activity	11q13	Various

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<i>H19</i>	Unknown	11p15.5	Kidney
<i>HIC1</i>	Transcription regulation	17p13.3	Various
<i>HOXA9</i>	Transcription regulation	7p14-15	Neuronal
<i>ID4</i>	Transcription regulation	6p21-22	Hematopoietic, stomach
<i>IGF2</i>	Cell proliferation	11p15.5	Colon, hematopoietic
<i>IGFBP3</i>	Apoptosis	7p12-13	Lung, skin
<i>IGFBP7</i>	Cell proliferation	4q12	Liver
<i>LMNA</i>	Morphogenesis	1q21.2-21.3	Hematopoietic
<i>LTB4R</i>	Cell signaling	14q11.2-12	Various
<i>MGMT</i>	DNA repair	10q26	Various
<i>MLH1</i>	DNA repair	3p21.3	Various
<i>MME</i>	Cell signaling	3q25.1-25.2	Prostate
<i>MT1A</i>	Ion binding	16q13	Liver
<i>MUC2</i>	Extracellular structure	11p15.5	Colon
<i>MYOD1</i>	Cell differentiation	11p15.4	Various
<i>NEFL</i>	Morphogenesis	8p21	Brain
<i>PAX6</i>	Transcription regulation	11p13	Various
<i>PGR</i>	Cell signaling	11q22-23	Breast
<i>PLAU</i>	Cell signaling	10q24	Breast
<i>PRDM2</i>	Transcription regulation	1p36.2	Various
<i>PRKCDBP</i>	Cell cycle	11p15.4	Breast, lung
<i>PRLR</i>	Cell signaling	5p13-14	Breast
<i>PTGS2</i>	Cell motility	1q25.2-25.3	Colon, stomach
<i>PYCARD</i>	Apoptosis	16p11.2-12	Breast
<i>P14ARF</i>	Cell cycle	9p21	Various
<i>RARB</i>	Transcription regulation	3p24	Various
<i>RASSF1</i>	Cell cycle	3p21.3	Various
<i>RASSF5</i>	Apoptosis	1q32.1	Lung
<i>RBI</i>	Cell cycle	13q14.2	Various
<i>RBP1</i>	Transport	3q23	Various
<i>SFN</i>	Apoptosis	1p36.1	Breast, colon
<i>SFRP1</i>	Cell signaling	8p11.1-12	Colon
<i>SLC5A5</i>	Ion transport	19p12-13.2	Thyroid
<i>SLC5A8</i>	Ion transport	12q23.2	Brain, colon
<i>SOCS1</i>	Cell signaling	16p13.1	Hematopoietic, liver
<i>SOCS3</i>	Cell signaling	17q25.3	Lung

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<i>STK11</i>	Cell cycle	19p13.3	Various
<i>SYK</i>	Cell signaling	9q22	Breast
<i>TERT</i>	Telomere maintenance	15p15.3	Various
<i>TES</i>	Ion binding	7q31.2	Hematopoietic
<i>THBS1</i>	Cell adhesion	15q15	Brain
<i>TIMP3</i>	Apoptosis	22q12.1-13.2	Various
<i>TMEFF2</i>	Unknown	2q32.3	Bladder, colon
<i>TP73</i>	Apoptosis	1p36.3	Hematopoietic
<i>TSHR</i>	Cell signaling	14q31	Thyroid
<i>TUSC3</i>	Glycosylation	8p22	Various
<i>VCAN</i>	Cell adhesion	5q14.3	Colon
<i>VHL</i>	Apoptosis	3p25-26	Various
<i>WIF1</i>	Cell signaling	12q14.3	Colon, lung
<i>WRN</i>	DNA repair	8p11.2-12	Various
<i>WT1</i>	Transcription regulation	11p13	Various

<sup>a</sup>Retrieved from the Genes Methylated in Cancer Website at MD Anderson Cancer Center, The University of Texas ([www.mdanderson.org/departments/methylation/](http://www.mdanderson.org/departments/methylation/)) and from a review by Esteller (1).

<sup>b</sup>Function and location are based on data in NCBI Entrez Gene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>). Only one major function per gene is given.

## Reference

1. Esteller, M. (2007) Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat. Rev. Genet.*, **8**, 286-298.



**Table S2.** Hypermethylated genes in B-lineage ALL<sup>a</sup>

Gene	Function <sup>b</sup>	Location <sup>c</sup>
<i>ABCB1</i>	Drug resistance	7q21.1
<i>ABL1</i>	Cell signaling	9q34.1
<i>ADAMTS1</i>	Proteolysis	21q21.2
<i>ADAMTS5</i>	Proteolysis	21q21.3
<i>AHR</i>	Apoptosis	7p15
<i>APAF1</i>	Apoptosis	12q23
<i>APC</i>	Cell cycle	5q21-22
<i>CADM1</i>	Apoptosis	11q23.2
<i>CALCA</i>	Cell signaling	11p15.1-15.2
<i>CCND2</i>	Cell cycle	12p13
<i>CD44</i>	Cell adhesion	11p13
<i>CDC2</i>	Cell cycle	10q21.1
<i>CDH1</i>	Cell adhesion	16q22.1
<i>CDH13</i>	Cell adhesion	16q24.2-24.3
<i>CDKN1A</i>	Cell cycle	6p21.2
<i>CDKN1C</i>	Cell cycle	11p15.5
<i>CDKN2A</i>	Cell cycle	9p21
<i>CDKN2B</i>	Cell cycle	9p21
<i>CHFR</i>	Cell cycle	12q24.33
<i>DACT1</i>	Cell signaling	14q23.1
<i>DAPK1</i>	Apoptosis	9q34.1
<i>DBC1</i>	Apoptosis	9q32-33
<i>DCC</i>	Apoptosis	18q21.3
<i>DDX51</i>	RNA processing	12q24.3
<i>DIABLO</i>	Apoptosis	12q24.3
<i>DKK3</i>	Cell signaling	11p15.2
<i>DLC1</i>	Cell adhesion	8p22
<i>EGR4</i>	Transcription regulation	2p13
<i>ESR1</i>	Cell signaling	6q25.1
<i>EXT1</i>	Cell cycle	8q24.1
<i>FHIT</i>	Cell cycle	3p14.2
<i>GATA5</i>	Transcription regulation	20q13.3
<i>GGH</i>	Peptidase	8q12.3
<i>GSTP1</i>	Transferase activity	11q13

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<i>HCK</i>	Morphogenesis	20q11-12
<i>HIC1</i>	Transcription regulation	17p13.3
<i>KCNK2</i>	Ion transport	1q41
<i>KLK10</i>	Cell cycle	19q13.3-13.4
<i>LATS1</i>	Cell cycle	6q24-25.1
<i>LATS2</i>	Cell cycle	13q11-12
<i>LMNA</i>	Morphogenesis	1q21.2-21.3
<i>LRP1B</i>	Proteolysis	2q21.2
<i>MGMT</i>	DNA repair	10q26
<i>MME</i>	Cell signaling	3q25.1-25.2
<i>MOS</i>	Cell cycle	8q11
<i>MSH6</i>	Apoptosis	2p16
<i>NKX6-1</i>	Morphogenesis	4q21.2-22
<i>NNAT</i>	Morphogenesis	20q11.2-12
<i>NOPE</i>	Unknown	15q22.3
<i>P14ARF</i>	Cell cycle	9p21
<i>PACRG</i>	Unknown	6q26
<i>PARK2</i>	Proteolysis	6q25.2-27
<i>PAX6</i>	Transcription regulation	11p13
<i>PCDHGA12</i>	Cell adhesion	5q31
<i>PGR</i>	Cell signaling	11q22-23
<i>PPP1R13B</i>	Apoptosis	14q32.3
<i>PRDM2</i>	Transcription regulation	1p36.2
<i>PTEN</i>	Cell cycle	10q23.3
<i>PTPN6</i>	Cell signaling	12p13
<i>PYCARD</i>	Apoptosis	16p11.2-12
<i>RARB</i>	Transcription regulation	3p24
<i>RPRM</i>	Cell cycle	2q23.3
<i>RUNDC3B</i>	Unknown	7q21.1
<i>SEPT4</i>	Apoptosis	17q22-23
<i>SFRP1</i>	Cell signaling	8p11.1-12
<i>SFRP2</i>	Cell signaling	4q31.3
<i>SFRP4</i>	Cell signaling	7p14.1
<i>SFRP5</i>	Cell signaling	10q24.1
<i>SLC2A14</i>	Transport	12p13.3
<i>SLC19A1</i>	Transport	21q22.3

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<i>SMC1A</i>	Cell cycle	Xp11.2
<i>SMC1B</i>	Cell cycle	22q13.3
<i>SYK</i>	Cell signaling	9q22
<i>THBS1</i>	Cell adhesion	15q15
<i>TMP3</i>	Apoptosis	22q12.1-13.2
<i>TP53</i>	Apoptosis	17p13.1
<i>TP73</i>	Apoptosis	1p36.3
<i>TUSC3</i>	Glycosylation	8p22
<i>WIF1</i>	Cell signaling	12q14.3
<i>WNT5A</i>	Cell signaling	3p14-21
<i>WT1</i>	Transcription regulation	11p13

<sup>a</sup>The genes were identified through a PubMed search using the terms “pediatric”, “ALL”, “CpG” and “methylation”. Initially, more than 56 articles were obtained, but when including only those that reported hypermethylated genes in B-lineage ALL only 40 remained (1-40). Based on these publications, a total of 81 genes have been shown to be hypermethylated in B-lineage ALL.

<sup>b</sup>Function is based on data produced by the EASE software (41) or found in NCBI Entrez Gene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>). Only one major function per gene is given.

<sup>c</sup>Location is based on information in NCBI Entrez Gene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>).

## References

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2. Taylor, K.H., Kramer, R.S., Davis, J.W., Guo, J., Duff, D.J., Xu, D., Caldwell, C.W. and Shi, H. (2007) Ultradeep bisulfite sequencing analysis of DNA methylation patterns in multiple gene promoters by 454 sequencing. *Cancer Res.*, **67**, 8511-8518.
3. Taylor, K.H., Pena-Hernandez, K.E., Davis, J.W., Arthur, G.L., Duff, D.J., Shi, H., Rahmatpanah, F.B., Sjahputera, O. and Caldwell, C.W. (2007) Large-scale CpG methylation analysis identifies novel candidate genes and reveals methylation hotspots in acute lymphoblastic leukemia. *Cancer Res.*, **67**, 2617-2625.

