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TRISOMIES IN HEMATOLOGIC MALIGNANCIES

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LUND UNIVERSITY
2005

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ORIGINAL ARTICLES

This thesis is based on the following original articles, referred to in the text by their Roman numerals:

- I. PAULSSON K, FIORETOS T, STRÖMBECK B, MAURITZSON N, TANKE HJ, and JOHANSSON B. Trisomy 8 as the sole chromosomal aberration in myelocytic malignancies: a multicolor and locus-specific fluorescence in situ hybridization study. *Cancer Genet Cytogenet* 2003;140:66-69.
- II. PAULSSON K, HEIDENBLAD M, STRÖMBECK B, STAAF J, JÖNSSON G, BORG Å, FIORETOS T, and JOHANSSON B. High-resolution genome-wide array-based comparative genome hybridization reveals cryptic chromosome changes in AML and MDS cases with trisomy 8 as the sole cytogenetic aberration. Manuscript.
- III. PAULSSON K, PANAGOPOULOS I, KNUUTILA S, JEE KJ, GARWICZ S, FIORETOS T, MITELMAN F, and JOHANSSON B. Formation of trisomies and their parental origin in hyperdiploid childhood acute lymphoblastic leukemia. *Blood* 2003;102:3010-3015.
- IV. PAULSSON K, MÖRSE H, FIORETOS T, BEHRENDTZ M, STRÖMBECK B, and JOHANSSON B. Evidence for a single-step mechanism in the origin of hyperdiploid childhood acute lymphoblastic leukemia. *Genes Chromosomes Cancer* 2005;44:113-122.

ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
array CGH	array-based comparative genome hybridization
BAC	bacterial artificial chromosome
CFU-GEMM	colony-forming unit of the erythrocyte-macrophage-megakaryocyte
CIN	chromosomal instability
CMD	chronic myeloproliferative disorder
CML	chronic myeloid leukemia
CNP	copy number polymorphism
CT21	constitutional trisomy 21
CT8M	constitutional trisomy 8 mosaicism
dmin	double minutes
EAD	equal allele dosage
FISH	fluorescence in situ hybridization
LOH	loss of heterozygosity
M-FISH	multicolor-FISH
MAC	morphology-antibody-chromosomes
MDS	myelodysplastic syndromes
miRNA	microRNA
MTX	methotrexate
PAC	P1 artificial chromosome
PCP	partial chromosome paint
QF-PCR	quantitative fluorescent polymerase chain reaction
RT-PCR	reverse transcriptase-polymerase chain reaction
SKY	spectral karyotyping
SNP	single nucleotide polymorphism
UAD	unequal allele dosage
UPD	uniparental disomy
WBC	white blood cell count
WCP	whole chromosome paint

PREFACE

Acquired clonal chromosome aberrations are found in a large proportion of malignant hematologic disorders. During the last decades, it has become increasingly clear that these genetic changes are closely associated with leukemogenesis, being involved in transformation as well as in neoplastic evolution. In hematologic malignancies, the balanced rearrangements have been successfully investigated, resulting in an increased understanding of the molecular mechanisms underlying the leukemogenic process, an improved stratification into prognostic and morphologic subgroups, and, recently, to novel treatment strategies. Gains of chromosomes – e.g., trisomies – are equally common. However, in contrast to the balanced rearrangements, little is known about the biologic significance and pathogenetic impact of these changes or how they arise. The aim of the present thesis was to find the answers to some of these questions.

This thesis is divided into four sections. The first is an introduction to chromosome abnormalities in malignant hematologic disorders, specifically focusing on trisomies, whereas the second is a brief summary of the present investigation, including the methods used and the most salient results. The third comprises reviews of the two chromosome aberrations/karyotypic abnormality patterns studied in articles I – IV: trisomy 8 in acute myeloid leukemia/myelodysplastic syndromes and hyperdiploidy in childhood acute lymphoblastic leukemia, including discussions of the results from the present investigation. The final section contains the original articles (I – IV) on which this thesis is based.

Lund, August 2005

INTRODUCTION

HEMATOLOGIC MALIGNANCIES

Hematologic malignancies are neoplastic disorders that affect the blood-forming bone marrow and the peripheral blood. They involve either the myeloid or the lymphoid lineages and comprise chronic proliferative or dysplasia-associated disorders, such as chronic myeloid leukemia (CML), chronic myeloproliferative disorders (CMD), and myelodysplastic syndromes (MDS), as well as the acute myeloid (AML) and the acute lymphoblastic leukemias (ALL), which are characterized by an accumulation of immature hematopoietic cells – i.e., blasts – in the bone marrow and peripheral blood.¹ The diagnosis and the prognostication of these disorders are based on clinical findings, such as white blood cell count (WBC) and age, the morphology and the immunophenotype of the neoplastic hematopoietic cells, and the presence of genetic aberrations.¹ The latter are seen in all types of hematologic malignancies, and are the subject of the present thesis.

CHROMOSOME ABERRATIONS IN HEMATOLOGIC MALIGNANCIES

Neoplasia is a genetic disorder

Acquired clonal chromosome aberrations are found in a wide range of neoplastic disorders (Fig. 1).² During the last century, it has become increasingly clear that these anomalies lead to deregulation of the genetic control systems of the cell, resulting in transformation, tumor development and tumor evolution.³ Today, we know that cancer is genetic disease – i.e., the occurrence of neoplasia, as well as the clonal evolution of malignant disorders, are the direct consequences of somatic mutations in the genome. This understanding has had profound ramifications, both for the clinical management of such disorders and for the understanding of the biologic mechanisms behind tumorigenesis. Thus, the questions why these abnormalities occur, how they arise, what their pathogenetic effects are, and, last but not least, how these effects may be counteracted, have received much attention during recent years.

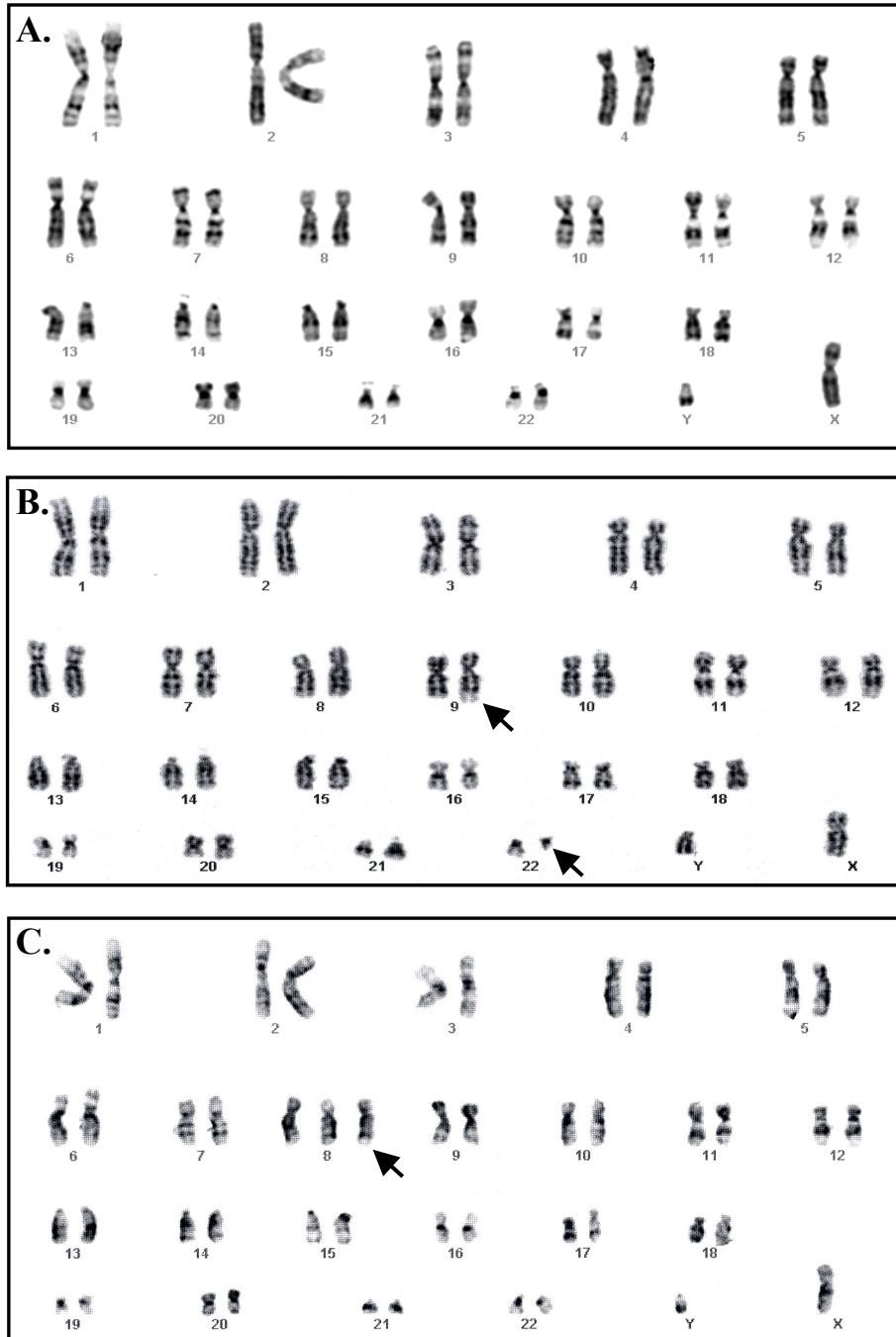


Figure 1. Examples of karyotypes. (A) Normal male karyotype, 46,XY. (B) Aberrant karyotype in a CML with the balanced rearrangement $t(9;22)(q34;q11)$. (C) Aberrant karyotype in an AML with the unbalanced chromosome abnormality trisomy 8.

Hematologic malignancies and chromosome abnormalities

More than 30,000 cases of hematologic malignancies with chromosome aberrations have been reported to date, making it the most thoroughly cytogenetically investigated group of all neoplastic disorders.⁴ This is partly due to the fact that the presence of different abnormalities has been shown to be of great clinical importance in hematologic malignancies.⁵ For example, chromosome analysis is utilized for the classification of AML cases to different subtypes of this disorder – e.g., the t(15;17)(q22;q12) is found almost exclusively in acute promyelocytic leukemia (AML M3)⁶ – and cases are assigned to prognostic risk groups and different treatment strategies based, in part, on the cytogenetic findings.⁵ Furthermore, the understanding of the molecular mechanisms underlying leukemogenesis may lead to new treatments. Thus, a drug aimed directly at the abnormal fusion protein resulting from the CML-specific t(9;22)(q34;q11) was recently developed and has shown excellent results in clinical practice.⁷ Taken together, cytogenetic and molecular genetic analyses are today invaluable for diagnostic, prognostic, and treatment-related purposes in malignant hematologic disorders.⁵

Types of genetic aberrations

The most characteristic karyotypic feature of hematologic malignancies – in particular of the acute leukemias and CML – is the presence of balanced translocations and inversions.⁸ The pathogenetic impact of these abnormalities is today relatively well understood; two, or possibly three, biologic consequences have been described. First, balanced rearrangements may lead to deregulation of a gene in one of the breakpoints by placing its coding sequence under the transcriptional control of regulatory elements in the other breakpoint.⁹ For example, the t(8;14)(q24;q32), characteristic for Burkitt's lymphoma/leukemia, fuses the oncogene *MYC* with *IGH@*, causing overexpression of the former gene.¹⁰ Secondly, they may result in fusion of the genes in the respective breakpoints, generating chimeric genes with novel functions.⁹ In hematologic malignancies, genes encoding transcription factors and tyrosine kinases are commonly involved, such as the hematopoietic transcription factor gene *RUNX1*, which has several different fusion partners in AML, and the *ABL1* tyrosine kinase, encoded by a gene which forms a chimera with *BCR* as a result of the t(9;22) in CML.^{3,11} Thirdly, *RUNX1* has been reported to be translocated to out-of-frame transcripts, leading to its truncation.^{12,13} Functional studies of one of these chimeras – the

RUNX1/AMP19 formed by a t(19;21)(q13;q22) in secondary AML – showed that it blocked maturation of immature myeloid cells.¹³ Thus, one possible pathogenetic impact of structural rearrangements is disruption of genes, presumably resulting in haploinsufficiency. However, further studies of this putative mechanism are needed. Although the above-mentioned chimeras frequently result from translocations and inversions, they may also be formed on extra-chromosomal episomes or through intra-chromosomal deletions.¹⁴⁻¹⁷ Because these may be cytogenetically cryptic, it is as yet unknown how common the latter mechanisms are.