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**Table S3.** Hypermethylated genes in pediatric B-lineage ALL<sup>a</sup>

Reference	Method	No. of genes		Genes validated to be hypermethylated
		investigated	No. of genes hypermethylated	
Paulsson <i>et al.</i> (1)	Multiplex ligation-dependent probe amplification + bisulfite sequencing	35	16	<i>APC, CADM1, CD44, CDH13, CDKN2B, CHFR, ESRI, FHIT, GATA5, MSH6, PAX6, RARB, THBS1, TMP3, TP73</i> and <i>WT1</i>
Taylor <i>et al.</i> (2)	454 bisulfite sequencing	25	Not given	Not given
Taylor <i>et al.</i> (3)	Microarray + bisulfite sequencing	2,267	262	<i>ABCBI, DCC, DDX51, DL1, KCNK2, LRP1B, NKX6-1, NOPE, PCDHGA12, RUNDC3B</i> and <i>SLC2A14</i>
Roman-Gomez <i>et al.</i> (4)	Bisulfite sequencing	1	1	<i>WNT5A</i>
Cheng <i>et al.</i> (5)	Bisulfite sequencing	1	1	<i>GGH</i>
Roman-Gomez <i>et al.</i> (6)	Bisulfite sequencing	38	38	<i>ADAMTS1, ADAMTS5, APAF1, CDH1, CDH13, CDKN1C, CDKN2A, CDKN2B, DACT1, DAPK1, DBCL, DIABLO, DKK3, FHIT, KLK10, LATS1, LATS2, PI4ARF, PACRG, PARK2, PGR, PPP1R13B, PRDM2, PTEN, PTPN6, PYCARD, RPRM, SEPT4, SFRP1, SFRP2, SFRP4, SFRP5, SLC19A1, SMC1A, SMC1B, SYK, TP73</i> and <i>WIFI</i>
Päxao <i>et al.</i> (7)	Bisulfite sequencing	6	6	<i>CALCA, CDH1, CDKN2A, GSTP1, HIC1</i> and <i>TP73</i>
Stam <i>et al.</i> (8)	Bisulfite sequencing	1	1	<i>FHIT</i>
Agirre <i>et al.</i> (9)	Bisulfite sequencing	2	2	<i>PACRG</i> and <i>PARK2</i>



Yang <i>et al.</i> (10)	Bisulfite sequencing	14	4	CDKN2A, CDKN2B, FHIT and RARB
Canalli <i>et al.</i> (11)	Bisulfite sequencing	3	3	CDKN1C, CDKN2B and TP73
Tsellou <i>et al.</i> (12)	Bisulfite sequencing	1	1	CDKN2B
Sahu and Das (13)	Bisulfite sequencing	1	1	TP73
Scholz <i>et al.</i> (14)	Microarray + bisulfite sequencing	57	5	CCND2, CDC2, EGR4, MOS and TUSC3
Roman-Gomez <i>et al.</i> (15)	Bisulfite sequencing	1	1	DKK3
Zheng <i>et al.</i> (16)	Bisulfite sequencing	1	1	FHIT
Gutierrez <i>et al.</i> (17)	Bisulfite sequencing	7	6	CDH1, DAPK1, MGMT, TP73, CDKN2A and CDKN2B
García-Manero <i>et al.</i> (18)	Bisulfite sequencing	7	5	ABCBI, ABLI, CDKN2B, ESRI and MME
Roman-Gomez <i>et al.</i> (19)	Restriction digestion	1	1	CDKN1A
Corn <i>et al.</i> (20)	Bisulfite sequencing	1	1	TP73
Iravani <i>et al.</i> (21)	Southern blot	2	1	CDKN2B

<sup>a</sup>Of the 40 publications on hypermethylated genes in B-lineage ALL (Supplementary Material, Table S2), 21 included samples from pediatric ALL.

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**Table S4.** Chromosomal distribution of the 8,662 genes with significant CpG island methylation scores

Chromosome	No. of genes only hypermethylated in <i>ETV6/RUNX1</i> - high hyperdiploid positive cases		No. of genes hypermethylated in both genetic subgroups		Total no. of hypermethylated genes		Total no. of genes hypermethylated in <i>ETV6/RUNX1</i> - high hyperdiploid positive cases	
	hypermethylated in <i>ETV6/RUNX1</i> - high hyperdiploid positive cases	hypermethylated in high hyperdiploid cases	hypermethylated in both genetic subgroups	hypermethylated in both genetic subgroups	hypermethylated in both genetic subgroups	hypermethylated in both genetic subgroups	hypermethylated in <i>ETV6/RUNX1</i> - high hyperdiploid positive cases	hypermethylated in <i>ETV6/RUNX1</i> - high hyperdiploid positive cases
1	88	453	261	802	349	714		
2	83	281	189	553	272	470		
3	49	224	128	401	177	352		
4	43	211	121	375	164	332		
5	62	184	119	365	181	303		
6	46	300	135	481	181	435		
7	46	191	180	417	226	371		
8	0	282	0	282	0	282		
9	60	173	131	364	191	304		
10	25	178	121	324	146	299		
11	62	327	174	563	236	501		
12	46	249	116	411	162	365		
13	18	64	58	140	76	122		
14	24	157	76	257	100	233		
15	39	122	85	246	124	207		
16	61	160	145	366	206	305		
17	60	229	176	465	237	404		

18	16	77	52	145	68	129
19	83	255	236	574	320	490
20	30	120	88	238	118	208
21	8	80	61	149	69	141
22	23	88	86	197	110	173
X	31	272	198	501	227	472
Y	9	2	35	46	43	38
Total	1012	4679	2971	8662	3983	7650

**Table S5.** The 30 genes displaying the highest methylation peak scores among the 8,662 genes with significant CpG island methylation scores

Gene <sup>a</sup>	Function <sup>b</sup>	Location <sup>b</sup>
<i>DNAJB8</i>	Heat shock protein	3q21.3
<i>LSP1</i>	Cell motility	11p15.5
<i>DPCR1</i>	Unknown	6p21.33
<i>GLI2</i>	Transcription regulation	2q14
<i>ZFX4</i>	Transcription regulation	8q21.11
<i>SLC5A3</i>	Ion transport	21q22.12
<i>SLC44A5</i>	Unknown	1p31.1
<i>AQP6</i>	Transport	12q13
<i>SSX7</i>	Transcription regulation	Xp11.23
<i>ATP9B</i>	Metabolism	18q23
<i>CD7</i>	Immune response	17q25.2-25.3
<i>OTUD4</i>	Unknown	4q31.21
<i>FTSJ1</i>	Methyltransferase	Xp11.23
<i>GJD4</i>	Cell signaling	10p11.21
<i>ANGPT2</i>	Angiogenesis	8p23.1
<i>HIST1H2AA</i>	Histone	6p22.2
<i>HIST1H2BA</i>	Histone	6p22.2
<i>WISP3</i>	Cell signaling	6q21
<i>MOBP</i>	Unknown	3p22.1
<i>LRRC37A</i>	Unknown	17q21.31
<i>AFAP1</i>	Morphogenesis	4p16
<i>ATP11A</i>	Metabolism	13q34
<i>SSX3</i>	Transcription regulation	Xp11.23
<i>GAS7</i>	Cell cycle	17p13.1
<i>RBMY1J</i>	RNA processing	Yq11.22
<i>IQSEC3</i>	Signal transduction	12p13.33
<i>SSX2</i>	Unknown	Xp11.22
<i>TULP4</i>	Transcription regulation	6q25-26
<i>MAGEB10</i>	Unknown	Xp21.3
<i>PHOX2B</i>	Transcription regulation	4p12

<sup>a</sup>The genes are listed in order of decreasing  $-\log_{10}$  p-values (see Materials and Methods).

<sup>b</sup>Function and location are based on data in NCBI Entrez Gene

(<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>). Only one major function per gene is given.



**Table S6.** The 30 genes displaying the highest methylation peak scores in *ETV6/RUNX1*-positive and high hyperdiploid cases

<i>ETV6/RUNX1</i> -positive cases			High hyperdiploid cases		
Gene <sup>a</sup>	Function <sup>b</sup>	Location <sup>b</sup>	Gene <sup>a</sup>	Function <sup>b</sup>	Location <sup>b</sup>
<i>OTUD4</i>	Unknown	4q31.21	<i>REXO1L1</i>	Exonuclease	8q21.2
<i>ATP9B</i>	Metabolism	18q23	<i>SLC17A4</i>	Ion transport	6p21.3-22
<i>GLI2</i>	Transcription regulation	2q14	<i>DEFB109</i>	Unknown	8p23.1
<i>MOBP</i>	Unknown	3p22.1	<i>DNAJB8</i>	Heat shock protein	3q21.3
<i>LRRC37A</i>	Unknown	17q21.31	<i>SPRYD5</i>	Unknown	11q11
<i>RBMY1J</i>	RNA processing	Yq11.22	<i>UGT2B11</i>	Metabolism	4q13.2
<i>SSX2</i>	Transcription regulation	Xp11.22	<i>PZP</i>	Pregnancy	12p12.2-13
<i>TULP4</i>	Transcription regulation	6q25-26	<i>DPCR1</i>	Unknown	6p21.33
<i>LSP1</i>	Cell motility	11p15.5	<i>SYT16</i>	Unknown	14q23.2
<i>LTBP1</i>	Unknown	2p21-22	<i>GLI2</i>	Transcription regulation	2q14
<i>MAGEB10</i>	Unknown	Xp21.3	<i>MAPK10</i>	Cell signaling	4q22.1-23
<i>SSX3</i>	Transcription regulation	Xp11.23	<i>LEMD1</i>	Unknown	1q32.1
<i>PHOX2B</i>	Transcription regulation	4p12	<i>ZFHX4</i>	Transcription regulation	8q21.11
<i>CD7</i>	Immune response	17q25.2-25.3	<i>SLC5A3</i>	Ion transport	21q22.12
<i>ATP11A</i>	Transport	13q34	<i>SLC44A5</i>	Unknown	1p31.1
<i>RAB27A</i>	Cell signaling	15q15-21.1	<i>AQP6</i>	Transport	12q13
<i>CPSF3L</i>	snRNA regulation	1p36.33	<i>CD7</i>	Immune response	17q25.2-25.3
<i>TAS1R3</i>	Cell signaling	1p36.33	<i>ATP9B</i>	Metabolism	18q23
<i>KCND2</i>	Transport	7q31	<i>FTSJ1</i>	Methyltransferase	Xp11.23

<i>TRAF7</i>	Transcription regulation	16p13.3	<i>GJD4</i>	Cell signaling	10p11.21
<i>HOXA3</i>	Transcription regulation	7p14-15	<i>ANGPT2</i>	Angiogenesis	8p23.1
<i>HOXA4</i>	Transcription regulation	7p14-15	<i>HIST1H2AA</i>	Histone	6p22.2
<i>PAGE2B</i>	Unknown	Xp11.21	<i>HIST1H2BA</i>	Histone	6p22.2
<i>ZNF609</i>	Unknown	15q22.31	<i>TRDN</i>	Muscle function	6q22-23
<i>UBE2U</i>	Protein degradation	1p31.3	<i>WISP3</i>	Cell signaling	6q21
<i>CSF2RA</i>	Cell signaling	Xp22.32, Yp11.3	<i>AFAP1</i>	Morphogenesis	4p16
<i>TSPY1</i>	Cell proliferation	Yp11.2	<i>ATP11A</i>	Transport	13q34
<i>TSPY2</i>	Cell differentiation	Yp11.2	<i>AMELX</i>	Mineralization	Xp22.1-22.31
<i>DNAJB8</i>	Heat shock protein	3q21.3	<i>CLCN5</i>	Transport	Xp11.23
<i>HIST1H2AA</i>	Histone	6p22.2	<i>AHRR</i>	Cell signaling	5p15.3

<sup>a</sup>The genes are listed in order of decreasing  $-\log_{10}$  p-values (see Methods section).

<sup>b</sup>Function and location are based on data in NCBI Entrez Gene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>). Only one major function per gene is given.