Imbalances, i.e., aberrations that result in gain or loss of genetic material, are even more common than translocations and inversions in hematologic malignancies. These include amplifications, duplications, hetero- or homozygous deletions, monosomies, and trisomies. Amplifications – multiple extra copies of a chromosome region – may occur in the form of heterogeneously staining regions or double minutes (dmin), and result in overexpression of one or more genes. Upregulation of genes is also a possible consequence of duplications, although these are low-copy number changes and are therefore expected to have a lesser impact on the gene expression patterns. Deletions may lead to loss of one or two copies of a tumor suppressor gene and also, as mentioned above, to fusion genes.¹⁵⁻¹⁸ The pathogenetic impact of monosomies and trisomies are more elusive. Presumably, they result in dosage effects for a large number of genes. The specific problems regarding trisomies are further discussed below.

Gene mutations, including point mutations or partial segmental duplications, have also been described in many malignant hematologic disorders. These may result in upregulation of the gene, such as activation of *FLT3* in AML,¹⁹ or in loss-of-function, e.g., haploinsufficiency for *RUNX1* in AML.²⁰

Recently, segmental uniparental disomies (UPDs) – i.e., parts of two homologues being derived from the same parental chromosome – have been described in AML. Two studies have identified such anomalies in 20% of investigated AML cases.^{21,22} The selective advantage that these anomalies may confer to the cell is as yet unknown, but loss of a wild-type tumor suppressor gene with subsequent duplication of a pre-existing mutated allele is one possibility; deregulation of imprinted loci another.²²

Epigenetic alterations have also been reported to be common in hematologic malignancies, resulting in silencing of important regulatory genes, such as the cyclin-dependent kinase inhibitors *CDKN2A*

(previously *P16*, *INK4A*) and *CDKN2B* (previously *P15*, *INK4B*), thereby promoting leukemogenesis.²³

Finally, recent findings implicate microRNAs (miRNA) in tumorigenesis. This family of non-coding RNAs negatively regulates the expression of genes, and their amplification or deletion could therefore result in deregulation of genes involved in the development of leukemia.²⁴ For example, underexpression of the miRNA genes *miR15* and *miR16* has been reported to result from deletions of the chromosome band 13q14 in chronic lymphocytic leukemia.²⁵

TRISOMIES IN HEMATOLOGIC MALIGNANCIES

Although balanced fusion gene-forming rearrangements may be the cytogenetic feature most commonly thought of in the context of hematologic malignancies, gains of chromosomes – e.g., trisomies – is an equally frequent finding. In AML, for example, +6, +8, +11, +13, +19, +21, and +22 are each found, as the single anomaly or in addition to other changes, in >2% of unselected cytogenetically abnormal cases – the by far most common being trisomy 8, which is present in approximately 15%.² Furthermore, in childhood ALL, the high hyperdiploid (>50 chromosomes) cytogenetic subgroup – characterized by a massive gain of specific chromosomes – constitutes almost half of all cytogenetically abnormal cases.²⁶ However, in spite of this frequent occurrence of trisomies – and in sharp contrast to the well-investigated biologic effects of fusion gene-forming rearrangements – next to nothing is known about their biologic outcome, their pathogenetic impact, or how they arise.

Gene dosage effects of trisomies

The occurrence of a trisomy results in duplication of hundreds of loci on the gained chromosome. It seems obvious that this must lead to dosage effects, not only for genes on the trisomic chromosome – which would be expected to display a 1.5 increase of expression – but also for loci on other chromosomes, which may be regulated by the protein products of the first group of genes. Thus, a specific chromosome-induced effect, as well as an overall deregulation of gene expression, should be seen.²⁷

In fact, microarray gene expression analyses have shown that both of the above-mentioned effects occur as a consequence of trisomies in malignant hematologic disorders.^{28,29} However, additional, cytogenetically cryptic, genetic aberrations may also be present in such cases,

and hence it cannot be excluded that hidden changes influence the observed gene expression signatures. Avoiding this problem, Upender et al.³⁰ used chromosome transfer to introduce trisomies 3, 7, and 13, respectively, into different cell lines, enabling investigation of the direct effect of gained chromosomes. Their results showed that introduction of an extra chromosome leads to a general increase in the expression of the genes residing on that chromosome, as well as to deregulation of a substantial proportion of the loci at other chromosomes. Investigations of cells from fetuses with constitutional trisomies 13 and 21 have also been performed.^{31,32} These cells are not expected to harbor additional genetic anomalies, and can therefore be utilized to investigate how trisomies affect gene expression. Microarray expression analyses showed a general upregulation of chromosome 13 and chromosome 21 genes, respectively, and additional gene deregulation was detected in at least one study.^{31,32}

Taken together, the presence of a trisomy results in large-scale gene dosage effects. However, although this gene deregulation is likely to be pathogenetically important, the question whether this is sufficient for leukemogenesis remains unanswered.

Trisomies as sole cytogenetic aberrations – additional cryptic changes?

Although trisomies are frequently seen as isolated chromosome aberrations in hematologic malignancies, this does not exclude the presence of additional, cytogenetically cryptic anomalies. For example, gene point mutations, uniparental disomies, and rearrangements involving telomeric sequences, such as the t(12;21)(p13;q22) in ALL,³³ are not detectable by standard G-banding analysis. In fact, it has been suggested that the gene dosage effects resulting from trisomies are not sufficient for leukemogenesis, but that specific primary rearrangements are also needed.⁸ These could be present at the duplicated chromosome, as described below, or they could be at any other chromosome.

In this context, AML in children with Down syndrome is enlightening. These patients, who have trisomy 21 constitutionally (CT21), have a highly increased risk of developing myeloid malignancies, especially of the AML M7 subtype.³⁴ The pathogenetic mechanism behind this is unknown; because not all individuals with CT21 develop AML, +21 by itself cannot be sufficient for leukemogenesis. Recently, mutations in the *GATA1* gene at Xp11 have been identified in the vast majority of these AML cases.³⁵ However, since *GATA1* mutations also are present in most CT21 patients with transient myeloproliferative disorder – a preleukemic state which sometimes precedes the AML, but

which often resolves by itself without transformation – these cannot be sufficient for AML transformation.^{34,36} Nevertheless, their detection confirms that hidden genetic aberrations may be present in cases with single trisomies.

Identification of additional genetic changes in hematologic malignancies with isolated trisomies would be important for several reasons. For example, cryptic anomalies may have a clinical impact. Also, the development of imatinib (Gleevec), which directly targets the BCR/ABL1 protein resulting from the t(9;22) in CML,⁷ shows that drugs aimed specifically at the products from genetic rearrangements may become a reality in future treatment of malignancies. Last but not least, the detection of additional genetic abnormalities in cases with trisomies as the sole cytogenetic change would undoubtedly increase our understanding of leukemogenesis.

Trisomies – ways to duplicate specific loci?

An intriguing possibility is that trisomies are associated with the duplication of a mutated allele, a cryptic rearrangement, or an imprinted locus. If so, gain of a whole chromosome is, at least in part, simply a way to obtain more copies of a specific aberration/allele on that chromosome. The first experimental evidence for this mechanism came from induced mouse skin squamous cell carcinomas, in which a nonrandom duplication of the chromosome carrying a mutated *Hras1* allele was noted.³⁷ A few years later, Caligiuri et al.³⁸ reported that trisomy 11 in AML was associated with partial tandem duplications of *MLL* (previously *ALL1*). Surprisingly, subsequent studies showed that it was the chromosome 11 containing the wild-type *MLL* allele that was duplicated, excluding that the pathogenetic outcome of the trisomy was duplication of the mutated copy.³⁹ However, the initial finding spurred other researchers to search for aberrant oncogenes in trisomic chromosomes. Hence, duplications of the mutated genes *MET* in hereditary papillary renal carcinoma with +7, *KIT* in AML with +4, and *RET* in multiple endocrine neoplasia type 2-associated pheochromocytomas with +10 were identified.⁴⁰⁻⁴³ In the latter disorder, some cases with disomy 10 displayed deletion of the wild-type allele, suggesting that the relative dosage of the mutated copy of *RET* was the pathogenetically important outcome.⁴³ In contrast, Kawakami et al.⁴⁴ found no changes in *MET* in testicular germ-cell tumors with +7 and Powell et al.⁴⁵ detected no internal tandem duplications of *FLT3* in AML with +13. Taken together, duplication of a mutated allele may be the functional outcome of some trisomies in malignant disorders. However,

they have so far only been identified in a subset of gained chromosomes, and many recurrent trisomies remain to be investigated.

It is also conceivable that a trisomy results in duplication of a cytogenetically undetectable fusion gene-forming rearrangement. A hypothetical example is the duplication of a cryptic der(8)t(8;8) with breakpoints in the same chromosome band, which may be misinterpreted as gain of a normal chromosome 8. The basis for this suggestion is that gain of an extra derivative chromosome containing a fusion gene is a common finding in hematologic malignancies, e.g., duplication of the Philadelphia chromosome – +der(22)t(9;22) – in CML.^{2,46} However, gains of derivatives containing the reciprocal – putatively without pathogenetic effects – chimeric genes are also frequent in some translocation-positive hematologic malignancies, suggesting that the resulting imbalance may be the biologically important outcome, and not the duplication of a fusion gene.⁴⁶ Nevertheless, a subset of the recurrent trisomies in hematologic malignancies may be derivatives containing cryptic rearrangements, although this mechanism has never been described.

Furthermore, the duplication of a chromosome may result in deregulation of imprinted loci, i.e., of genes that are differentially expressed depending on whether they are maternally or paternally inherited.⁴⁷ This would be detectable as a preferential parental origin of the gained chromosome. Haas⁴⁸ suggested that this mechanism may be of importance in hyperdiploid childhood ALL; something that will be further discussed in the “Hyperdiploidy in childhood ALL” section below. Apart from investigations of the latter disease subgroup,^{49,III,IV} no studies have specifically addressed the possibility of preferential parental duplications in hematologic malignancies with trisomies. However, the fact that some other genetic aberrations display skewed parental origin in neoplastic disorders – such as preferential loss of the maternally derived chromosome band 11p15 in Wilms’ tumors and of the paternally inherited 19q in oligodendrogliomas^{50,51} – as well as the recent reports of segmental UPDs in AML,^{21,22} suggest that imprinting effects may be involved in tumorigenesis.

The formation of trisomies

Solid tumors are often characterized by massive aneuploidy, possibly resulting from a higher rate of obtaining chromosome aberrations; a phenomenon known as chromosomal instability (CIN).⁵² In many instances, this has been associated with mitotic spindle aberrations and mutations in genes encoding proteins involved in the mitotic check-

points.⁵² In contrast, hematologic malignancies are generally cytogenetically stable, harboring only a few, nonrandom numerical abnormalities; they do not display CIN. It is, however, possible that the same pathways are involved in the formation of some trisomies in malignant hematologic disorders as in the occurrence of CIN in solid tumors. Thus, centrosome aberrations have been detected in AML and CML in blast crisis with cytogenetic abnormalities, including chromosome rearrangements as well as numerical changes.^{53,54} Furthermore, single cases of AML and ALL have been reported in patients with mosaic variegated aneuploidy, a condition caused by constitutional mutations in the mitotic spindle checkpoint gene *BUB1B*.⁵⁵⁻⁵⁷ This gene has also been shown to be mutated in some T-cell leukemias with complex karyotypes.⁵⁸ Finally, occasional mutations in the mitotic checkpoint genes *CHEK2* (previously *CHK2*) in AML/MDS and *MAD1L1* in B-cell lymphomas have been described, but at low frequencies and without concordant numerical abnormalities, at least in the study by Hofmann et al.⁵⁹⁻⁶¹ The latter suggests that mutations in these genes are not associated with aneuploidy in hematologic malignancies. Taken together, the mechanisms behind the occurrence of trisomies in hematologic malignancies remain largely unknown.

THE PRESENT INVESTIGATION

The articles constituting the basis of this thesis may be divided into two parts: studies I and II, in which we attempted to identify cryptic chromosome changes in AML and MDS with trisomy 8 as the sole cytogenetic aberration, and studies III and IV, in which the mechanisms behind the occurrence of hyperdiploidy and the possibility of imprinting effects in this karyotypic subgroup were investigated in childhood ALL. Below is a brief introduction to the methods used, as well as a short summary of the most salient findings. For details on how the individual studies were performed, see articles I – IV.

MATERIALS AND METHODS

Patients

All patients had been analyzed with standard cytogenetic techniques at the Department of Clinical Genetics, Lund University Hospital, Sweden as part of the clinical evaluation of their respective disorders. The present investigation comprised a total of 17 AML and MDS cases and one CMD with trisomy 8 as the apparently sole aberration. Furthermore, 37 childhood ALLs with hyperdiploidy were included. For 22 of these cases, samples from the patients' parents were obtained and analyzed. All studies were approved by the Research Ethics Committee of Lund University.

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) is a technique in which fluorescently labeled probes are hybridized to target sequences in interphase or metaphase cells and visualized in a fluorescence microscope (Fig. 2).⁶² The resolution of FISH depends on the utilized probes. For example, multicolor-FISH (M-FISH) – such as the combined ratio labeling probe set used in the present investigation – and spectral karyotyping (SKY) enable detection of each chromosome pair in a single hybridization,^{63,64} generating information of the whole genome, whereas gene-specific and subtelomeric probes only provide data for the investigated loci – sometimes with a resolution as high as 10 kb – and the subtelomeric sequences of a chromosome arm, respectively (Fig. 2A, C). In between these extremes are FISH analyses performed with whole and partial

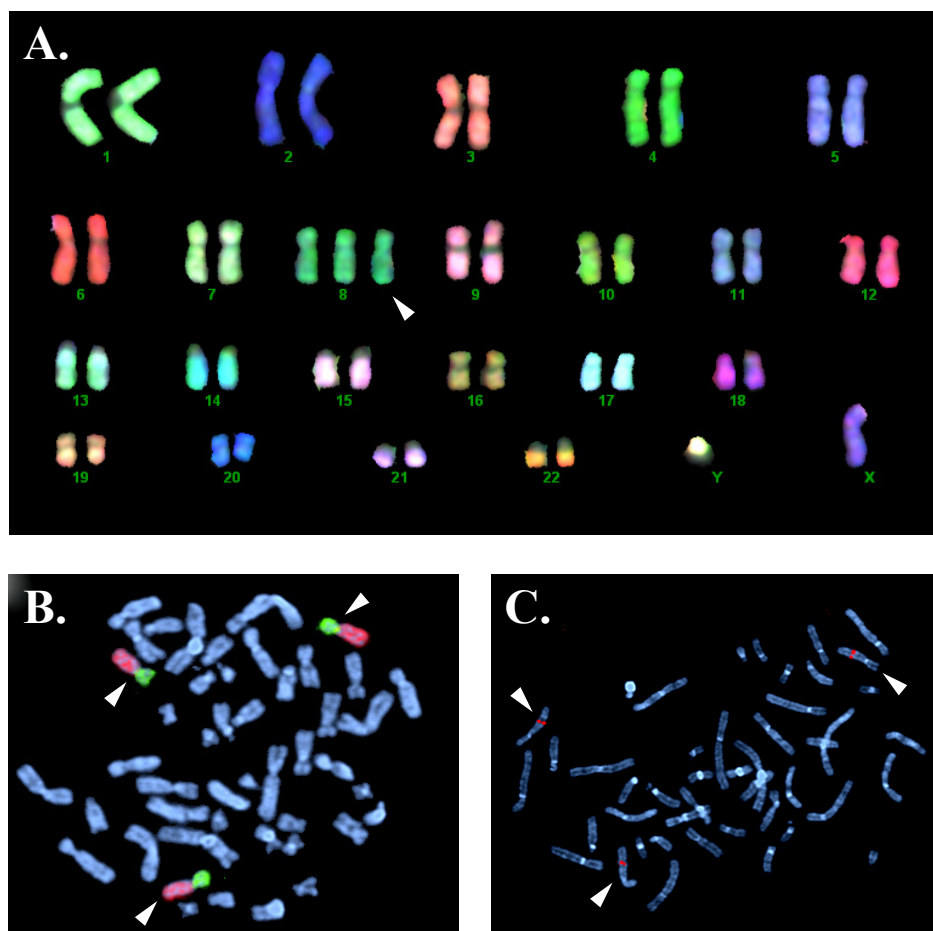


Figure 2. Examples of FISH findings in +8-positive AML/MDS in article I. Trisomy 8 is the only abnormality detected. Chromosomes 8 are indicated by arrows. (A) M-FISH. (B) FISH analysis with partial chromosome paint probes for 8p (green) and 8q (red). (C) FISH analysis with a gene-specific probe for *RUNX1T1* (red).

chromosome paint (WCP and PCP, respectively) probes, resulting in identification of chromosomes and chromosome parts (Fig. 2B), and multicolor banding/RxFISH, which generates chromosome-specific banding patterns, enabling detection of structural rearrangements within chromosomes.^{68,69}

In hematologic malignancies, FISH-based techniques have been used for identification and characterization of chromosome aberrations. This method was utilized in investigations I, II, and IV. In study I, FISH analyses with probes for M-FISH, PCP and subtelomeric probes, and

locus-specific FISH for four genes were performed. In study II, bacterial and P1 artificial chromosomes (BACs and PACs, respectively) and gene-specific probes were utilized for FISH confirmation of intra-chromosomal gains and losses. In study IV, determination of chromosome copy numbers was done with M-FISH and interphase FISH.

Array-based comparative genome hybridization

In array-based comparative genome hybridization (array CGH) analyses, test and reference DNA is labeled with different fluorescent dyes and hybridized to slides containing genomic sequences, for example BAC clones.⁶⁵ This enables detection of gains and losses of genetic material, with a resolution level depending on the size of the clones and their spacing across the genome. In neoplastic disorders, array CGH may be used as a supplementary method to G-banding and FISH for detection and characterization of chromosome aberrations and for identification of cytogenetically cryptic abnormalities. In study II of the present investigation, array CGH was utilized for the latter of these purposes.

Quantitative fluorescent PCR

Quantitative fluorescent polymerase chain reaction (QF-PCR) with polymorphic microsatellite markers is a rapid method for DNA identification and determination of chromosome copy numbers.⁶⁶ Primers for microsatellite markers are fluorescently labeled and used in a semiquantitative PCR; the resulting products are separated by capillary electrophoresis. Alleles are visualized as peaks: heterozygotes display two allelic peaks, whereas homozygotes display one peak. Copy number changes of the chromosome region including the marker are detectable as increased or decreased peak-to-peak ratios. Originally, this method was used for paternity testing and forensic DNA analyses. During recent years, QF-PCR has emerged as an alternative to G-banding and FISH for prenatal detection of constitutional whole-chromosome imbalances, utilizing markers localized to the commonly aneuploid chromosomes.^{66,67}

Because QF-PCR may be used for both detection of chromosome gains and (if parental samples are available) for determination of the parental origin of the chromosomes, this method was ideal for addressing the objectives of studies III and IV.

RESULTS ARTICLES I/II

The aim of studies I and II was to identify cryptic genetic aberrations in AML and MDS with trisomy 8 as the apparently sole chromosome abnormality. In the first of these investigations, FISH was used to search for rearrangements between chromosomes or within chromosome 8. No cytogenetically cryptic changes were found with M-FISH or metaphase FISH utilizing PCP and subtelomeric probes for 8p and 8q and probes specific for the chromosome 8-genes *FGFR1*, *MYST3* (previously *MOZ*), *RUNX1T1* (previously *ETO*), and *MYC* in thirteen cases (Fig. 2).

Because small imbalances involving loci other than the above-mentioned genes would not be detectable by the FISH assays described in article I, the investigation was extended utilizing array CGH in study II. Analyses with this method revealed imbalances of 0.2 – 4.9 Mb, comprising segmental duplications and hemizygous deletions, in 9/10 AMLs/MDSs. In total, 23 different changes were identified, but eleven of these overlapped with previously described genomic copy number polymorphisms (CNPs) and were hence most likely constitutional. The remaining twelve abnormalities were present in altogether five AMLs/MDSs. Although some of these imbalances may have been novel CNPs, at least two were undoubtedly leukemia-associated: a hemizygous deletion at 12p13, including the *ETV6* gene and similar to previously described losses in AML, and a hemizygous del(7)(p14p14), which was shown to have occurred prior to the trisomy 8. Thus, the findings presented in article II showed that cryptic imbalances are frequent in trisomy 8-positive AML and MDS, and that +8 may not be the primary event even when found as the sole cytogenetic aberration.

RESULTS ARTICLES III/IV

Studies III and IV had two aims: to investigate the mechanisms behind the formation of hyperdiploidy in childhood ALL and to address the possibility of imprinting effects related to the parental origin of the gained chromosomes or to the presence of UPDs.

In study III, QF-PCR with polymorphic markers was utilized to investigate ten hyperdiploid childhood ALLs. We showed that the hyperdiploid pattern in these cases most likely had arisen by a simultaneous gain of chromosomes in a single abnormal cell division. However, a prerequisite for definite determination of the basis of multiple chromo-

some gains with this method is that all disomic and tetrasomic chromosomes are identified and investigated. Because the G-banding morphology was poor in the included cases – as it usually is in this cytogenetic subtype – and samples for FISH were not available, this requirement could not be fulfilled. An attempt was made to use conventional CGH for identification of gains, but the results were ambiguous. Therefore, we could not exclude that the hyperdiploidy had arisen via a tetraploid state or by sequential gains of chromosomes in consecutive mitoses based on the results presented in article III, although these alternatives were less likely. Hence, study IV was designed to resolve this issue: cases were selected on the basis of material being available for FISH and were investigated with M-FISH if analyzable metaphases were present and otherwise with interphase FISH for the commonly gained chromosomes. This approach resulted in identification of all disomies and tetrasomies in ten cases and of all tetrasomies in the remaining seventeen, enabling definite conclusions regarding the origin of the hyperdiploidy in the first ten cases and strong indications of the most likely mechanism in the remaining. The results provided strong evidence for the simultaneous gain of chromosomes suggested by study III.