**Table S7.** The 30 unique genes displaying the highest methylation peak scores in *ETV6/RUNX1*-positive and high hyperdiploid cases

<i>ETV6/RUNX1</i> -positive cases				High hyperdiploid cases			
Gene <sup>a</sup>	Function <sup>b</sup>	Location <sup>b</sup>	Gene <sup>a</sup>	Function <sup>b</sup>	Location <sup>b</sup>		
<i>OTUD4</i>	Unknown	4q31.21	<i>KLHDC7B</i>	Unknown	22q13.33		
<i>SSX7</i>	Transcription regulation	Xp11.23	<i>GP1BB</i>	Signal transduction	22q11.21		
<i>BDNF</i>	Growth factor	11p13	<i>BRP44L</i>	Unknown	6q27		
<i>LSP1</i>	Cell motility	11p15.5	<i>GZF1</i>	Transcription regulation	20p11.21-12.3		
<i>MS4A12</i>	Cell signaling	11q12	<i>MFSD3</i>	Unknown	8q24.3		
<i>PTPN3</i>	Cell signaling	9q31	<i>TMEM200B</i>	Unknown	1p35		
<i>SSX3</i>	Transcription regulation	Xp11.23	<i>CSAG1</i>	Tumor antigen	Xq28		
<i>GGTL4</i>	Catalytic activity	22q11.22	<i>MAGEA12</i>	Unknown	Xq28		
<i>SSX2</i>	Transcription regulation	Xp11.22	<i>TCEB3C</i>	Transcription regulation	18q21.1		
<i>SYNE2</i>	Morphogenesis	14q23.2	<i>RPL36</i>	Translation	19p13.3		
<i>CPNI</i>	Proteolysis	10q24.2	<i>FGF20</i>	Cell growth	8p21.3-22		
<i>LRRC37A</i>	Unknown	17q21.31	<i>GAS2</i>	Apoptosis	11p14.3-15.2		
<i>SPANXB2</i>	Spermatoid development	Xq27.1	<i>GNL2</i>	GTPase	1p34.3		
<i>ANP32D</i>	Tumor suppressor	12q13.11	<i>LCK</i>	Hematopoiesis	1p34.3		
<i>PCDHAI2</i>	Cell adhesion	5q31	<i>PGA5</i>	Unknown	11q13		
<i>TSPY1</i>	Cell proliferation	Yp11.2	<i>SLC25A25</i>	Unknown	9q34.11		
<i>TSPY2</i>	Cell proliferation	Yp11.2	<i>TNS3</i>	Cell proliferation	7p12.3		
<i>NALP4</i>	Apoptosis	19q13.42	<i>TRPV1</i>	Ion transport	17p13.3		
<i>FAM156A</i>	Unknown	Xp11.22	<i>AQP5</i>	Water transport	12q13		

<i>TP53TG3</i>	Unknown	16p13	<i>C4A</i>	Inflammatory response	6p21.3
<i>RBMY1A1</i>	RNA binding	Yq11.22	<i>C4B</i>	Inflammatory response	6p21.3
<i>RBMY1B</i>	RNA binding	Yq11.22	<i>NCAN</i>	Cell adhesion	19p12
<i>RBMY1F</i>	RNA binding	Yq11.22	<i>EIF4G1</i>	Translation	3q27-29
<i>ANAPC11</i>	Cell cycle	17q25.3	<i>FHL2</i>	Transcription regulation	2q12-14
<i>GPR109B</i>	Signal transduction	12q24.31	<i>TMEM176A</i>	Unknown	7q36.1
<i>FOXD4LI</i>	Transcription regulation	2q14.1	<i>HEXDC</i>	Unknown	17q25.3
<i>HOXA3</i>	Transcription regulation	7p14-15	<i>TMEM176B</i>	Morphogenesis	7q36.1
<i>IQSEC3</i>	Signal transduction	12p13.33	<i>LYPD5</i>	Unknown	19q13.31
<i>SPANXE</i>	Spermatid development	Xq27.1	<i>ODF3</i>	Sperm component	11p15.5
<i>HOXA4</i>	Transcription regulation	7p14-15	<i>RPUSD1</i>	Unknown	16p13.3

<sup>a</sup>The genes are listed in order of decreasing -log10 p-values (see Methods section).

<sup>b</sup>Function and location are based on data in NCBI Entrez Gene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>). Only one major function per gene is given.

**Table S8.** The genes shown by segmental analysis to be highly enriched for methylation but that did not display decreased expression or for which no expression data were available. The 45 genes associated with decreased expression are given in Table 2 in the article.

Gene	Function <sup>a</sup>	Location <sup>a</sup>	Expression
<i>FOXA2</i>	Transcription regulation	20p11	Not available
<i>FOXD4L3</i>	Transcription regulation	9q13	Not available
<i>FUSSEL18</i>	Transcription regulation	18q21.1	Not available
<i>GLIS1</i>	Transcription regulation	1p32.3	Not available
<i>HDAC9</i>	Transcription regulation	7p21.1	Not available
<i>PHOX2B</i>	Transcription regulation	4p12	Not available
<i>SNAPC4</i>	Transcription regulation	9q34.3	Not available
<i>SSBP3</i>	Transcription regulation	1p32.3	Not available
<i>SSX2</i>	Transcription regulation	Xp11.22	Not available
<i>SSX5</i>	Transcription regulation	Xp11.23	Not available
<i>SSX7</i>	Transcription regulation	Xp11.23	Not available
<i>YY2</i>	Transcription regulation	Xp22.1-22.2	Not available
<i>ZNF384</i>	Transcription regulation	12p12	Not available
<i>ZNF502</i>	Transcription regulation	3p21.31	Not available
<i>CAMK1G</i>	Cell signaling	1q32-41	Not available
<i>CCR5</i>	Cell signaling	3p21.31	Not available
<i>ERAS</i>	Cell signaling	Xp11.23	Not available
<i>FYN</i>	Cell signaling	6q21	Not available
<i>GDF9</i>	Cell signaling	5q31.1	Not available
<i>ICOS</i>	Cell signaling	2q33	Not available
<i>IL2</i>	Cell signaling	4q26-27	Not available
<i>MS4A8B</i>	Cell signaling	11q12.2	Not available
<i>TLR3</i>	Cell signaling	4q35	Not available
<i>TRPV1</i>	Cell signaling	17p13.3	Not available
<i>WISP3</i>	Cell signaling	6q21	Not available
<i>AFAP1</i>	Apoptosis	4p16	Not available
<i>CASP5</i>	Apoptosis	11q22.2-22.3	Not available
<i>HUS1B</i>	Apoptosis	6p25.3	Not available
<i>ICEBERG</i>	Apoptosis	11q21-22	Not available
<i>MAGED1</i>	Apoptosis	Xp11.23	Not available
<i>SYNE1</i>	Apoptosis	6q25	Not available

<i>TRAF7</i>	Apoptosis	16p13.3	Not available
<i>CTNNA3</i>	Cell adhesion	10q22.2	Not available
<i>DSC2</i>	Cell adhesion	18q12.1	Not available
<i>LSP1</i>	Cell adhesion	11p15.5	Not available
<i>HIST1H1B</i>	Nucleosome assembly	6p21.3-22	Not available
<i>HIST1H2AA</i>	Nucleosome assembly	6p22.2	Not available
<i>HIST1H3E</i>	Nucleosome assembly	6p21.3	Not available
<i>CABLES1</i>	Cell cycle	18q11.2	Not available
<i>DMBT1</i>	Cell cycle	10q25.3-26.1	Not available
<i>CD7</i>	Immune response	17q25.2-25.3	Not available
<i>FCRL3</i>	Immune response	1q21-22	Not available
<i>DCN</i>	Morphogenesis	12q21.33	Not available
<i>ITIH5</i>	Morphogenesis	10p14	Not available
<i>MAS1</i>	Cell proliferation	6q25.3-26	Not available
<i>TNS3</i>	Cell proliferation	7p12.3	Not available
<i>ELA2B</i>	Proteolysis	1p36.21	Not available
<i>DNPEP</i>	Proteolysis	2q35	Not available
<i>B3GALT2</i>	Glycosylation	1q31	Not available
<i>CES1</i>	Metabolism	16q13-22.1	Not available
<i>CMT5</i>	Chemotaxis	14q11.2	Not available
<i>IL21</i>	Cell maturation	4q26-27	Not available
<i>SETD2</i>	Chromatin modification	3p21.31	Not available
<i>SLC44A5</i>	Transport	1p31.1	Not available
<i>TRPC7</i>	Ion transport	5q31.2	Not available
<i>DNTT</i>	Various	10q23-24	Not available
<i>GLI2</i>	Various	2q14	Not available
<i>MATK</i>	Various	19p13.3	Not available
<i>RAG2</i>	Various	11p13	Not available
<i>ANP32D</i>	Unknown	12q13.11	Not available
<i>DIRC1</i>	Unknown	2q33	Not available
<i>LEMD1</i>	Unknown	1q32.1	Not available
<i>REG4</i>	Unknown	1p12-13.1	Not available
<i>CD69</i>	Cell signaling	12p12-13	Not differential
<i>GJB1</i>	Cell signaling	Xq13.1	Not differential
<i>LYNX1</i>	Cell signaling	8q24.3	Not differential
<i>PTPRF</i>	Cell signaling	1p34	Not differential

<i>PTPRU</i>	Cell signaling	1p35.1-35.3	Not differential
<i>RGS10</i>	Cell signaling	10q25	Not differential
<i>IL1A</i>	Apoptosis	2q14	Not differential
<i>PRMT2</i>	Apoptosis	21q22.3	Not differential
<i>EWSR1</i>	Transcription regulation	22q12.2	Not differential
<i>SSX3</i>	Transcription regulation	Xp11.23	Not differential
<i>APTX</i>	DNA repair	9p13.3	Not differential
<i>ATP2B2</i>	Ion transport	3p25.3	Not differential
<i>ATRX</i>	Methylation regulation	Xq13.1-21.1	Not differential
<i>CD53</i>	Signal transduction	1p13	Not differential
<i>DDX59</i>	ATP binding	1q32.1	Not differential
<i>DNAJB8</i>	Protein folding	3q21.3	Not differential
<i>RBMY1A1</i>	Cell differentiation	Yq11.22	Not differential
<i>SELE</i>	Cell adhesion	1q22-25	Not differential
<i>TCL6</i>	Unknown	14q32.1	Not differential
<i>MLL3</i>	Transcription regulation	7q36.1	Upregulated
<i>TIGD2</i>	Transcription regulation	4q22.1	Upregulated
<i>FGFR1</i>	Cell signaling	8p11.1-11.2	Upregulated
<i>IL2RG</i>	Cell signaling	Xq13.1	Upregulated
<i>ANGPTL7</i>	Signal transduction	1p36.2-36.3	Upregulated
<i>KIFAP3</i>	Signal transduction	1q24.2	Upregulated
<i>ALKBH1</i>	DNA repair	14q24.3	Upregulated
<i>CTNNA1</i>	Transport	5q31	Upregulated
<i>LRMP</i>	Vesicle function	12p12.1	Upregulated
<i>MS4A1</i>	B-cell differentiation	11q12	Upregulated
<i>NEK7</i>	Mitotic control	1q31.3	Upregulated

<sup>a</sup>Function and location are based on data in NCBI Entrez Gene

(<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>). Only one major function per gene is given.

**Table S9.** Genes involved in B-cell development and regulation<sup>a</sup>

Gene	Function <sup>b</sup>	Location <sup>c</sup>
<i>ABL1</i>	Cell signaling	9q34.1
<i>BAD</i>	Apoptosis	11q13.1
<i>BCL2</i>	Apoptosis	18q21.3
<i>BCL2L11</i>	Apoptosis	2q13
<i>BCL6</i>	Transcription regulation	3q27
<i>BCL10</i>	Apoptosis	1p22
<i>BCL11A</i>	Transcription regulation	2p16.1
<i>BLK</i>	Cell signaling	8p22-23
<i>BLNK</i>	Cell signaling	10q23.2-23.3
<i>BTK</i>	Apoptosis	Xq21.3-22
<i>BTLA</i>	Immune response	3q13.2
<i>CD19</i>	Cell signaling	16p11.2
<i>CD79A</i>	Cell signaling	19q13.2
<i>CD79B</i>	Cell signaling	17q23
<i>CD40LG</i>	Immune response	Xq26
<i>CSF1R</i>	Cell signaling	5q33-35
<i>DNTT</i>	DNA synthesis	10q23-24
<i>EBF1</i>	Transcription regulation	5q34
<i>FCGR2B</i>	Immune response	1q23
<i>FLT3</i>	Cell signaling	13q12
<i>FLT3LG</i>	Cell signaling	19q13.3
<i>IFNAR1</i>	Cell signaling	21q22.1
<i>IFNG</i>	Immune response	12q14
<i>IGH@</i>	Immune response	14q32.3
<i>IGK@</i>	Immune response	2p12
<i>IGL@</i>	Immune response	22q11.1-11.2
<i>IKBKB</i>	Transcription regulation	8p11.2
<i>IKZF1</i>	Transcription regulation	7p11.1-13
<i>IKZF2</i>	Transcription regulation	2q34
<i>IKZF3</i>	Transcription regulation	17q21
<i>IL2RA</i>	Immune response	10p14-15
<i>IL2RB</i>	Immune response	22q13.1
<i>IL2RG</i>	Immune response	Xq13.1



<i>IL4R</i>	Immune response	16p11.2-12.1
<i>IL7R</i>	Immune response	5p13
<i>IRF4</i>	Transcription regulation	6p23-25
<i>IRF8</i>	Transcription regulation	16q24.1
<i>LAG3</i>	Immune response	12p13.3
<i>LAX1</i>	Immune response	1q32.1
<i>LEF1</i>	Cell signaling	4q23-25
<i>LILRB4</i>	Immune response	19q13.4
<i>LYN</i>	Cell signaling	8q13
<i>NFKB1</i>	Transcription regulation	4q24
<i>NFKB2</i>	Transcription regulation	10q24
<i>NOTCH1</i>	Cell signaling	9q34.3
<i>NOTCH2</i>	Cell cycle	1p11-13
<i>PAX5</i>	Transcription regulation	9p13
<i>PLCG2</i>	Cell signaling	16q24.1
<i>RAG1</i>	DNA recombination	11p13
<i>RAG2</i>	DNA recombination	11p13
<i>SOX4</i>	Transcription regulation	6p22.3
<i>SPI1</i>	Transcription regulation	11p11.2
<i>SPIB</i>	Transcription regulation	19q13.3-13.4
<i>SYK</i>	Cell signaling	9q22
<i>TCF3</i>	Transcription regulation	19p13.3
<i>VPREB1</i>	Immune response	22q11.22

<sup>a</sup>In order to identify putative targets for hypermethylation in B-lineage ALL, we compiled genes known to be involved in B-cell development and regulation using the pathway analysis software provided by Ingenuity Systems (Ingenuity Systems, Redwood, CA) and, to a lesser extent, PubMed. A total of 56 genes strongly associated with these features were identified.

<sup>b</sup>Function is based on data produced by the EASE software (1) or found in NCBI Entrez Gene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>). Only one major function per gene is given.

<sup>c</sup>Location is based on information in NCBI Entrez Gene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>).

## Reference

1. Hosack, D.A., Dennis, Jr G., Sherman, B.T., Lane, H.C. and Lempicki, R.A. (2003) Identifying biological themes within lists of genes with EASE. *Genome Biol.*, **4**, R70.

**Table S10.** Genes involved in gene fusions in B-lineage ALL<sup>a</sup>

Gene	Function <sup>b</sup>	Location <sup>c</sup>
<i>ABL1</i>	Cell signaling	9q34.1
<i>AFF1</i>	Transcription regulation	4q21
<i>AFF3</i>	Transcription regulation	2q11.2-12
<i>AFF4</i>	Transcription regulation	5q31
<i>BAZ2A</i>	Transcription regulation	12q24.3
<i>BCL2</i>	Apoptosis	18q21.3
<i>BCL6</i>	Transcription regulation	3q27
<i>BCL7A</i>	Transcription regulation	12q24.13
<i>BCL9</i>	Cell signaling	1q21
<i>BCR</i>	Cell signaling	22q11.2
<i>CDKN2A</i>	Cell cycle	9p21
<i>CEBPA</i>	Transcription regulation	19q13.1
<i>CEBPB</i>	Transcription regulation	20q13.1
<i>CEBPD</i>	Transcription regulation	8q11.2
<i>CEBPE</i>	Transcription regulation	14q11.2
<i>CEBPG</i>	Transcription regulation	19q13.11
<i>CREBBP</i>	Transcription regulation	16p13.3
<i>DAZAP1</i>	Morphogenesis	19p13.3
<i>DDX6</i>	RNA binding	11q23.3
<i>ELN</i>	Morphogenesis	7q11.2
<i>ERG</i>	Transcription regulation	21q22.3
<i>ETV6</i>	Transcription regulation	12p13
<i>EWSR1</i>	Transcription regulation	22q12.2
<i>FGFR1</i>	Cell signaling	8p11.1-p11.2
<i>FGFR1OP</i>	Cell proliferation	6q27
<i>FOXP1</i>	Transcription regulation	3p14.1
<i>FRYL</i>	Transcription regulation	4p12
<i>FUS</i>	RNA binding	16p11.2
<i>GAS7</i>	Cell cycle	17p13.1
<i>HLF</i>	Transcription regulation	17q22
<i>ID4</i>	Transcription regulation	6p21-22
<i>IGH@</i>	Immune response	14q32.3
<i>IGK@</i>	Immune response	2p12
<i>IGL@</i>	Immune response	22q11.1-11.2

<i>IL3</i>	Cell signaling	5q31.1
<i>JAK2</i>	Cell signaling	9p24
<i>LHX4</i>	Transcription regulation	1q25.2
<i>MAPRE1</i>	Cell cycle	20q11.1-11.2
<i>MEF2D</i>	Transcription regulation	1q12-23
<i>MLL</i>	Transcription regulation	11q23
<i>MLLT1</i>	Transcription regulation	19p13.3
<i>MLLT4</i>	Cell signaling	6q27
<i>MLLT10</i>	Transcription regulation	10p12
<i>MYC</i>	Transcription regulation	8q24.2
<i>PAX5</i>	Transcription regulation	9p13
<i>PBX1</i>	Transcription regulation	1q23
<i>PCMI</i>	Morphogenesis	8p21.3-22
<i>PML</i>	Transcription regulation	15q22
<i>POU2AF1</i>	Transcription regulation	11q23.1
<i>PVT1</i>	Unknown	8q24
<i>RANBP17</i>	Transport	5q34
<i>RUNX1</i>	Transcription regulation	21q22.3
<i>SFPQ</i>	RNA processing	1p34.3
<i>STL</i>	Unknown	6q22
<i>TAF15</i>	Transcription regulation	17q11.1-11.2
<i>TCF3</i>	Transcription regulation	19p13.3
<i>TFPT</i>	Transcription regulation	19q13
<i>TTL</i>	Unknown	13q14.11
<i>ZNF384</i>	Transcription regulation	12p12
<i>ZNF521</i>	Unknown	18q11.2

<sup>a</sup>Genes known to be involved in gene fusions in B-lineage ALL may also be putative targets for differential methylation, and all 60 such genes were retrieved from the Mitelman Database of Chromosome Aberrations in Cancer (<http://cgap.nci.nih.gov/Chromosomes/Mitelman>).

<sup>b</sup>Function is based on data produced by the EASE software (1) or found in NCBI Entrez Gene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>). Only one major function per gene is given.

<sup>c</sup>Location is based on information in NCBI Entrez Gene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>).

## Reference

1. Hosack, D.A., Dennis, Jr G., Sherman, B.T., Lane, H.C. and Lempicki, R.A. (2003)  
Identifying biological themes within lists of genes with EASE. *Genome Biol.*, **4**, R70.

**Table S11.** Genes mutated in B-lineage ALL <sup>a</sup>

Gene	Type of cancer gene	Location <sup>b</sup>
<i>ATM</i>	Tumor suppressor	11q22-23
<i>BRAF</i>	Oncogene	7q34
<i>CDKN2A</i>	Tumor suppressor	9p21
<i>FLT3</i>	Oncogene	13q12
<i>JAK2</i>	Oncogene	9p24
<i>KRAS</i>	Oncogene	12p12.1
<i>NRAS</i>	Oncogene	1p13.2
<i>PTEN</i>	Tumor suppressor	10q23.3
<i>PTPN11</i>	Oncogene	12q24
<i>RUNX1</i>	Oncogene	21q22.3
<i>TP53</i>	Tumor suppressor	17p13.1

<sup>a</sup>The Catalogue of Somatic Mutations in Cancer (COSMIC;

[www.sanger.ac.uk/genetics/CGP/cosmic/](http://www.sanger.ac.uk/genetics/CGP/cosmic/)) was used to identify genes that have been shown to be mutated in B-lineage ALL. A total of 11 genes were found.

<sup>b</sup>Location is based on information in NCBI Entrez Gene

(<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>).

**Table S12.** The 131 hypermethylated genes (out of 167 shown to be recurrently methylated and/or involved in B-cell development or mutated or rearranged in B-lineage ALL) that did not display decreased expression or for which no expression data were available. The 36 genes associated with decreased expression are given in Table 3 in the article.

Gene	Function <sup>a</sup>	Location <sup>a</sup>	Expression
<i>BCL11A</i>	Transcription regulation	2p16.1	Not available
<i>EGR4</i>	Transcription regulation	2p13	Not available
<i>HIC1</i>	Transcription regulation	17p13.3	Not available
<i>LHX1</i>	Transcription regulation	17q12	Not available
<i>MATK</i>	Transcription regulation	19p13.3	Not available
<i>PAX6</i>	Transcription regulation	11p13	Not available
<i>TAL1</i>	Transcription regulation	1p32	Not available
<i>TFAP2C</i>	Transcription regulation	20q13.2	Not available
<i>TRAF7</i>	Transcription regulation	16p13.3	Not available
<i>BCL7C</i>	Apoptosis	16p11	Not available
<i>CDC2L2</i>	Apoptosis	1p36.3	Not available
<i>DIABLO</i>	Apoptosis	12q24.31	Not available
<i>HTRA2</i>	Apoptosis	2p12	Not available
<i>P53AIP1</i>	Apoptosis	11q24	Not available
<i>SFN</i>	Apoptosis	1p35.3	Not available
<i>TRADD</i>	Apoptosis	16q22	Not available
<i>TSC22D3</i>	Apoptosis	Xq22.3	Not available
<i>CAVI</i>	Transport	7q31.1	Not available
<i>KCNK2</i>	Transport	1q41	Not available
<i>RANBP17</i>	Transport	5q34	Not available
<i>SLC5A3</i>	Transport	21q22.12	Not available
<i>SLC5A8</i>	Transport	12q23.3	Not available
<i>SLC19A1</i>	Transport	21q22.3	Not available
<i>SLC22A18</i>	Transport	11p15.5	Not available
<i>TSSC4</i>	Transport	11p15.5	Not available
<i>BLK</i>	Cell signaling	8p22-23	Not available
<i>EPHA3</i>	Cell signaling	3p11.2	Not available
<i>IL3</i>	Cell signaling	5q31.1	Not available
<i>LRIG3</i>	Cell signaling	12q14.1	Not available
<i>LTB4R</i>	Cell signaling	14q11.2-12	Not available