Possible imprinting effects for the gained chromosomes were addressed by investigation of hyperdiploid childhood ALLs and samples obtained from the patients' parents in study III. These parental samples had been collected in the early 1990's but not investigated previously because of lack of a rapid technique for identification of the origin of chromosomes until QF-PCR with polymorphic markers became available. Although no statistically significant ratios of parental origin were detected, trends toward preferential duplication of the paternal chromosome 8 and the maternal chromosome 14 were seen. However, the limited number of cases precluded definitive conclusions. In study IV, samples were collected from the parents to an additional twelve patients with hyperdiploid childhood ALL and trisomies 8 or 14. The subsequent QF-PCR analyses revealed no preferential paternal or maternal origin, respectively, excluding imprinting effects related to the parental origin of gained chromosomes in hyperdiploid childhood ALL.

Finally, the possibility of a pathogenetic impact of UPDs for the frequently gained chromosomes in hyperdiploid childhood ALL was addressed in study IV. Only one UPD, not associated with the formation mechanism, was identified among the 27 cases. Thus, UPDs are not common for these chromosomes in hyperdiploid cases.

REVIEWS

TRISOMY 8 IN AML AND MDS

Trisomy 8 is one of the most common cytogenetic findings in myeloid malignancies, including AML, MDS, CMD, and CML.² In spite of this, the biologic importance and the functional – and pathogenetic – outcome of this aberration remain elusive. Below, our present knowledge of +8 is summarized, focusing on trisomy 8 as the sole anomaly in AML and MDS and including the results presented in articles I and II.

Epidemiology

A survey of published, unselected karyotypically aberrant AML and MDS cases shows that trisomy 8 as the single cytogenetic change is present in approximately 6% and 11%, respectively (Tables 1, 2). Similar incidences have previously been reported in studies of cytogenetic subgroups in AML and MDS.⁷⁰⁻⁷⁵ This makes +8 the most common numerical abnormality in these disorders, and one of the most frequent chromosome changes overall.² Isolated trisomy 8 displays no gender-related frequency differences in AML or MDS.⁷² However, it is more common among elderly AML patients, with a similar trend being present, albeit not statistically significant, in MDS.^{72,76}

Clinical features

Although +8 is found in all morphologic subgroups of AML and MDS, its frequency has been shown to vary among the different subtypes.^{72,77} Previous studies have found the highest incidence of isolated trisomy 8 in the AML M1, M4 and M5 subtypes.^{72,77,78} As regards the latter morphology, Haferlach et al.⁷⁷ reported that +8 as the sole chromosome aberration was significantly more common in the M5a subgroup (22%) than in M5b (3%). However, a survey of published, unselected cases with trisomy 8 as the sole aberration in relation to different AML subtypes

Table 1. Frequency of +8 as the sole aberration in unselected cytogenetically abnormal AML cases reported in the literature²

<i>M0</i>	<i>M1</i>	<i>M2</i>	<i>M3</i>	<i>M4</i>	<i>M5</i>	<i>M5a</i>	<i>M5b</i>	<i>M6</i>	<i>M7</i>	<i>Spec</i>	<i>NOS</i>	<i>Total</i>
5.2	6.1	5.8	1.8	6.8	10	11	11	6.3	4.4	0	8.0	6.4

AML, acute myeloid leukemia; M0 – M7, AML subtypes; Spec, special type; NOS, not otherwise specified.

Table 2. Frequency of +8 as the sole aberration in unselected cytogenetically abnormal MDS cases reported in the literature²

<i>RA</i>	<i>RARS</i>	<i>RAEB</i>	<i>CMML</i>	<i>RAEBt</i>	<i>Spec</i>	<i>NOS</i>	<i>Total</i>
10	13	12	14	9.6	0	7.4	11

MDS, myelodysplastic syndromes; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, RA with excess of blasts; CMML, chronic myelomonocytic leukemia; RAEBt, RAEB in transformation; Spec, special type; NOS, not otherwise specified.

does not reveal a particularly high frequency of +8 in AML M1 and M4, and identical incidences of isolated +8 are seen in the M5a and M5b subgroups (11%; Table 1). In MDS, +8 has been reported to be most common in chronic myelomonocytic leukemia, refractory anemia, and refractory anemia with excess of blasts,^{72,73,75} but no statistically significant differences in the frequency between different morphologic subtypes are detected among published cases (Table 2). Thus, different studies have yielded conflicting results as regards the occurrence of +8 in the various morphologic subgroups, especially in AML. Although these discrepancies may be spurious, reflecting various ascertainment procedures for included cases, it cannot be excluded that, e.g., geographic differences exist in the incidence of trisomy 8 as the sole aberration in AML and MDS. Hence, further studies are needed to clarify this issue.

No specific immunophenotype has been described for AML and MDS with isolated trisomy 8.⁷⁹ However, Casasnovas et al.⁸⁰ reported that AML cases with +8 mainly expressed CD13 and CD33, and that this cytogenetic subgroup differed from AMLs with abnormal karyotypes by fewer cases expressing CD34.

Etiology

Next to nothing is known about environmental risk factors for AML/MDS with +8 as the sole aberration. In fact, trisomy 8 is negatively associated with AML and MDS following iatrogenic exposure, i.e., prior treatment with radio- or chemotherapy; it is significantly more frequent in de novo disease.^{72,81} In AML, trisomy 8 is positively associated with previous exposure to organic solvents, in particular benzene.⁸² Although the latter compound has been suggested to induce specifically aneuploidy of chromosome 8,⁸³ more investigations are needed to address this issue. Smoking has also been reported to increase the risk for +8,⁸⁴ but this finding could not be confirmed in a later study which included more cases.⁸⁵ Taken together, the epidemiologic data are scant, but the fact that +8 is more common among elderly patients^{72,76} suggests that prolonged

exposure to one or several as yet unknown environmental factors is involved in the genesis of trisomy 8-positive AML and MDS.

Constitutional trisomy 8 and myeloid malignancies

Constitutional trisomy 8, which is almost always associated with mosaicism (CT8M), is a rare condition, estimated to occur in less than 0.1% of all recognized pregnancies.⁸⁶ In contrast to other congenital trisomies, such as +21, the nondisjunction event resulting in gain of chromosome 8 in liveborn individuals with CT8M seems to have occurred almost exclusively as a post-zygotic error, and as expected for gains arising through this mechanism, there is no preferential parental origin of the extra chromosome.^{87,88} CT8M is associated with mild to moderate mental retardation, characteristic facial dysmorphisms, deep palmar and plantar grooves, bone and joint abnormalities, and cardiovascular and urogenital malformations.⁸⁹ However, the severity of the clinical picture is very variable, and some individuals present with an apparently normal phenotype, including normal intelligence.⁸⁹

CT8M seems to be associated with an increased risk for neoplasia, in particular myeloid malignancies.⁹⁰⁻⁹² Taken together with the fact that some individuals with this mosaicism have a normal phenotype, this has led several investigators to suggest that a subset of AMLs and MDSs with +8 may in fact represent constitutional cases.^{90,93-95} In line with this, Maserati et al.⁹⁶ reported that two of fourteen trisomy 8-positive myeloid malignancies were previously undetected CT8M. Although isolated +8 in leukemia is hence constitutional in some instances, this rather high frequency (14%) remains to be confirmed in other studies. However, even if the incidence of undetected CT8M in myeloid malignancies proves to be significant, this congenital mosaicism is highly unlikely to be an underlying factor in most cases of AML and MDS with trisomy 8.

Prognostic impact

There is some controversy as to the prognostic impact of trisomy 8 as the sole aberration in AML. Although several investigators have included cases with isolated +8 in the intermediate risk group,^{71,97,98} others have reported a poor outcome.^{70,99-101} In MDS, cases with +8 as the sole change are included in the intermediate risk group,⁷³⁻⁷⁵ although an increased incidence of transformation to AML as compared to other cytogenetic subtypes in MDS has been noted.^{73,75} Considering that the intermediate risk groups in AML and MDS are large, additional

prognostic factors are clearly needed to stratify cases with +8 as the sole aberration.

Cell of origin

An issue that has received much attention during recent years is the involvement of the hematopoietic stem cell pool in AML and MDS. For AML, the transforming event usually seems to occur in a CD34⁺/CD38⁻ leukemic “stem cell”.¹⁰² Such cells have also been shown to harbor trisomy 8 in AML with this abnormality as the sole cytogenetic aberration.¹⁰³

The hematopoietic cell in which trisomy 8 arises in MDS is more controversial. This is a clinically important question; if the stem cell pool is not involved, autologous stem cell transplantation may become an alternative treatment for this disorder. Early studies, in which morphology-antibody-chromosomes (MAC) techniques were used to identify different cell populations, showed that +8 sometimes was present in all myeloid lineages, including erythroid precursor cells, granulocytes, megakaryocytes, and monocytes, whereas it was not detected in cells of the lymphoid lineages.^{104,105} This provided evidence for its occurrence at the colony-forming unit of granulocyte-erythrocyte-macrophage-megakaryocyte (CFU-GEMM) or pluripotent stem cell level.¹⁰⁴ However, some investigators reported single cases of MDS with clonal involvement of lymphoid cells.^{106,107} More recent studies have utilized cell populations obtained by fluorescence-activated cell sorting to analyze the presence of trisomy 8. Thus, Saitoh et al.¹⁰⁸ investigated the occurrence of trisomy 8 – both as the sole aberration and in addition to other changes – in pluripotent stem cells and progenitor cells from seven MDS cases. Trisomy 8 was detected at the level of the CFU-GEMM (CD34⁺CD33⁺), but not in cells of the lymphoid lineages or in hematopoietic stem cells (CD34⁺Thy1⁺). In contrast, Nilsson et al.¹⁰⁹ reported that a variable part of the CD34⁺CD38⁻ (including both Thy1⁻ and Thy1⁺) cells carried +8, although a sizeable fraction still had disomy for this chromosome. Interestingly, these disomic cells were functionally abnormal, suggesting that they were part of the MDS clone. Furthermore, in four patients with +8 in addition to 5q-, the latter aberration was shown to precede the extra chromosome 8.¹⁰⁹ They concluded that MDS with trisomy 8 seems to involve the hematopoietic stem cell pool, although +8 may not be present initially.¹⁰⁹ Taken together, more studies of trisomy 8-positive MDS are definitely warranted to resolve the important issue of in which cell this chromosome aberration originates.

Trisomy 8 as a secondary aberration

In addition to being common as the sole chromosome change in AML and MDS, +8 is a frequent secondary aberration, occurring in association with other abnormalities in approximately 10% of cytogenetically aberrant AML and MDS cases.² Secondary +8 is found in all morphologic subtypes of AML and MDS, and is not specifically associated with a certain primary rearrangement,^{2,110} although it is particularly frequent in, e.g., AML with t(7;12)(q36;p13) and t(1;11)(p32;q23) and in MDS with der(1;7)(q10;p10) (Tables 3, 4). Schoch et al.⁹⁷ reported that trisomy 8 secondary to chromosome abnormalities classified as favorable or poor risk factors in AML did not alter the prognosis based on the primary rearrangements. However, in a subsequent, larger study, Wolman et al.⁷¹ found a significantly worsened outcome in cases assigned to the poor risk group when +8 accompanied the primary abnormalities. Thus, the prognostic impact of trisomy 8 as a secondary change is debated, and more studies are hence needed to determine this clinically important issue.

Table 3. Frequency of +8 in addition to well-known primary abnormalities in AML²

<i>Primary change</i>	<i>+8 (%)</i>
der(1;7)(q10;p10)	18
inv(3)(q21q26)/t(3;3)(q21;q26)	2.1
inv(16)(p13q22)/t(16;16)(p13;q22)	9.6
t(1;3)(p36;q21)	0
t(1;11)(p32;q23)	20
t(1;11)(q21;q23)	0
t(1;22)(p13;q13)	0
t(2;11)(p21;q23)	9.1
t(3;12)(q26;p13)	0
t(3;21)(q26;q22)	10
t(4;12)(q11-12;p13)	5.0
t(6;9)(p23;q34)	6.2
t(6;11)(q27;q23)	4.9
t(7;11)(p15;p15)	5.0
t(7;12)(q36;p13)	25
t(8;16)(p11;p13)	5.2
t(8;21)(q22;q22)	4.9
t(9;11)(p21-22;q23)	19
t(9;22)(q34;q11)	16
t(10;11)(p11-13;q23)	9.7
t(11;17)(q23;q21)	9.7
t(11;17)(q23;q25)	11
t(11;19)(q23;p13)	12
t(15;17)(q22;q11-21)	12
t(16;21)(p11;q22)	10

AML, acute myeloid leukemia.

Table 4. Frequency of +8 in addition to other well-known anomalies in MDS²

<i>Other change</i>	<i>+8 (%)</i>
del(5q)	11
del(7q)	11
del(11q)	16
del(12p)	10
del(20q)	8.2
der(1;7)(q10;p10)	22
idic(X)(q12-13)	0
inv(3)(q21q26)/t(3;3)(q21q26)	0
t(1;3)(p36;q21)	0
t(3;12)(q26;p13)	0
t(3;21)(q26;q22)	5.6

MDS, myelodysplastic syndromes.

What is the pathogenetic impact of trisomy 8 as the sole aberration in AML and MDS?

Next to nothing is known about the pathogenetic consequences of +8 in AML and MDS. Possible explanations include global gene deregulation, resulting from the putative gene dosage effects that the duplication of chromosome 8 may confer, deregulation of imprinted loci, or duplication of a cytogenetically cryptic rearrangement/mutation present in the chromosome. However, the latter mechanism is less likely for trisomy 8 in AML and MDS because the occurrence of myeloid malignancies in CT8M cases suggests that additional abnormalities would have to occur after the trisomy.

Is trisomy 8 sufficient for initiating leukemia? The answer to this question is not known. It is possible that additional genetic changes are needed for leukemogenesis; aberrations that would have to be cryptic in AMLs/MDSs with +8 as the sole cytogenetic anomaly. Such hidden changes could be located at chromosome 8, or they could involve other chromosomes. Their identification would be important, not only for the understanding of the biology of trisomy 8-positive AML/MDS, but also clinically, with possible diagnostic, prognostic, and treatment-related ramifications.

Pathogenetic impact: Gene dosage effects associated with trisomy 8?

The pathogenetic impact of +8 has in some instances been considered to equal duplication of the *MYC* oncogene at 8q24, at least in CML.^{111,112} However, because chromosome 8 contains approximately 800 genes (the Ensembl Genome Browser; <http://www.ensembl.org/>), it seems unlikely that the functional outcome of this trisomy is contributable to a single

locus. Furthermore, Mertens et al.¹¹³, by mapping chromosome 8 gains in close to 10,000 cases of myeloid malignant disorders, showed that such imbalances almost always occur in the form of a trisomy and could not be reduced to a single band. Thus, they concluded that the pathogenetic effect of trisomy 8 was unlikely to be upregulation of only one gene on this chromosome, including *MYC*. Also, it has been reported that *MYC* is not overexpressed in trisomy 8-positive AML.¹¹⁴ In fact, recent studies have shown that *MYC* is not even upregulated in AML/MDS in which it is highly amplified on dmin, suggesting that overexpression of this gene is not a pathogenetic event in these disorders.^{115,116} Instead, duplication of chromosome 8 seems to be associated with global changes in the gene expression patterns, as shown in several microarray analyses of AML cases with isolated trisomy 8.^{28,114,117,118}

Four microarray studies of trisomy 8 as the sole aberration in AML have been reported. Virtaneva et al.¹¹⁴ specifically compared cases with +8 to AML with a normal karyotype, whereas the remaining investigations included various additional cytogenetic subgroups. Unsupervised analyses, utilizing all transcripts in the array, did not detect clustering of trisomy 8-positive cases.^{114,117,118} However, characteristic gene expression patterns, i.e., clustering, were seen for AML with trisomy 8 in two of three supervised – including only pre-selected genes – analyses.^{28,117,118} This suggests that the +8-subgroup has a heterogeneous gene expression profile compared with AML with well-known primary rearrangements.¹¹⁷ Furthermore, different genes have been shown to be over- or underexpressed in the investigations. Obviously, this discrepancy could be due to the different array platforms used, but it could also reflect an underlying heterogeneity of trisomy 8-positive AML. A general overexpression of genes on chromosome 8 was noted in three of the analyses, corresponding to 1.27, 1.32, and 1.13, and times the level in AMLs with normal karyotypes, respectively.^{28,114,117} However, a substantial proportion of the chromosome 8 genes was not upregulated, demonstrating that gain of one allelic copy does not automatically confer a higher expression of the gene. As regards the biologic function of the differentially expressed genes in trisomy 8-positive AML, Virtaneva et al.¹¹⁴ found a downregulation of genes involved in apoptosis.

Only one microarray expression study of MDS with isolated +8 has been reported. Chen et al.¹¹⁹ compared the gene expression profiles of purified CD34-positive cells from patients with trisomy 8 with such cells from monosomy 7 cases. In contrast to the findings in AML, no general overexpression of genes on chromosome 8 was noted in +8-

positive MDSs, although a specific gene expression signature was identified. This included upregulation of genes involved in immune and inflammatory responses.¹¹⁹

In summary, microarray expression analyses have shown discrepant results among different AML analyses of trisomy 8 as the sole cytogenetic aberration as well as between such AMLs and MDSs. More studies are needed to determine if these differences are the result of the different experimental procedures, or if a true heterogeneity exists at the gene expression level.

Pathogenetic impact: Imprinting?

Although no studies have addressed specifically the parental origin of the gained chromosome 8 in AML and MDS, information is available from a handful of CT8M patients with such disorders and from families with high incidences of AML and MDS. In total, 4 cases with maternal origin of the +8 and two with paternal origin have been reported ($P = 0.69$),^{90,120-122} indicating that no gender-specific duplication occurs. Taken together with the facts that no known chromosome 8 gene has been shown to be imprinted, that no cases of segmental UPD involving chromosome 8 loci have been reported, and that constitutional UPD for chromosome 8 seems to be associated with a normal phenotype,^{21,22,123-125} it is unlikely that imprinting effects related to the parental origin of the gained chromosome are of importance in trisomy 8-positive AML and MDS.

Pathogenetic impact: Is trisomy 8 associated with cryptic changes in AML and MDS?

Several lines of evidence indicate that cryptic anomalies are present in AML/MDS in which an extra chromosome 8 is the only cytogenetically detectable abnormality. These include: (1) CT8M. Although individuals with this condition have an increased risk of myeloid malignancies, only a minority develop such disorders.⁹¹ Furthermore, AML/MDS do not occur until the affected persons are several years of age,^{92,96} strongly suggesting that a latency period, during which additional genetic changes may arise, is necessary for leukemogenesis. (2) The clonal origin of MDS with trisomy 8 as the sole aberration. Although the hematopoietic stem cell pool in +8-positive MDS cases contains a sizable fraction of cells with disomy 8, these cells carry intrinsic deficiencies, indicating that they are part of the malignant clone. Thus, trisomy 8 appears to occur late in MDS and is hence not the primary transformation event.¹⁰⁹ (3) The gene

expression signature. Schoch et al.²⁸ reported that the discriminating gene expression pattern of AML with isolated trisomy 8 was not dependent on the upregulation of chromosome 8 genes alone, implying the presence of additional genetic changes. (4) +8 as a secondary change. The fact that trisomy 8 is a common secondary aberration in AML and MDS, as well as frequently constituting one of the abnormal clones in polyclonal cases,^{2,110,126} suggests that it may be involved in leukemia progression rather than in transformation. (5) Finally, the heterogeneity of +8-positive leukemia. AML/MDS cases with trisomy 8 as the sole cytogenetic aberration differ with regard to clinical factors, morphology, and gene expression patterns.^{72,117} This heterogeneity may well be explained by different underlying cryptic genetic changes.

Studies to identify hidden abnormalities

Several different FISH investigations have been focused on finding cryptic chromosome aberrations in AML and MDS with trisomy 8 as the sole cytogenetic abnormality. In total, 20 AMLs and MDSs with isolated +8 have been investigated with M-FISH or SKY,^{1,127-129} including the 12 cases in article I; only one of these was shown to harbor an additional anomaly. This case was an AML displaying poor G-banding morphology, in which a t(7;14)(q3?1;q2?2) was detected by SKY analysis, but not further characterized.¹²⁷ Also, Brown et al.¹³⁰ studied one AML with +8 with their subtelomeric multicolor-FISH analysis, utilizing probes for all subtelomeric chromosome regions, but no cryptic abnormality was detected. To address the possibility of structural anomalies within chromosome 8 itself, FISH assays with PCP and subtelomeric probes for 8p and 8q, as well as with gene-specific probes for *FGFR1*, *MYST3* (previously *MOZ*), *RUNX1T1* (previously *ETO*), and *MYC* were performed in the twelve AML/MDS cases included in article I. No hidden aberrations were detected. Finally, Heller et al.¹³¹ used multicolor banding to study chromosome 8 in 11 AMLs/MDSs with this trisomy, of which eight had it as the sole aberration, but all homologues were apparently normal. Thus, various FISH techniques have not yielded any evidence for cytogenetically cryptic rearrangements in the vast majority of +8-positive AML and MDS.

Molecular methods aimed at finding additional abnormalities include Southern blot, reverse transcriptase-PCR (RT-PCR), and single nucleotide polymorphism (SNP) arrays. For example, Diaz et al.¹³² used the first of these techniques to investigate the *MYC* and *MOS* genes in 6 MDS cases with isolated trisomy 8 and 4 AMLs with +8 in addition to

other changes, whereas Langabeer et al.¹³³, recognizing that +8 is a common secondary change in AML with t(15;17) (Table 3), analyzed 54 trisomy 8-positive AMLs for the presence of cryptic *PML/RARA* fusions with RT-PCR. Also, the possibility of partial UPD was addressed by Raghavan et al.²¹ in two AMLs with trisomy 8. Apart from one MDS showing a rearranged *HindIII* fragment for *MYC* – not further investigated – in the study by Diaz et al.¹³², none of these studies identified any hidden abnormalities.

Studies of the presence of somatic point mutations of leukemia-associated genes have proved somewhat more fruitful. Thus, several AMLs/MDSs with +8 as the sole cytogenetic aberration have been reported to harbor, e.g., *CEBPA*, *FLT3*, *KRAS*, *NRAS*, and *RUNX1* mutations.¹³⁴⁻¹³⁷ However, none of these abnormalities have been specifically associated with, or particularly frequent in, +8 cases.

Finally, array CGH analysis has been used to investigate 10 AMLs/MDSs with trisomy 8 as the sole cytogenetic aberration.¹¹ Interestingly, this assay detected cryptic intra-chromosomal imbalances, not corresponding to known CNPs in the human genome, in 5 of the 10 cases (in 1 MDS and 4 AMLs). These changes, some of which were confirmed by FISH, included both segmental duplications and hemizygous deletions and involved several different chromosomes, although not chromosome 8. Most notably, at least two of the identified imbalances were almost certainly leukemia-associated: a del(7)(p14p14), shown to have occurred before the trisomy 8, and a hemizygous deletion of the region surrounding *ETV6* in 12p13; an aberration which previously has been described in AML. Hence, this study indicated that additional cryptic chromosome abnormalities may indeed be present at a high frequency in AML and MDS with +8 as the sole cytogenetic aberration.