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<i>PGR</i>	Cell signaling	11q22-23	Not available
<i>THEM4</i>	Cell signaling	1q21	Not available
<i>ATM</i>	Cell cycle	11q22-23	Not available
<i>CDK2AP1</i>	Cell cycle	12q24.31	Not available
<i>CDKN1C</i>	Cell cycle	11p15.5	Not available
<i>CIRBP</i>	Cell cycle	19p13.3	Not available
<i>KLK10</i>	Cell cycle	19q13.3-13.4	Not available
<i>MOS</i>	Cell cycle	8q11	Not available
<i>PRKCDBP</i>	Cell cycle	11p15.4	Not available
<i>ADAMTS1</i>	Proteolysis	21q21.2	Not available
<i>CST3</i>	Proteolysis	20p11.21	Not available
<i>DUB3</i>	Proteolysis	8p23.1	Not available
<i>FBXW7</i>	Proteolysis	4q31.3	Not available
<i>LRP1B</i>	Proteolysis	2q21.2	Not available
<i>MMP21</i>	Proteolysis	10q26.2	Not available
<i>SERPINB13</i>	Proteolysis	18q21.3-22	Not available
<i>GP1BB</i>	Cell adhesion	22q11.21	Not available
<i>PARVB</i>	Cell adhesion	22q13.2-13.33	Not available
<i>PCDHGA12</i>	Cell adhesion	5q31	Not available
<i>ADPRH</i>	Hydrolase	3q13.31-13.33	Not available
<i>B4GALT2</i>	Transferase	1p33-34	Not available
<i>DNTT</i>	DNA replication	10q23-24	Not available
<i>FRK</i>	Cell proliferation	6q21-22.3	Not available
<i>HOXD3</i>	Morphogenesis	2q31.1	Not available
<i>TMOD1</i>	Morphogenesis	9q22.3	Not available
<i>RAG2</i>	Various	11p13	Not available
<i>TAPBP</i>	Various	6p21.3	Not available
<i>H19</i>	Unknown	11p15.5	Not available
<i>LYPD1</i>	Unknown	2q21.2	Not available
<i>RHBDD3</i>	Unknown	22q12.1-12.2	Not available
<i>ARID3A</i>	Transcription regulation	19p13.3	Not differential
<i>EWSR1</i>	Transcription regulation	22q12.2	Not differential
<i>HOPX</i>	Transcription regulation	4q11-12	Not differential
<i>HOXA9</i>	Transcription regulation	7p14-15	Not differential
<i>ID4</i>	Transcription regulation	6p21-22	Not differential
<i>IRF7</i>	Transcription regulation	11p15.5	Not differential

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<i>MBD1</i>	Transcription regulation	18q21	Not differential
<i>MYT1</i>	Transcription regulation	20q13.33	Not differential
<i>PRDM2</i>	Transcription regulation	1p36	Not differential
<i>RARB</i>	Transcription regulation	3p24	Not differential
<i>SIN3A</i>	Transcription regulation	15q23	Not differential
<i>SMAD4</i>	Transcription regulation	18q21.1	Not differential
<i>SSBP2</i>	Transcription regulation	5q14.1	Not differential
<i>TCF3</i>	Transcription regulation	19p13.3	Not differential
<i>TGIF1</i>	Transcription regulation	18p11.3	Not differential
<i>BAD</i>	Apoptosis	11q13.1	Not differential
<i>BCL2</i>	Apoptosis	18q21.3	Not differential
<i>CASP3</i>	Apoptosis	4q34	Not differential
<i>DAPK3</i>	Apoptosis	19p13.3	Not differential
<i>DCC</i>	Apoptosis	18q21.3	Not differential
<i>FASTK</i>	Apoptosis	7q35	Not differential
<i>GAS2</i>	Apoptosis	11p14.3-p15.2	Not differential
<i>LCK</i>	Apoptosis	1p34.3	Not differential
<i>PHF17</i>	Apoptosis	4q26-27	Not differential
<i>VHL</i>	Apoptosis	3p26-25	Not differential
<i>CADM4</i>	Cell cycle	19q13.31	Not differential
<i>CDKN2A</i>	Cell cycle	9p21	Not differential
<i>CGREF1</i>	Cell cycle	2p23.3	Not differential
<i>EXT1</i>	Cell cycle	8q24.11-24.13	Not differential
<i>FHIT</i>	Cell cycle	3p14.2	Not differential
<i>FOXN3</i>	Cell cycle	14q31.3	Not differential
<i>LIG4</i>	Cell cycle	13q33-34	Not differential
<i>CD19</i>	Cell signaling	16p11.2	Not differential
<i>DKK3</i>	Cell signaling	11p15.2	Not differential
<i>EDNRB</i>	Cell signaling	13q22	Not differential
<i>MLLT4</i>	Cell signaling	6q27	Not differential
<i>AXIN2</i>	Signal transduction	17q23-24	Not differential
<i>IGFBP1</i>	Signal transduction	7p12-13	Not differential
<i>LILRB4</i>	Signal transduction	19q13.4	Not differential
<i>PRMT2</i>	Signal transduction	21q22.3	Not differential
<i>CDH1</i>	Cell adhesion	16q22.1	Not differential
<i>SRPX</i>	Cell adhesion	Xp21.1	Not differential

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<i>CYR61</i>	Morphogenesis	1p22-31	Not differential
<i>GPC3</i>	Morphogenesis	Xq26.1	Not differential
<i>SELENBP1</i>	Transport	1q21-22	Not differential
<i>SLC12A4</i>	Transport	16q22.1	Not differential
<i>ADAMTS5</i>	Proteolysis	21q21.3	Not differential
<i>CTF8</i>	DNA replication	16q22.1	Not differential
<i>MGAT4A</i>	Carbohydrate processing	2q12	Not differential
<i>NPM1</i>	Cell proliferation	5q35	Not differential
<i>CRMP1</i>	Various	4p15-16.1	Not differential
<i>IGSF8</i>	Various	1q23.1	Not differential
<i>IL7</i>	Various	8q12-13	Not differential
<i>LYNX1</i>	Various	8q24.3	Not differential
<i>PRKCD</i>	Various	3p21.31	Not differential
<i>SST</i>	Various	3q28	Not differential
<i>MEG3</i>	Unknown	14q32	Not differential
<i>MSMB</i>	Unknown	10q11.2	Not differential
<i>JDP2</i>	Transcription regulation	14q24.3	Upregulated
<i>SOX4</i>	Transcription regulation	6p22.3	Upregulated
<i>WT1</i>	Transcription regulation	11p13	Upregulated
<i>BCL2L11</i>	Morphogenesis	2q13	Upregulated
<i>NDRG2</i>	Morphogenesis	14q11.2	Upregulated
<i>NNAT</i>	Morphogenesis	20q11.2-12	Upregulated
<i>BLNK</i>	Cell signaling	10q23.2-23.33	Upregulated
<i>FGFR1</i>	Cell signaling	8p11.1-11.2	Upregulated
<i>CTNNA1</i>	Apoptosis	5q31	Upregulated
<i>GAS7</i>	Cell cycle	17p13.1	Upregulated
<i>TUSC3</i>	Glycosylation	8p22	Upregulated
<i>ADAMTS9</i>	Various	3p14.2-14.3	Upregulated
<i>IL2RB</i>	Various	22q13.1	Upregulated

<sup>a</sup>Function and location are based on data in NCBI Entrez Gene

(<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>). Only one major function per gene is given.

**Table S13.** The genomic positions of the methylation peaks, the peak sizes, the probe densities and the positions of the peaks in relation to the transcription start sites of all genes listed Tables 2 and 3 in the manuscript

Gene	Median no. of probes per peak	Median peak size (bp)	Position of the peak center (bp)	Position of the peak center in relation to transcription start site
<i>AR</i>	3	239	ChrX: 66705329	Covering <sup>a</sup>
<i>ARHGAP25</i>	7	660	Chr2: 68814972	499 bp upstream
<i>ARTN</i>	7	559	Chr1: 44170190	579 bp upstream
<i>BBC3</i>	5	539	Chr19: 52426792	501 bp upstream
<i>BCL10</i>	3	250	Chr1: 85514796	Covering
<i>BOK</i>	5	459	Chr2: 242146277	571 bp upstream
<i>BRCA1</i>	6	529	Chr17: 38531918	924 bp upstream
<i>BRDT</i>	7	664	Chr1: 92187236	483 bp upstream
<i>CASP2</i>	5	1288	Chr7: 142695721	Covering
<i>CASP9</i>	5	449	Chr1: 15723633	351 bp upstream
<i>CCND2</i>	6	650	Chr12: 4252950	248 bp upstream
<i>CD151</i>	3	349	Chr11: 822388	563 bp upstream
<i>CD2</i>	8	539	Chr1: 117098036	588 bp upstream
<i>CD36</i>	8	766	Chr7: 80069158	300 bp upstream
<i>CD44</i>	7	444	Chr11: 35116512	480 bp upstream
<i>CDC14B</i>	4	454	Chr9: 98186329	3727 bp upstream
<i>CDH13</i>	5	444	Chr16: 81218103	Covering
<i>CDK2API</i>	6	533	Chr12: 122323116	476 bp upstream
<i>CEBPA</i>	3	264	Chr19 38485123	Covering
<i>CES2</i>	4	339	Chr16: 65525257	590 bp upstream
<i>CLDN1</i>	5	459	Chr3: 191523556	400 bp upstream
<i>CPSF3L</i>	3	359	Chr1: 1234785	1966 bp upstream
<i>CSF1R</i>	8	832	Chr5: 149473399	Covering
<i>CSF2RA</i>	9	954	ChrY: 1347318	Covering
<i>DBC1</i>	6	534	Chr9: 3170345	Covering
<i>DMP1</i>	9	855	Chr4: 88790089	393 bp upstream
<i>DUSP10</i>	9	835	Chr1: 219977791	366 bp upstream
<i>ETV1</i>	6	534	Chr7: 13995314	Covering
<i>FAT</i>	8	738	Chr4: 187883487	1506 bp upstream

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<i>FGFBP1</i>	8	739	Chr4: 15549318	259 bp upstream
<i>FOXO3A</i>	7	634	Chr6: 109091532	340 bp upstream
<i>GAK</i>	4	149	Chr4: 831913	1232 bp upstream
<i>GALNAC4S-6ST</i>	4	359	Chr10: 125841514	1579 bp downstream
<i>GNG4</i>	7	644	Chr1: 233881213	536 bp upstream
<i>HCK</i>	2	159	Chr20: 30104375	658 bp downstream
<i>HIST1H1A</i>	4	455	Chr6: 26126462	446 bp upstream
<i>HLF</i>	4	425	Chr17: 50696793	576 bp upstream
<i>HRH1</i>	8	749	Chr3: 11269000	384 bp upstream
<i>IGF2</i>	8	757	Chr11: 2110104	251 upstream
<i>IGFBP6</i>	6	448	Chr12: 51777179	547 bp upstream
<i>LDOC1</i>	4	468	ChrX: 140099530	580 bp upstream
<i>LTBP1</i>	10	994	Chr2: 33212849	Covering
<i>MSC</i>	3	472	Chr8: 72919760	509 bp upstream
<i>NEFL</i>	4	454	Chr8: 24870630	587 bp upstream
<i>NFKB2</i>	5	469	Chr10: 104144243	Covering
<i>NOTCH1</i>	4	429	Chr9: 138532307	13747 bp downstream
<i>PAQR3</i>	6	559	Chr4: 80080121	540 bp upstream
<i>PARP14</i>	10	946	Chr3: 123900580	Covering
<i>PDPN</i>	9	864	Chr1: 13784260	1422 bp downstream
<i>PLGLB1</i>	11	1040	Chr2: 87102776	317 bp upstream
<i>PPFIA4</i>	9	855	Chr1: 201286646	287 bp upstream
<i>PRKCDBP</i>	13	1229	Chr3: 6298431	Covering
<i>PRKD3</i>	7	641	Chr2: 37398132	406 bp upstream
<i>PRLR</i>	4	363	Chr5: 35266751	417 bp upstream
<i>PTPN3</i>	9	832	Chr9: 111265756	199 bp upstream
<i>RBP1</i>	2	116	Chr3: 140741526	246 bp upstream
<i>RGS12</i>	8	662	Chr4: 3344629	3108 bp upstream
<i>SDHC</i>	7	629	Chr1: 159550339	450 bp upstream
<i>SFRP1</i>	2	129	Chr8: 41286179	Covering
<i>SMURF1</i>	8	749	Chr7: 98471133	4511 bp downstream
<i>SOX30</i>	5	442	Chr5: 157012157	151 bp upstream
<i>SSX1</i>	4	349	ChrX: 47999562	246 bp upstream
<i>SSX4</i>	7	651	ChrX: 48127734	Covering
<i>TES</i>	2	149	Chr7: 115637067	749 bp upstream
<i>TGFBR1</i>	6	554	Chr9: 100906692	521 bp upstream

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<i>TP53I3</i>	4	354	Chr2: 24161877	645 bp upstream
<i>TPD52</i>	4	334	Chr8: 81247048	657 bp upstream
<i>TRIM10</i>	4	369	Chr6: 30238327	1659 bp upstream
<i>TULP4</i>	10	933	Chr6: 158653332	347 bp upstream
<i>UCHL1</i>	6	546	Chr4: 40953156	529 bp upstream
<i>WRN</i>	2	136	Chr8: 31010340	Covering

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<sup>a</sup>Covering indicates a peak center position within 50 bp up- or downstream of the transcription start site.







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## Relapsed childhood high hyperdiploid acute lymphoblastic leukemia: presence of preleukemic ancestral clones and the secondary nature of microdeletions and RTK-RAS mutations

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**Although childhood high hyperdiploid acute lymphoblastic leukemia is associated with a favorable outcome, 20% of patients still relapse. It is important to identify these patients already at diagnosis to ensure proper risk-stratification. We have investigated 11 paired diagnostic/relapse samples with SNP array and mutation analyses of *FLT3*, *KRAS*, *NRAS*, and *PTPN11* in order to identify changes associated with relapse and to ascertain the genetic evolution patterns. Structural changes, mainly cryptic hemizygous deletions, were significantly more common at relapse ( $P<0.05$ ). No single aberration was linked to relapse, but four deletions, involving *IKZF1*, *PAX5*, *CDKN2A/B* or *AK3*, were recurrent. Based on the genetic relationship between the paired samples, three groups were delineated: 1) identical genetic changes at diagnosis and relapse (18%), 2) clonal evolution with all changes at diagnosis being present at relapse (18%), and 3) clonal evolution with some changes conserved, lost or gained (64%), suggesting the presence of a preleukemic clone. This ancestral clone was characterized by numerical changes only, with structural changes and RTK-RAS mutations being secondary to the high hyperdiploid pattern.**

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## Introduction

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High hyperdiploid (51-67 chromosomes) B-cell precursor acute lymphoblastic leukemia (ALL) is the largest genetic subgroup of childhood ALL, comprising 25% of all such cases. Cytogenetically, high hyperdiploid ALLs are characterized by a nonrandom gain of chromosomes, in particular X, 4, 6, 10, 14, 17, 18, and 21. In addition, microdeletions of *CDKN2A*, *ETV6*, *IKZF1*, *PAX5*, *RBI*, and *TCF3*, and mutations in the *FLT3*, *KRAS*, *NRAS*, and *PTPN11* genes, all involved in the RTK-RAS signaling pathway, are relatively frequent.<sup>1</sup>

In recent larger series of children with high hyperdiploid ALL, the event-free survival and overall survival rates have been approximately 80% and 90%, respectively.<sup>2-4</sup> Thus, despite the generally very good prognosis, a substantial proportion of the cases relapse and some patients succumb to the disease. It is of utmost clinical importance to identify these patients up-front in order to assign them to correct risk groups and hence proper treatment regimens. One approach would be to identify specific relapse-associated genetic features already at the time of diagnosis. It has previously been suggested that high hyperdiploid ALLs with structural chromosome

changes are associated with a poor outcome; however, this has not been confirmed in later studies.<sup>3,5-7</sup>

In fact, there are no genetic changes, including microdeletions and mutated genes, known to correlate with relapse in high hyperdiploid ALL. In contrast, several investigators have identified “favorable” aberrations, reporting a superior outcome for cases harboring certain trisomies, that is +4, +10,+17, and +18;<sup>2,3,8,9</sup> findings that are now used for risk stratification, at least by the Children’s Oncology Group (COG) as regards the triple trisomies +4, +10, and +17.<sup>10</sup>

Another approach to identify genetic changes associated with relapse of high hyperdiploid ALL is to compare the genetic features of paired diagnostic and relapse samples. This would also provide valuable information as regards the temporal order of the various chromosome abnormalities and gene mutations and thus enable identification of “preleukemic” ancestral clones as well as secondary, progression-related changes. Considering the high incidence of high hyperdiploid ALL, surprisingly few patients have been analyzed in this respect. In fact, only two studies, both using SNP array analyses, of paired

diagnostic and relapse samples have been published, comprising a total of eight high hyperdiploid ALLs.<sup>11,12</sup> The two patients analyzed by Yang et al.<sup>11</sup> displayed identical numbers of gains and losses at diagnosis and relapse, providing no evidence for genetic clonal evolution at disease recurrence, whereas Mullighan et al.<sup>12</sup> observed differences in copy number alterations in diagnostic and relapse samples in five of the six cases analyzed. Thus, available data are contradictory as regards genetic heterogeneity/homogeneity between high hyperdiploid ALLs at

diagnosis and relapse, and further studies are clearly needed to clarify this biologically and clinically important issue. In the present investigation, we used 500k SNP arrays to analyze paired samples from 11 children with high hyperdiploid ALL, the largest series to date, and also screened for mutations in the RTK-RAS genes *FLT3*, *KRAS*, *NRAS*, and *PTPN11*. Apart from a very recent study on the secondary nature of *PTPN11* mutations in high hyperdiploid ALLs,<sup>13</sup> a similar screening has previously not been performed in relapse samples.

## Methods

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### Patient samples

Diagnostic and relapse samples from 11 children with high hyperdiploid ALL were included in the study (Table 1). All patients had been diagnosed and treated according to the NOPHO-ALL 1992 or NOPHO-ALL 2000 protocols at the Departments of Pediatric Oncology and Hematology, Lund University Hospital, Lund, Sweden; Karolinska Institute, Stockholm, Sweden; Linköping University Hospital, Linköping, Sweden; Umeå University Hospital, Umeå, Sweden; Uppsala University Hospital, Uppsala, Sweden; and

Rigshospitalet, Copenhagen, Denmark. Cytogenetic analyses of bone marrow samples obtained at the time of diagnosis and relapse were performed using conventional methods. High hyperdiploidy was defined as 51-67 chromosomes.<sup>1</sup> None of the cases had the well-known ALL-associated translocations t(1;19)(q23;p13), t(9;22)(q34;q11), 11q23/*MLL* rearrangements, or t(12;21)(p13;q22). DNA was extracted using standard methods and purified further with the DNeasy Blood & Tissue Kit (Qiagen, Solna, Sweden) and quantified using a Nanodrop

Spectrophotometer (Nanodrop Technologies, Wilmington, DE). The study was reviewed and approved by the research ethics

board at Lund University, and informed consent was obtained in accordance with the Declaration of Helsinki.

### SNP array analyses

The SNP array analyses were performed using the Affymetrix GeneChip Human 250k *Nsp*, 250k *Sty*, and 10k 2.0 array systems. These arrays cover approximately 510,000 SNPs, with a median physical distance between the SNPs of <2.5 kb. Hybridization and washes were performed as described previously.<sup>14</sup> The Affymetrix GTYPE software was used for analysis of signal intensity and for genotype calling. Apart from copy number analysis, the genotype data were also used to confirm that the diagnostic and relapse samples were derived from the same individual. As an initial step to identify putative genomic imbalances, log<sub>2</sub> ratios were segmented with the CBS algorithm<sup>15</sup> using the DNA copy package in R (<http://www.bioconductor.org>). For further and detailed copy number alteration (CNA) and uniparental isodisomy (UPD) analyses the dChip<sup>16</sup> and the in-house Genome Orientated Laboratory File (GOLF) software packages

were used. When applying the former software for CNA detection, the median smoothing was set to a 21 SNP window and the sample trimming to 20%, with the function “scale copy number mode to 2 copy” being used.

Classification as a CNA was based on visual inspection of the inferred log<sub>2</sub> ratio versus the pooled signal intensity of 10 control samples. Ratios +/- ≥0.5 were classified as abnormal, with ratios between +/- 0.5 and 1.0 interpreted as duplications or hemizygous deletions and +/- ≥1.0 classified as amplifications or homozygous deletions. Imbalances representing copy number polymorphisms, listed in the Database of Genomic Variants (<http://projects.tcag.ca/variation/>), or somatic immunoglobulin and T cell receptor gene rearrangements were excluded from further analysis.

## Mutation analyses of *FLT3*, *KRAS*, *NRAS*, and *PTPN11*

For analyses of codons 835 and 836 in the second tyrosine kinase domain and of internal tandem duplication of exons 14 and 15 in *FLT3*, codons 12, 13, and 61 in *NRAS* and *KRAS* and of exons 3 and 13 in *PTPN11* eight different PCR reactions were performed, as described previously.<sup>17</sup> For each PCR reaction, approximately 100

ng DNA was used as a template. The PCR products were directly sequenced using the McLAB sequencing service (San Francisco, CA) and both the sense and the antisense strands were sequenced and analyzed using the Seqscape software (PE Applied Biosystems, Foster City, CA).

## Results

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### Comparison of paired diagnostic and relapse samples identifies ancestral clones

Based on the genetic relationship between the diagnostic and relapse samples in the 11 patients (Table 1), three distinct groups were identified (Figure 1): 1) **identical** genetic changes at diagnosis and relapse (#1 and 3; 18%), 2) **clonal evolution** with all changes at diagnosis being present at relapse (#6 and 9; 18%), and 3) **ancestral clones** with some changes present at diagnosis but not at relapse (#2, 4, 5, 7, 8, 10, and 11; 64%), suggesting the presence of a

preleukemic clone.<sup>12</sup> As seen in Table 1, it can be deduced that the ancestral clones were characterized by numerical changes only, with structural changes and mutated RTK-RAS genes differing between the paired samples.

There were no apparent differences as regards time from diagnosis to relapse among the three groups (4 years, 1-4 years, and 2-6 years, respectively; Table 1).