Conclusions and future directions

What are the functional outcome and the pathogenetic effect of trisomy 8 as the sole aberration in AML and MDS? Recent microarray studies suggest that +8 is associated with specific gene expression patterns, at least partly associated with dosage effects of chromosome 8 genes.^{28,114,117} It is conceivable that such a deregulation is associated with leukemia, and this may well be the direct biologic effect of the trisomy. However, the above-mentioned possibility of cryptic additional anomalies in myeloid malignancies with isolated trisomy 8 is an issue of great clinical and pathogenetic importance. In this context, the findings described in article II are important. Although none of the identified

aberrations in that study were obvious primary, leukemia-inducing anomalies, their identification nevertheless provided the first solid evidence for a high frequency of additional chromosome abnormalities in AML/MDS with trisomy 8 as the sole cytogenetic aberration. Future investigations using array-based CGH or similar methods will determine whether the detected incidence – 4/6 (67%) AMLs and 1/4 (25%) MDSs – of cases having hidden intra-chromosomal imbalances is representative for this cytogenetic subgroup. Furthermore, although rearrangements involving large chromosome parts can be excluded to be common in AML/MDS with isolated +8, based on previous FISH investigations,^{1,127-129} intra-chromosomal rearrangements and translocations involving subtelomeric parts remain a possibility. Also, the recent findings of partial UPD in AML cases with other abnormalities is intriguing,²¹ warranting further studies of AMLs and MDSs with trisomy 8 as the seemingly sole anomaly. In summary, many important issues remain to be addressed regarding this frequent chromosome aberration.

HYPERDIPLOIDY IN CHILDHOOD ALL

High hyperdiploidy, i.e., more than 50 chromosomes, is the most common cytogenetic pattern in childhood ALL.² Hence, the elucidation of its genetic and clinical features, origin, and pathogenetic effects is of great importance. Below, the present knowledge of hyperdiploid childhood ALL is summarized, including the results presented in articles III and IV.

Cytogenetic findings

The hyperdiploid cytogenetic subgroup in childhood ALL is characterized by the presence of extra chromosomes, resulting in modal numbers of >50 chromosomes. The gains are nonrandom, and typically involve chromosomes X, 4, 6, 8, 10, 14, 17, 18, and 21. Chromosome 21 is frequently tetrasomic and sometimes pentasomic, whereas the other imbalances are usually found in the form of trisomies, although occasional tetrasomies are seen. Not all tri-/tetrasomies are equally common: gain of chromosome 21 is seen in virtually all hyperdiploid pediatric ALLs, whereas the remaining imbalances are present in 60 – 90%, except +8, which occurs with a frequency of 40% (Table 5).

The G-banding morphology is often poor in the hyperdiploid subgroup; thus, misclassification of chromosomes is quite common. A

comparison between the frequencies of gained chromosomes in cytogenetic and FISH investigations shows similar incidences, but with a trend towards higher rates of most trisomies/tetrasomies in cases analyzed with FISH (Table 5). Also, the G-banding karyotypes reported by Moorman et al.¹³⁹ – including more than half of the cytogenetically investigated cases in Table 5 – were reviewed centrally, with cases displaying incomplete karyotypes being excluded; G-banding results in general may be expected to be less accurate. Thus, it seems safe to conclude that FISH analyses are usually superior for identification of gained chromosomes in hyperdiploid cases.

In addition to the tri- and tetrasomies, G-banding analysis of hyperdiploid childhood ALL reveals structural changes in approximately half of the cases (Table 5). Although such abnormalities may go undetected due to poor chromosome morphology, this frequency is not increased when cases are investigated with SKY or M-FISH (Table 5). The most common structural change in hyperdiploid childhood ALL is duplication of 1q, which is present in 8 – 10% of the cases, followed by other 1q abnormalities (6%), deletions involving 6q (5 – 7%), and isochromosome 17q (2 – 5%).^{2,139} Translocations are seen in 20% of the cases, including a small subset of cases with well-known primary ALL rearrangements such as t(1;19)(q23;p13) and t(9;22)(q34;q11).¹⁴⁰

Hyperdiploid childhood ALL is generally karyotypically stable, i.e., these cases display little or no cell-to-cell variation when investigated with cytogenetic techniques. Teixeira and Heim¹⁴⁵ found evidence for clonal evolution in only 5% of the cases in a review of all published hyperdiploid ALLs. However, because of the poor chromosome morphology in many of the reviewed studies, the actual frequency may be higher.¹⁴⁵ Approximately 20% of the cases reported by Raimondi et al.¹⁴⁰ harbored subclones, which usually differed from the major clone by two to three additional chromosomes or structural rearrangements. This is in line with the frequencies detected by SKY/M-FISH; two such studies have reported cytogenetic subclones in 2/18 (11%)¹⁴² and 8/27 (30%)^{IV} cases, respectively. However, it should be stressed that hyperdiploid childhood ALL does not display the general CIN seen in many aneuploid solid tumors.

Epidemiology and etiology

Hyperdiploidy has been reported to occur in 20 – 30% of all pediatric ALL cases, making it the largest cytogenetic subgroup.^{26,139,146-149} It has been suggested that the frequencies of hyperdiploid childhood ALL vary

Table 5. Comparison between chromosome aberrations detected in G-banding and FISH analyses of hyperdiploid childhood ALL^a

<i>Chromosome aberration</i>	<i>Cytogenetic investigations^b</i> (% of the cases)				<i>FISH investigations^b</i> (% of the cases)				
	<i>Raimondi et al.¹⁴⁰ (n=182)</i>	<i>Heerema et al.¹⁴¹ (n=480)</i>	<i>Moorman et al.¹³⁹ (n=700)</i>	<i>Mean</i>	<i>Moorman et al.¹³⁸ (n=146)</i>	<i>Elghezel et al.¹⁴² (n=18)</i>	<i>Nordgren et al.¹⁴³ (n=15)</i>	<i>Nordgren et al.¹⁴⁴ (n=13)</i>	<i>Paulsson et al.^{IV} (n=27)</i>
Gain of X	81	88	89	86	95	100	100	100	81
Gain of 4	76	74	78	76	75	67	60	54	74
Gain of 6	86	85	85	85	86	78	87	69	81
Gain of 8	34	37	-	36	-	39	33	31	52
Gain of 10	56	63	63	61	61	78	47	54	74
Gain of 14	80	81	84	82	90	100	93	100	93
Gain of 17	68	67	68	68	65	67	93	92	70
Gain of 18	68	73	76	72	78	78	73	54	81
Gain of 21	97	98	99	98	100	100	100	100	100
Tetrasomy 21 ^c	66	69	70	68	-	83	53	77	85
Structural changes	54	-	53	54	-	39	33	38	50 ^d

FISH, fluorescence in situ hybridization; ALL, acute lymphoblastic leukemia; SKY, spectral karyotyping; M-FISH, multicolor-FISH.

^aIncluding cases with 51 – 65 chromosomes, except for Raimondi et al.¹⁴⁰ (51 – 67 chromosomes) and Heerema et al.¹⁴¹ (>50 chromosomes).

^bAnalyzed with SKY, except for Moorman et al.¹³⁸ (analyzed with interphase FISH using probes for chromosomes X, 4, 6, 10, 14, 17, 18, and 21) and Paulsson et al.^{IV} (analyzed with M-FISH in ten cases and with interphase FISH using probes for chromosomes X, 4, 6, 8, 10, 14, 17, 18, and 21 in seventeen cases).

^cMay include occasional cases with pentasomy 21.

^d% of the ten cases investigated with M-FISH.

among different populations; for example, a higher incidence has been noted in the Nordic countries.^{26,146,150} Hyperdiploidy is strongly associated with young age, being most common in children below the age of ten, with a median age of 4 years at diagnosis.^{140,147,148,151,152} There is no gender-related frequency difference.^{140,148}

Little is known about environmental risk factors for hyperdiploid childhood ALL. As described below, this cytogenetic feature appears to arise already in utero,¹⁵³⁻¹⁵⁷ suggesting that exposure to transforming agents during pregnancy may be of importance. Wiemels et al.¹⁵⁸ reported that hyperdiploidy is significantly less common in children prenatally exposed to paternal smoking. They hence speculated that smoking-associated mutagens may be toxic to hyperdiploid clones,¹⁵⁸ but further studies are needed to clarify this issue. Also, Hjalgrim et al.¹⁵⁹ reported that an elevated birth weight increased the risk for hyperdiploid childhood ALL. However, this risk factor seemed to be associated with pediatric ALL in general and not specifically with the hyperdiploid subtype.¹⁵⁹

An increased risk for childhood ALL, albeit not specifically for the hyperdiploid subtype, has been associated with low folate intake during pregnancy.¹⁶⁰ Interestingly, folate deficiency has been shown to induce aneuploidy of chromosomes 17 and 21 in cultured lymphocytes, suggesting a possible association between disturbances in this metabolic pathway and hyperdiploidy.¹⁶¹ Furthermore, results by Wiemels et al.¹⁶² indicated that constitutional homozygosity for polymorphisms in the *MTHFR* gene, encoding an enzyme active in the folate pathway, may be less common among hyperdiploid childhood ALL cases. Taken together, the possibility of an association between folate metabolism and hyperdiploidy is interesting, warranting further investigations.

Clinical features

Hyperdiploidy in childhood ALL is associated with favorable prognostic factors, including low WBC ($<10 \times 10^9/l$) and age between 1 to 9 years.^{140,146-149,151,152} Involvement of the central nervous system is seen in less than 5% of the cases and the presence of a mediastinal mass is rare.^{140,141,148,152,163,164} Furthermore, hyperdiploidy is associated with an early pre-B immunophenotype,¹⁵¹ typically $CD34^+CD10^+CD19^+CD45^-CD22^+CD24^+TdT^+IgM^-CD66c^+$.⁷⁹

Prognostic impact

The hyperdiploid cytogenetic subgroup is associated with a favorable outcome in childhood ALL, first described by Lampert¹⁶⁵ as early as in 1967 and confirmed in later studies.^{147,149,163,166-169} Thus, patients within this karyotypic subgroup have an overall survival of approximately 90%.¹³⁹

Several studies have addressed the possibility that specific trisomies may have a prognostic impact. In the first of these, Jackson et al.¹⁷⁰ found that +6 was associated with a favorable prognosis; however, that investigation also included cases with 47 – 50 chromosomes, and was hence not restricted to the hyperdiploid subgroup. Subsequently, Harris et al.¹⁷¹, analyzing 1,000 children with pre-B ALL, detected increased event-free survival for cases with +4 and +10; an association that was not seen in a later study of 182 hyperdiploid ALLs by Raimondi et al.¹⁴⁰ Heerema et al.¹⁴¹ concluded, in an investigation of 480 ALLs with >50 chromosomes, that whereas +4 and +6 did not affect the prognosis, concurrent +10 and +17 were associated with a superior outcome and +5 conferred a poor prognosis. Furthermore, Moorman et al.¹³⁹ reported an association between improved outcome and trisomies 4 and 18 among 700 cases of hyperdiploid childhood ALL, and Sutcliffe et al.¹⁷², investigating +4, +10, and +17 in a large study of >5,000 hyperdiploid cases, found that these trisomies were associated with a favorable prognosis. Although these results seem conflicting, most studies have detected specific effects of trisomies 4, 10, 17, and 18 in univariate analyses, but the conclusions drawn from the multivariate analyses have differed.¹³⁹ Thus, it seems likely that certain trisomies might affect the prognosis in hyperdiploid childhood ALL, but more investigations are needed to clarify this clinically important issue.