### Patterns of gained chromosomes

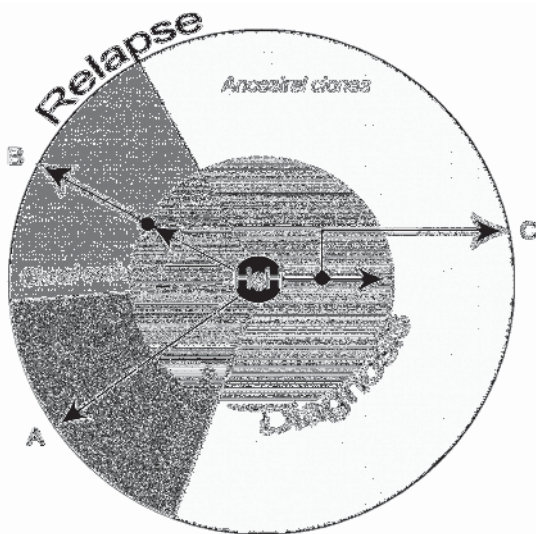
The number of gains ranged between 7 and 13 (median 9) among the 11 cases (22 samples), and involved, in decreasing

frequency order, chromosomes 6 (100%), 21 (100%), 4 (91%), 17 (91%), 18 (91%), X (86%), 10 (82%), 8 (77%), 14 (45%), 5

(23%), 2 (9%), 7 (9%), 9 (9%), 15 (9%), and 16 (5%). In seven cases (#1, 3-6, 9, and 10), the gains were identical at diagnosis and relapse, whereas they differed by 1-5 trisomies/tetrasomies, involving chromosomes X, 5, 8, 14, 16, and 21, in four (36%) of the 11 patients. There were no major modal shifts in the latter four cases (55→54 in case 2, 55→58 in case 7, 59→58 in case 8, and 57→55 in

case 11; Table 1). An acquired UPD was detected in one (9%) of the 11 cases, comprising a UPD8 at diagnosis and relapse in case 8.

Simultaneous trisomies of chromosomes 4, 10, and 17 – the triple trisomies used for stratification of patients into a lower-risk group by the COG<sup>10</sup> – were found in seven (64%) of the 11 patients both at diagnosis and at relapse (Table 1).



**Figure 1. Three genetic groups of relapsed high hyperdiploid ALLs.** Based on the genetic relationship between the diagnostic and relapse samples three distinct groups could be delineated. (A) Identical clones (18%) at diagnosis and relapse, (B) Clonal evolution (18%) with all changes at diagnosis being present at relapse, and (C) Ancestral clones (64%), that is some changes were conserved and others lost or gained in the diagnostic and relapse samples, respectively. HeH indicates high hyperdiploidy.

### Microdeletions are significantly more common in relapses

The number of structural changes varied between 0 and 6 (median 3) among the 22 samples. A total of 34 different hemizygous deletions, two homozygous deletions, and five copy-number gains (whole chromosomes excluded) were

identified. The median size of the 36 hemi- and homozygous deletions was 0.156 Mb (range 0.019-66.579 Mb), with both the homozygous deletions being <1 Mb. The median size of the copy-number gains was 40.127 Mb

(range 0.142-100.895 Mb). Among all 41 structural changes detected, nine (22%) were >10 Mb in size; the remaining 32 (78%) imbalances would be considered cytogenetically cryptic (Table 2).

None of the five copy-number gains involved the same chromosome regions (Table 1). Of the 36 hemi- and homozygous deletions identified, four were overlapping (albeit with different breakpoints) in more than one case and were hence recurrent (Table 2): 7p12.2/*IKZF1* (#4D and 7R), 9p13.2/*PAX5* (#4D and 10D/R), 9p21.3/*CDKN2A/B* (#2D and 4R), and 9p24.1/*AK3* (#8R and 10D) (Figure 2).

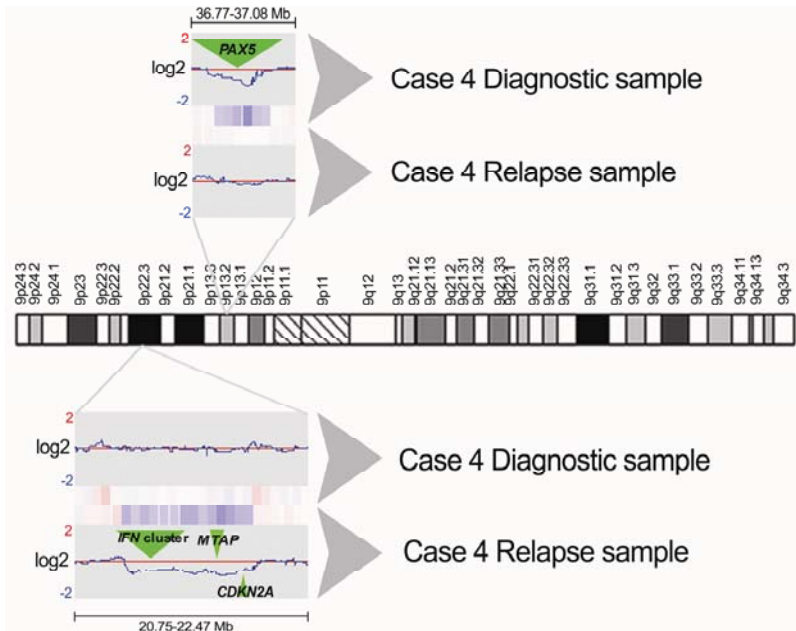
None of the homozygous deletions or copy-number gains

was shared in paired diagnostic and relapse samples, and apart from a homozygous 9p deletion at diagnosis in case 2, these changes were only found at relapse. Among the 34 hemizygous deletions, nine were seen both at diagnosis and relapse in five patients (#1, 4, 5, 10, and 11). Of the remaining 25 hemizygous deletions, 17 (68%) were found at relapse and 8 (32%) at diagnosis (Tables 1 and 2). Taken together, the mean frequency of structural changes at diagnosis was 1.6 (median 1.0; range 0-4), whereas the corresponding frequency at relapse was 2.9 (median 3.0; range 0-6) ( $P<0.05$ ; Wilcoxon matched pairs signed rank sum test).

### **Most *FLT3*, *KRAS*, *NRAS*, and *PTPN11* mutations are found at relapses**

Five (45%) of the 11 cases harbored a total of six mutated RTK-RAS genes (Table 1): one *FLT3* mutation (#8R), two *KRAS* mutations (#6R and 9R), and three *PTPN11* mutations (#5D, 8D/R, and 10R).

Among the six different mutations, one was detected both at diagnosis and relapse (*PTPN11* in case 8). Of the remaining five mutations, one was found at diagnosis only (#5) and four at relapse only (#6, 8, 9, and 10) (Table 1).



**Figure 2. Different 9p deletions in case 4 at diagnosis and at relapse.** The SNP array analyses at diagnosis revealed a hemizygous deletion between positions 36.843 Mb and 36.999 Mb at 9p13.2, involving *PAX5*; this deletion was not seen at relapse. In contrast, a hemizygous deletion between positions 21.064 Mb and 22.089 Mb at 9p21.3, involving several genes, such as *CDKN2A*, was identified only at relapse.

## Discussion

The salient findings in the present study of paired diagnostic and relapse samples from childhood high hyperdiploid ALLs are 1) that structural changes were significantly more common in relapse samples, 2) that no single, recurrent aberration could be directly linked to relapse and, 3) that there were genetic differences, involving mainly structural abnormalities and mutated RTK-RAS genes, between diagnostic and relapse samples in the vast majority

of patients, suggesting the presence of an ancestral high hyperdiploid clone in many of the cases and that structural changes and mutations are secondary to the high hyperdiploid pattern.

Despite the fact that simultaneous occurrence of +4, +10, and +17 in childhood ALL has been associated with low-risk pediatric ALL,<sup>10</sup> we nevertheless found these triple trisomies in the majority (64%) of the patients that later relapsed (Table 1), a

frequency on a par with the incidence of 55% of these triple trisomies in close to 60 high hyperdiploid cases also analyzed by high resolution arrays at the time of diagnosis.<sup>18-21</sup> Thus, it is clear that the triple trisomies are seen in a substantial proportion of relapses of high hyperdiploid ALLs, a finding that may question their favorable prognostic impact.

The frequencies of structural aberrations, mainly cytogenetically cryptic hemizygous deletions, were significantly higher in the relapse samples. This is not unexpected considering that several previous studies, using conventional chromosome banding techniques, have shown increased numbers of structural chromosome abnormalities in ALL, including high hyperdiploid cases, at relapse.<sup>22-27</sup> Using SNP array analysis, Mullighan et al.<sup>12</sup> also observed significantly higher frequencies of deletions in relapse samples. In the present study, most RTK-RAS mutations were detected at relapse. Although not statistically significant, it agrees well with the notion that additional genetic changes are acquired prior to relapse. Whether these are caused by the previous genotoxic therapy or are a consequence of inherent clonal evolution over time is unknown, and may well be impossible to clarify because ALL

treatment is solely based on chemotherapy.

There were no structural aberrations or mutated RTK-RAS genes common to all cases (Tables 1 and 2), showing that there is no single aberration directly linked to relapse, at least not identifiable by the methods used herein. However, four structural abnormalities, namely the deletions involving *AK3*, *CDKN2A/B*, *IKZF1*, or *PAX5*, were recurrent in the present series, being found in two cases each (Table 2). Submicroscopic deletions of these genes have previously been identified at diagnosis as well as at relapse of high hyperdiploid ALLs,<sup>1,12,21</sup> but their prognostic impact remains to be clarified, at least in the context of high hyperdiploidy.<sup>28,29</sup> Recently, deletions of *IKZF1* were shown to be a predictor of poor outcome in high-risk pediatric B-cell progenitor ALL.<sup>30</sup> However, high hyperdiploid ALLs are rarely grouped as high-risk.<sup>1</sup> Furthermore, of the four high hyperdiploid cases included in the high-risk group reported by Mullighan et al.<sup>30</sup>, none had *IKZF1* deletions, whereas the frequency of such deletions was approximately 10% (4/44) in the validation cohort. Also, among the present 11 patients and the six informative paired samples previously reported<sup>12</sup> three (18%) harbored



*IKZF1* deletions, with the deletions being present both at diagnosis and relapse in one case, only at diagnosis in one, and only at relapse in one. Taken together, it seems unlikely that *IKZF1* deletions have a major prognostic impact in high hyperdiploid ALL.

For many years, childhood high hyperdiploid childhood ALLs were thought to be genetically characterized only by the nonrandom tri- and tetrasomies and a handful of well-defined structural chromosome abnormalities. However, with the recent advent of array-based screening methods, revealing a more complex pattern with several and heterogeneous submicroscopic aberrations, this view is now changing.<sup>1</sup> In addition, and as clearly seen in the present series (Tables 1 and 2) and in the study by Mullighan et al.<sup>30</sup>, the complexity increases even further when comparing diagnostic and relapse samples. In fact, the genetic relationship between diagnostic and relapse samples can be divided into three distinct groups (Figure 1): identical clones (~20%), unidirectional clonal evolution

(~20%), and ancestral clones (~65%). The latter were identifiable by displaying a complex genetic evolution pattern with some changes conserved and others lost or gained and probably correspond to early preleukemic clones from which both the diagnostic and relapse clones emanate (Figure 2).<sup>12</sup>

Although the ancestral clone cannot be directly observed, it is possible to deduce its basic genetic constitution by comparing diagnostic and relapse samples (Tables 1 and 2). It then becomes clear that both structural chromosome changes and mutated RTK-RAS genes are secondary to the high hyperdiploid pattern. Considering that high hyperdiploid cells have been detected already at birth in children who subsequently developed high hyperdiploid ALL,<sup>31,32</sup> the present results indicate that structural chromosome abnormalities and *FLT3*, *KRAS*, *NRAS*, and *PTPN11* mutations may represent the secondary changes necessary for overt leukemia.

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**Table 1. Clinical and genetic features at the time of diagnosis and relapse of childhood high hyperdiploid acute lymphoblastic leukemia**

Sample	Sex/ age	Genomic imbalances/UPDs identified by SNP array analyses <sup>a</sup>	<i>FLT3</i> , <i>KRAS</i> , <i>NRAS</i> , and <i>PTPN11</i> mutations <sup>a</sup>	Genetic relationship between diagnosis and relapse
1D	F/2	+X,+4,del(4)(p16.3),del(5)(q35.1q35.1),+6,del(6)(p21.1p22.1),del(11)(q12.1q12.1),+14,+17,+21,+21	No	Identical clones
1R	F/6	+X,+4,del(4)(p16.3),del(5)(q35.1q35.1),+6,del(6)(p21.1p22.1),del(11)(q12.1q12.1),+14,+17,+21,+21	No	Identical clones
3D	F/16	+X,+4,+6,+8,+10,+17,+18,+21,+21	No	Identical clones
3R	F/20	+X,+4,+6,+8,+10,+17,+18,+21,+21	No	Identical clones
6D	F/3	+X,+4,+6,+8,+10,+17,+18,+21,+21	No	Clonal evolution
6R	F/7	+X,+4,dup(5)(q31.3q35.3),+6,+8,del(9)(q21.1q21.1),+10,del(12)(p12.3p13.3),+17,+18,+21,+21	<b>KRAS: c.34G&gt;A (p.Gly12Ser)</b>	Clonal evolution
9D	F/2	+4,+6,+8,+10,+15,+17,+18,+21,+21	No	Clonal evolution
9R	F/3	+4,+6,+8,+10,+15,+17,+18,+21,+21	<b>KRAS: c.35G&gt;A (p.Gly12Asp)</b>	Clonal evolution
2D	M/5	+X,+2,+4,+6,+8,del(9)(p21.3p21.3)x1-2,+10,+18,+21,+21	No	Ancestral clone
2R	M/7	+X,+2,+4,del(4)(p15.3p15.3),+6,del(7)(q33q33),+8,del(9)(q33.1q33.1),+10,+18,+21	No	Ancestral clone
4D	M/2	+X,+X,+4,+6,del(7)(p12.2p12.2),del(9)(p13.2p13.2),del(10)(q21.1q21.1),+14,+17,+18,-19,+21,del(21)(q22.3q22.3)	No	Ancestral clone
4R	M/4	+X,+X,+4,+6,del(9)(p21.3p21.3),+14,+17,+18,del(20)(q11.2q11.2),+21,del(21)(q22.3q22.3)	No	Ancestral clone

5D	M/14	+X,+X,del(3)(q23q25.1),+6,+8,+10,+14,+17,+18,+21,+21	<b>PTPNI1: c.214G&gt;A (p.Ala72Thr)</b>	Ancestral clone
5R	M/19	+X,+X,del(3)(q23q25.1),+6,+8,+10, <b>del(10)(q22.1q26.3)</b> ,+14,+17,+18, <b>dup(20)(q11.1q11.2)</b> ,+21,+21	No	
7D	M/11	+4,+6,+8,+9,+10,+14,+17,+18,+21	No	Ancestral clone
7R	M/16	+X,+X,+4,+5,+6, <b>del(7)(p11.2p22.3)</b> , <b>dup(7)(q11.1q36.3)</b> ,+8,+9,+10, <b>del(11)(p15.1p15.1)</b> , <b>del(13)(q14.1q14.3)x1-2</b> ,+17,+18,+21,+21	No	
8D	M/5	+X,+X,+4,+5,+6,+7,+8,+10,+14,+17,+18,+21,+21	<b>PTPNI1: c.215C&gt;T (p.Ala72Val)</b>	Ancestral clone
		UPD: 8		
8R	M/10	+X,+4,+5,+6,+7,+8, <b>del(9)(p24.1p24.1)</b> ,+10, <b>del(11)(q24.2q24.2)</b> , <b>del(13)(q12.1q12.1)</b> ,+14,+17,+18,+21,+21	<b>FLT3: c.2505-2507delATC (p.Ile836del)</b> <b>PTPNI1: c.215C&gt;T (p.Ala72Val)</b>	
		UPD: 8		
10D	F/5	+X,+4,+6,+8,del(9)(p13.2p13.2), <b>del(9)(p24.1p24.1)</b> ,+10,del(13)(q12.2q12.2),+17,+18,+21,+21	No	Ancestral clone
10R	F/9	+X,+4,+6,+8,del(9)(p13.2p13.2),+10,del(13)(q12.2q12.2),+17,+18,+21,+21	<b>PTPNI1: c.218C&gt;T (p.Thr73Ile)</b>	
11D	M/3	+X, <b>del(1)(q21.1q21.1)</b> ,del(3)(q26.3q26.3),+4,+5,+6,+8,+10,+14,+17, <b>del(17)(q11.2q11.2)</b> ,+18, <b>del(19)(q13.3q13.3)</b> ,+21,+21	No	Ancestral clone
11R	M/9	+X, <b>dup(1)(q11q32.2)</b> , <b>del(2)(q23.3q23.3)</b> ,del(3)(q26.3q26.3),+4,+5,+6,+10, <b>del(11)(q23.1q23.1)</b> ,+16, <b>del(16)(q21q22.1)</b> ,+17,+18, <b>dup(19)(p13.1p13.1)</b> ,+21	No	

Abbreviations: D, sample from the time of diagnosis; R, sample from the time relapse; F, female; M, male; and UPD, uniparental disomy.

<sup>a</sup>Genomic imbalances and mutations differing between diagnostic and relapse samples are indicated in bold type.

**Table 2. Partial chromosomal imbalances in paired diagnostic and relapsed samples from childhood high hyperdiploid acute lymphoblastic leukemias**

Case	Chr	Region	Type/ copies <sup>a</sup>	Position (Mb) <sup>b</sup>	Size (Mb)	Genes <sup>c</sup>
1D/R	4	4p16.1-16.3	He L/2	0-9.820	9.820	Several
1D/R	5	5q35.1	He L/1	169.244-169.539	0.295	<i>DOCK2/FOXO1/LOC345430</i>
1D/R	6	6p21.1-22.1	He L/2	29.683-44.190	14.507	Several
1D/R	11	11q12.1	He L/1	56.798-57.135	0.337	<i>TNKS1BP1/SSRP1/P2RX3/PRG3/PRG2/SLC43A3/RTN4RL2/SLC43A1/TIMM10/SMTNL1/UBE2L6/LOC390183/SERPING1</i>
2R	4	4p15.3	He L/2	20.146-20.245	0.099	<i>SLIT2</i>
2R	7	7q33	He L/1	134.009-134.144	0.135	<i>CALD1/LOC340351</i>
2D	9	9p21.3	He L/1	20.675-22.583	1.908	<i>KIAA1797/LOC401494/IFNB1/IFNW1/IFNA21/LOC392289/IFNA4/LOC392290/IFNA7/IFNA10/G13P1/LOC392291/IFNA16/IFNA17/LOC392292/IFNA14/IFNA22/IFNA5/IFNA10/G13P1/LOC392291/IFNA16/IFNA8/LOC402357/IFNA1/LOC402358/IFNE1/KLHL9/IFNA6/IFNA13/IFNA2/IFNA8/LOC402357/IFNA1/LOC402358/IFNE1/LOC441388/LOC402359/MTAP/CDKN2A/CDKN2B/LOC401495/DMRTA1</i>
2R	9	9q33.1	He L/1	115.116-115.206	0.090	<i>DEC1</i>
4D	7	7p12.2	He L/1	50.143-50.320	0.177	<i>IKZF1</i>
4R	9	9p21.3	He L/1	21.064-22.089	1.025	<i>IFNB1/IFNW1/IFNA21/LOC392289/IFNA4/LOC392290/IFNA7/IFNA10/G13P1/LOC392291/IFNA16/IFNA17/LOC392292/IFNA14/IFNA22/IFNA5/KLHL9/IFNA6/IFNA13/IFNA2/IFNA8/LOC402357/IFNA1/LOC402358/IFNE1/LOC441388/LOC402359/MTAP/CDKN2A/CDKN2B/LOC401495</i>
4D	9	9p13.2	He L/1	36.843-36.999	0.156	<i>PAX5</i>
4D	10	10q21.1	He L/1	53.702-53.783	0.081	<i>PRKG1/DKK1</i>
4R	20	20q11.2	He L/1	32.635-32.805	0.170	<i>CDC91LI/TP53INP2/NCOA6</i>



4D/R	21	21q22.3	He L/2	42.059-46.885	4.826	Several	
5D/R	3	3q23-25.1	He L/1	141.219-153.246	12.027	Several	
5R	10	10q22.1-26.3	He L/2	68.743-135.322	66.579	Several	
5R	20	20q11.1-11.2	G/3	28.120-36.379	8.259	Several	
6R	5	5q31.3-35.3	G/3	140.498-180.625	40.127	Several	
6R	9	9q21.1	He L/1	70.605-70.656	0.051	TRMP3	
6R	12	12p12.3-13.3	He L/1	0-18.253	18.253	Several	
7R	7	7p11.2-22.3	He L/1	0-57.731	57.731	Several (including IKZF1)	
7R	7	7q11.1-36.3	G/3	57.731-158.626	100.895	Several	
7R	11	11p15.1	He L/1	18.906-18.939	0.033	LOC390098/LOC390099/MRGX1/LOC390101	
7R	13	13q14.1-14.3	He L/1	45.175-51.859	6.684	Several	
		13q14.2	Ho L/0	47.809-47.973	0.164	<b>RB1/1P2RY5/RCBTB2</b>	
8R	9	9p24.1	He L/1	4.694-4.786	0.092	CDC37LI/AK3/LOC392282/RCLI	
8R	11	11q24.2	He L/1	126.201-126.258	0.057	KIRREL3	
8R	13	13q12.1	He L/1	21.127-21.211	0.084	FGF9	
10D	9	9p24.1	He L/1	4.694-4.784	0.090	CDC37LI/AK3/LOC392282/RCLI	
10D/R	9	9p13.2	He L/1	36.875-37.002	0.127	PAX5	
10D/R	13	13q12.2	He L/1	27.529-27.684	0.155	FLT3/PAN3	
11D	1	1q21.1	He L/1	142.379-142.504	0.125	PDE4DIP	
11R	1	1q11-32.2	G/3	141.484-206.994	65.510	Several	
11R	2	2q23.3	He L/1	152.918-153.025	0.107	FMNL2	
11D/R	3	3q26.3	He L/1	178.381-178.583	0.202	TBL1XR1	
11R	11	11q23.1	He L/1	112.332-112.351	0.019	NCAM1	
11R	16	16q21-22.1	He L/2	56.856-67.106	10.250	Several	
11D	17	17q11.2	He L/2	26.171-26.693	0.522	CRLF3/FLJ12735/FLJ22729/CENTA2/RNF135/LOC400590/NF1/OMG/EVI2B/EVI2A	

11R	19	19p13.1	G/3	15.974-16.116	0.142	<i>LOC126536/FLJ25328/ORIAB1/PTPM4/RAB8A/HSH2D</i>
11D	19	19q13.3	He L/1	53.246-53.344	0.098	<i>PLA2G4C/LIG1</i>

Abbreviations: D, indicates sample from the time of diagnosis; R, sample from the time relapse; Chr, chromosome; Mb, megabase.

<sup>a</sup>Type: hemizygous loss (He L), homozygous loss (Ho L) or copy-number gain (G). Copy: number of alleles present in the region involved (note that 2 copies represent a loss if the chromosome was trisomic).

<sup>b</sup>The SNP array platform is based on NCBI Build 35.1.

<sup>c</sup>Genes only listed if the region involved was <2 Mb, in which case all known genes are listed from pter to qter. Bold type indicates homozygously deleted genes.