It has also been suggested that the modal number may be prognostically important in hyperdiploid cases. Thus, patients with >53 or >55 chromosomes have been reported to fare better than those with 51 – 53/55 chromosomes.¹³⁹⁻¹⁴¹ However, a recent study have shown that this effect cannot be separated from the above-mentioned impact of specific trisomies.¹³⁹

Pui et al.¹⁶⁴ reported an inferior outcome for cases with structural chromosome changes in addition to the hyperdiploid karyotype. This has not, however, been confirmed in subsequent, larger studies, with the exception of the rare hyperdiploid cases carrying t(1;19), t(8;14), t(9;22), or translocations involving 11q23.^{139,140,163} Specifically, isochromosome 17q has been suggested to be an adverse

cytogenetic feature in hyperdiploid childhood ALL, but in recent investigations, no such effect has been seen.^{139,140,163,173}

What is the reason for the favorable outcome of hyperdiploid childhood ALLs as compared to other cases? Several investigators have shown that the blast cells from these ALLs are particularly sensitive to chemotherapy, especially to methotrexate (MTX).¹⁷⁴⁻¹⁷⁸ This sensitivity may be associated with gains of chromosome 21, because the *SLC19A1* (previously *RFC1*) gene, encoding a protein that transports MTX into the cell, is located at 21q22.¹⁷⁷⁻¹⁷⁹ Trisomy/tetrasomy 21 has been shown to upregulate *SLC19A1*, presumably resulting in higher concentrations of MTX in hyperdiploid blast cells.^{177,178} Furthermore, it has been shown that hyperdiploid cells have an increased propensity to undergo apoptosis, both in vitro in stromal cultures and in vivo in bone marrows.¹⁸⁰⁻¹⁸² Thus, the superior prognosis of this cytogenetic subgroup in childhood ALL may be due to a combination of high sensitivity to chemotherapy in association with an increased incidence of spontaneous apoptosis.

Cell of origin

Larramendy et al.¹⁸³ investigated five cases of hyperdiploid childhood ALL by the MAC technique and found that the extra chromosomes were restricted to cells of the lymphoid lineages in all but one of the cases. The latter ALL displayed hyperdiploidy in a subset of the erythroid cells, indicating that this cytogenetic feature may have arisen in a pluripotent hematopoietic stem cell.¹⁸³ However, Kasprzyk et al.¹⁸⁴, investigating B-lineage (CD19⁺), T-lineage (CD3⁺), myeloid (CD13⁺), and erythroid (glycophorin A⁺) cells in nine cases, detected hyperdiploidy only in the first of these groups. They hence suggested that the chromosome gain arose in a lymphoid committed progenitor cell, although occurrence at the pluripotent stem cell level could not be excluded.¹⁸⁴ Quijano et al.¹⁸⁵ reported that hyperdiploidy was present in a subset of the CD34⁺CD33⁻CD38⁻CD19⁻ compartment – i.e., in CD34⁺, but lineage negative, cells – in 3/12 investigated cases, thus indicating the latter. Taken together, the analyses performed so far suggest that hyperdiploidy arises in a lineage uncommitted stem cell, possibly without the ability to form myeloid cells.¹⁸⁴ However, this issue is not yet definitively clarified.

Hyperdiploidy arises in utero

That some childhood leukemias may arise already in utero was first suggested by reports of twins with concordant ALL,¹⁸⁶ and later directly

demonstrated by Gale et al.¹⁸⁷, who showed that the *MLL/AFF1* (previously *AF4*) fusion transcript, resulting from the t(4;11)(q21;q23), was present in neonatal blood spots (Guthrie cards) from patients who later developed ALL with this translocation. Subsequently, several cytogenetic abnormalities associated with childhood leukemia have been shown to occur before birth.¹⁸⁶

The first indication that hyperdiploidy may be included in this group came from an investigation by Yagi et al.¹⁵³, who screened neonatal blood spots from patients who later developed ALL for the presence of the clonotypic somatic recombination of *IGH@*, the heavy immunoglobulin gene at 14q32. One of the positive cases was a hyperdiploid ALL, diagnosed when the patient was two years old.¹⁵³ Similar results were subsequently reported by Taub et al.¹⁵⁴, who found clonotypic *IGH@* rearrangements in 6/6 Guthrie cards from patients with such ALL. However, from these findings, it could not be confirmed that the detected clones harbored hyperdiploidy at the time of birth; this cytogenetic feature could have arisen later.¹⁵⁴

Szczpansky et al.¹⁸⁸ reported that ALL with trisomy 14 sometimes displayed three different *IGH@* rearrangements, but they did not investigate the possibility of a prenatal event. Instead, this was done by Panzer-Grümayer et al.¹⁵⁵, who detected three clonotypic *IGH@* rearrangements, all derived from a single abnormal clone, in the Guthrie card from one patient with hyperdiploid, +14-positive ALL. Thus, they provided the first solid evidence for a prenatal origin of hyperdiploidy – or at least for trisomy 14 – albeit in a single case. Subsequently, a pair of monozygotic twins with concordant hyperdiploid ALL, having identical chromosome aberrations and shared *TRDJ1/TRDD3* (previously *TCRD*) and *IGH@* rearrangements, was reported by Maia et al.¹⁵⁶, again demonstrating that the hyperdiploidy arose in utero. More recently, the presence of +15 and +17 was demonstrated in CD34⁺CD19⁺ cord blood cells from a child who later developed a hyperdiploid ALL with these trisomies.¹⁵⁷ Taken together, these findings show that hyperdiploidy is a prenatal event in at least some cases of childhood ALL, and provide strong support for the possibility that all such cases arise in utero.

How does hyperdiploidy arise?

As described above, hyperdiploidy in pediatric ALL is probably acquired already in utero, in an immature B-lineage cell. How, then, do these multiple gains of chromosomes happen? Theoretically, hyperdiploidy may arise by four different mechanisms: (1) by initial near-haploidy

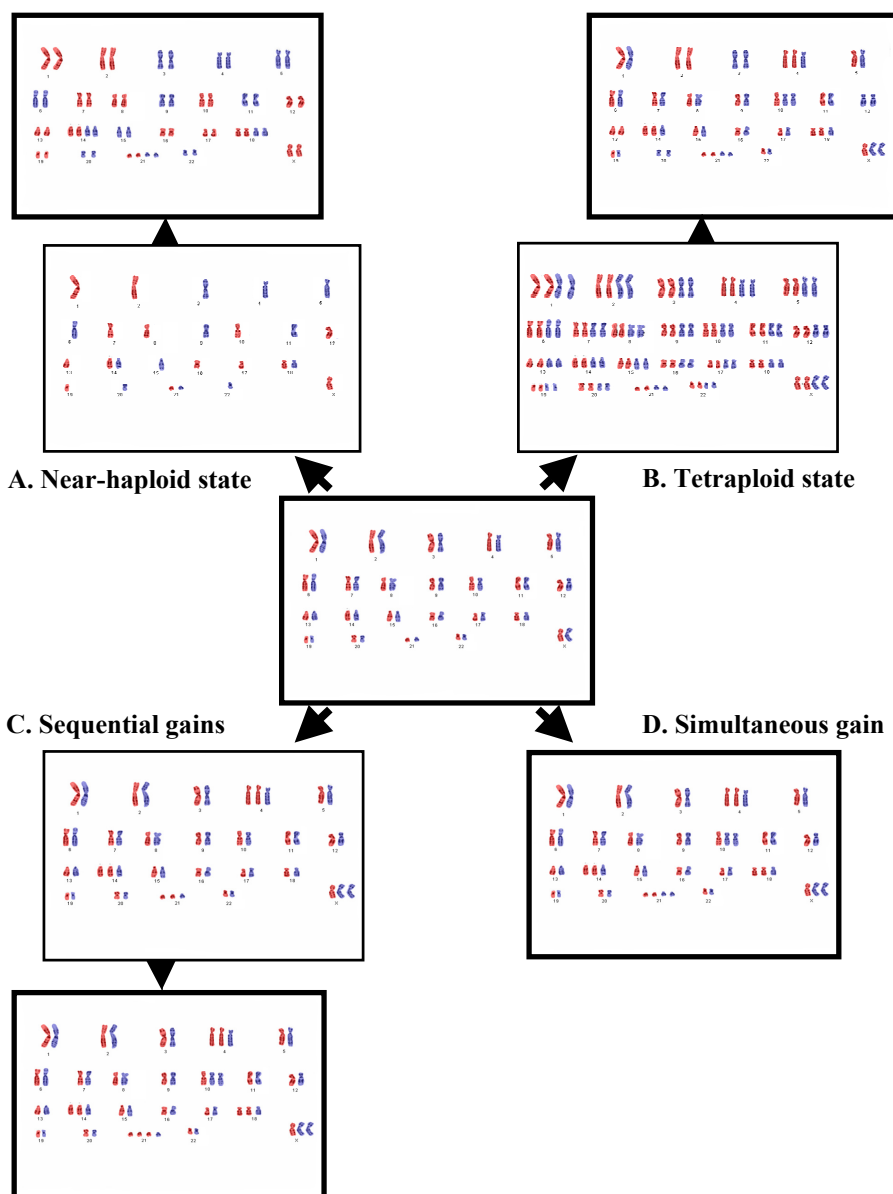


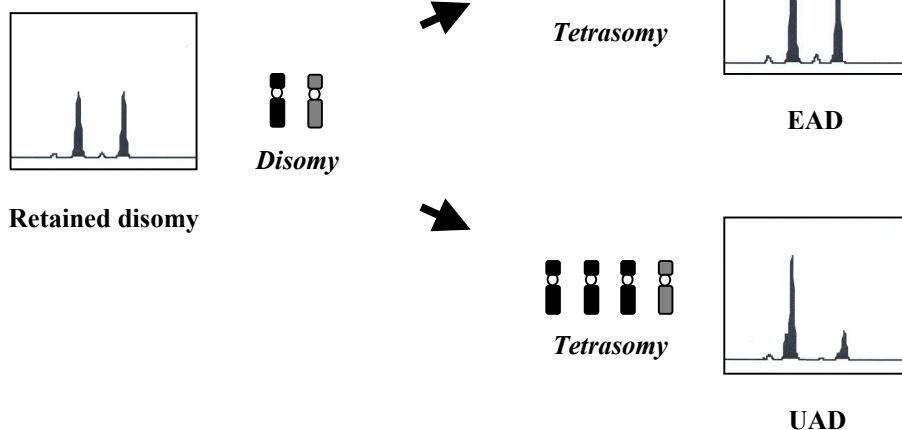
Figure 3. Four possible mechanisms for the formation of hyperdiploidy. UPD, uniparental disomy; EAD, equal allele dosage; UAD, unequal allele dosage. (A) Doubling of a near-haploid set of chromosomes, resulting in UPDs for all disomic chromosomes and EAD for tetrasomies. (B) Initial tetraploidization with subsequent losses of chromosomes, resulting in UPDs for approximately one third of the disomic chromosomes and EAD for tetrasomies. (C) Sequential gains of individual chromosomes in consecutive cell divisions, resulting in no UPDs and UAD for two thirds of the tetrasomies. (D) Simultaneous gain of chromosomes in a single abnormal mitosis, resulting in no UPDs and EAD for all tetrasomies.

followed by doubling of the chromosomes, (2) by tetraploidization with subsequent chromosome losses, (3) by sequential gains of chromosomes in consecutive cell divisions, or (4) by a simultaneous gain of chromosomes in a single abnormal mitosis (Fig. 3).¹⁸⁹ Considering that we cannot directly observe the transformation event, the evidence for and against these mechanisms is indirect. However, investigations of the allele dosage for genes in tetrasomic chromosomes, as well as of the presence of UPDs, may provide valuable clues (Fig. 4). In total, three studies addressing the mechanism behind hyperdiploidy have been published. These include one investigation by Onodera et al.¹⁸⁹ of 15 hyperdiploid childhood ALLs using restriction fragment length polymorphisms and articles III and IV, utilizing QF-PCR with polymorphic microsatellite markers to study 10 and 27 cases, respectively.

Starting with the tetrasomies, these may display either equal (EAD) or unequal (UAD) allele dosage (Fig. 4A). The former is a 2:2 allele ratio for all loci at that chromosome, resulting from duplication of both homologues, whereas the latter is a 3:1 ratio for all loci, because of triplication of one homologue and retention of the other (Fig. 4A). Experimental evidence indicates that EAD for tetrasomies is an almost universal feature of hyperdiploid childhood ALL.^{189,III,IV} Thus, tetrasomy 21, which is the most common tetrasomy and also the most well-investigated, has been shown to display EAD in 31 of 33 (94%) cases.^{189,III,IV} It should be noted that the +21,+21 in the two cases that had UAD were identified with G-banding only and it is hence possible that they had an unrecognized pentasomy for chromosome 21. The only study that included FISH to confirm all gains – article IV – did not detect any cases with unequal allele dosage for +21,+21. As regards other tetrasomies, fifteen such changes were investigated in article IV, involving chromosomes X, 8, 10, 14, and 18. Thirteen (87%) displayed equal allele dosage. The two exceptions – +8,+8 and +14,+14 – were present in the same case.^{IV} It should also be mentioned that Haas et al.¹⁹⁰ investigated seven ALLs with tetrasomy X by a FISH X inactivation assay; all had duplicated both the active and the inactive X, i.e., the equivalent of EAD. Thus, of the altogether 55 tetrasomies that have been analyzed in hyperdiploid childhood ALL cases, 51 (93%) have displayed duplication of both homologues, as opposed to triplication of one homologue (Fig. 4A).^{189,190,III,IV}

Clues to the mechanism behind hyperdiploidy from disomic chromosomes are given by the possible occurrence of UPDs, i.e., both

A. Tetrasomies



B. Disomies

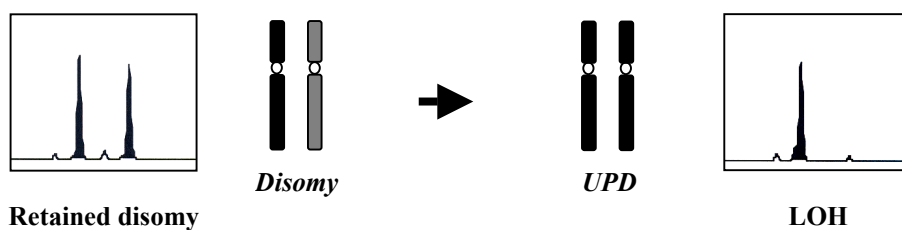


Figure 4. QF-PCR findings for aberrant chromosomes. Representative electropherograms are shown next to chromosomes. Each peak represents an allele for a polymorphic microsatellite marker. EAD, equal allele dosage; UAD, unequal allele dosage; UPD, uniparental disomy; LOH, loss of heterozygosity. (A) Tetrasomies may result either from the duplication of both homologues or from the triplication of one homologue. The former leads to EAD, visible as two peaks with a 2:2 ratio. The latter results in UAD, visible as two peaks with a 3:1 ratio. (B) UPD – both homologues being derived from the same parental chromosome – is visible as LOH, i.e., complete loss of one peak.

homologues being derived from a single parental chromosome (Fig. 4B). This is detectable as loss of heterozygosity (LOH) for all loci at that chromosome pair (Fig. 4B). In the study by Onodera et al.¹⁸⁹ and in articles III and IV, a subset of all disomic chromosomes was investigated for the presence of UPD. Apart from three cases, further discussed

below, in which UPDs were seen for all disomic chromosomes, only a handful of UPDs were detected among the 52 cases included in these three studies.^{189,III,IV} Furthermore, in article IV, a systematic investigation of all disomic chromosomes in ten cases was performed, revealing one case with UPDs for 4/9 disomies, one with UPDs for 2/14 disomies, and one with UPD for 1/10 disomies. Finally, Irving et al.¹⁹¹ studied all chromosomes in three cases of hyperdiploid childhood ALL with SNP arrays. They detected UPDs for 4/19 disomic chromosomes in one case.¹⁹¹ Taken together, hyperdiploid childhood ALLs display different frequencies of UPDs, with the majority of the cases having no UPDs, a subset harboring a few UPDs, and a subset displaying UPDs for all disomic chromosomes.^{189,191,III,IV}

The mechanism behind hyperdiploidy

The first of the above-mentioned mechanisms for formation of a hyperdiploid karyotype involves a near-haploid step (Fig. 3A). Near-haploidy of 23 – 29 chromosomes is a rare but recurrent cytogenetic feature of childhood ALL, occurring in less than 1% of the cases.^{2,192} It is characterized by a specific chromosome pattern, with most cases retaining disomies 21, X, Y, 14, and 18, and having monosomies for the remaining chromosomes.¹⁹² This cytogenetic subgroup is associated with a poor prognosis.¹⁹² Already in 1977, Oshimura et al.¹⁹³ reported a case of near-haploid ALL with 27 chromosomes that harbored a second clone containing an exact duplicate of the chromosomes present in the first. Subsequent investigations have shown that such related clones are common in the near-haploid subgroup of childhood ALL; e.g., in 9/14 (64%) of the cases reported by Harrison et al.¹⁹² This raised the possibility that hyperdiploid cases may have originated as near-haploid, with subsequent duplication of chromosomes and loss of the original stem line, at least at the cytogenetic level.¹⁹⁴

By molecular genetic techniques, hyperdiploidy arising via a near-haploid state would be detectable by having UPDs for all disomic chromosomes and EAD for all tetrasomic chromosomes (Fig. 3A).¹⁸⁹ The first experimental evidence for occurrence of this mechanism was presented by Stamberg et al.¹⁹⁵, who described a hyperdiploid childhood ALL characterized by tetrasomies only and with a reduction from heterozygosity to homozygosity for a chromosome 15 polymorphism. Subsequently, Onodera et al.¹⁹⁶ reported that all loci at disomic chromosomes displayed LOH, indicative of UPDs, in two hyperdiploid cases; a similar case is also described in article IV. Furthermore, FISH studies of

apparently hyperdiploid cases have revealed near-haploid clones in inter-phase cells in at least four cases.^{197,198} Hence, a subset of hyperdiploid childhood ALLs originates through near-haploidy.

Does the above mechanism explain all hyperdiploid cases in this disorder? The short answer to that question is “no”. As described above, molecular studies performed by Onodera et al.¹⁸⁹ and Irving et al.¹⁹¹ and those presented in articles III and IV have shown that the vast majority of disomic chromosomes in hyperdiploid cases display retained heterozygosity, excluding a near-haploid pathway. Only three of a total of 55 investigated cases had UPD for all disomies.^{189,III,IV} Thus, most hyperdiploid cases must be formed by another mechanism. It should also be stressed that the few cases that arise via a near-haploid state should probably be assigned to the near-haploid cytogenetic subgroup and not associated with the “true” hyperdiploid cases. Considering the inferior prognosis of near-haploid pediatric ALL,¹⁹² it is extremely important to identify these cases, e.g., by being observant for hyperdiploid ALLs displaying only tetrasomies and no trisomies.

This leaves three possible mechanisms for the occurrence of “true” hyperdiploidy in childhood ALL (Fig. 3B – D). In the first of these alternatives, the cell initially becomes tetraploid and then loses chromosomes until it is hyperdiploid (Fig. 3B). Hyperdiploidy formed via a tetraploid state would display EAD for all tetrasomies and UPDs for approximately one third of the disomic chromosomes (Fig. 3B). As mentioned above, almost all tetrasomic chromosomes in hyperdiploid childhood ALL display EAD,^{189,190,III,IV} in agreement with what be expected for this pathway. UPDs are less common.^{189,191,III,IV} However, in studies including all disomic chromosomes, a subset of cases has been shown to harbor some UPDs.^{191,IV} Thus, in article IV and the study by Irving et al.¹⁹⁹, this mechanism could not be excluded in 3/10 and 1/3 cases, respectively. Taken together, it is possible that some cases of hyperdiploidy arise by initial tetraploidization with subsequent losses of chromosomes.

Another putative pathway to hyperdiploidy is by sequential gains of chromosomes in consecutive mitoses (Fig. 3C). This is an attractive possibility, because it would fit well with our current view of cytogenetic clonal evolution in malignancies. However, it is not supported by findings at the cytogenetic level, i.e., hyperdiploid ALL rarely harbor subclones when investigated with G-banding.^{140,145} At the molecular level, hyperdiploidy formed by this pathway is expected to display UAD for approximately two thirds of the tetrasomic chromo-

somes and no UPDs (Fig. 3C). Although the latter agrees well with the findings in the majority of the analyzed cases, the former does not; UAD has only been detected for 7% of tetrasomies in hyperdiploid childhood ALL, and not all of these chromosomes have actually been confirmed to be tetrasomic by FISH.^{189-191,III,IV} Thus, this mechanism is highly unlikely for most cases. However, a possible exception exists; the one case described in article IV in which 2/2 tetrasomies displayed UAD. In this single case, formation by sequential gains of chromosomes may have occurred.^{IV}

The final possible pathway for formation of hyperdiploidy is by a simultaneous gain of all extra chromosomes in a single abnormal cell division (Fig. 3D). Such cases would display EAD for all tetrasomies and no UPDs. This pattern agrees well with most molecularly investigated hyperdiploid childhood ALLs; 51/55 (93%) tetrasomic chromosomes have shown EAD and the vast majority of the disomies have not been UPDs.^{189-191,III,IV} Hence, it is likely that most cases of hyperdiploidy in childhood ALL arise through a simultaneous gain of chromosomes.^{189,III,IV}

Taken together, 55 cases of hyperdiploid childhood ALL have been sufficiently analyzed with molecular techniques to determine by which pathway they were formed. Three of these cases (5%) displayed all the expected features of hyperdiploidy arising via a near-haploid state. Four cases (7%) may have arisen through a tetraploid state, although sequential gains or a simultaneous gain are also possible in at least two of these. One case (2%) most likely originated by a sequential gain of chromosomes. Seven cases (13%) were shown to have arisen by a simultaneous gain of chromosomes. Finally, the remaining 40 cases (77%) displayed patterns of EAD/UAD and UPDs strongly suggesting a simultaneous gain. However, formation via a tetraploid state or by sequential gains could not be definitely excluded, although they were highly unlikely based on the combined molecular findings. Thus, different childhood ALL cases may have hyperdiploidy originating by different mechanisms. This may be clinically important – for example, the prognosis for hyperdiploid cases arising via a tetraploid state or by sequential gains could possibly be inferior to that of cases occurring through a simultaneous gain of chromosomes in a single abnormal mitosis. Studies of clinical features associated with hyperdiploidy originating by different pathways are therefore warranted.

What is the pathogenetic impact of hyperdiploidy?

Like for other trisomies in hematologic malignancies, little is known about the biologic importance of hyperdiploidy in leukemogenesis. Possible pathogenetic effects include duplication of mutated loci on the gained chromosomes, deregulation of imprinted loci, and gene dosage effects. Furthermore, additional genetic alterations besides the hyperdiploidy may be needed for the development of ALL.

Hyperdiploidy – sufficient for leukemogenesis?

As described in detail above, several lines of evidence – including clonotypic *IGH@* rearrangements, concordant hyperdiploid ALL in twins, and the presence of hyperdiploid cells in cord blood – strongly suggest that hyperdiploidy frequently, possibly always, arises before birth.¹⁵³⁻¹⁵⁷ However, the twelve patients in whom a prenatal origin has been demonstrated have had a median age at diagnosis of 2.4 years, with the oldest being 9 years old.¹⁵³⁻¹⁵⁷ Also, the incidence of hyperdiploidy in childhood ALL is highest in the 1 – 9 years age group.^{140,148,151} This strongly suggests that hyperdiploidy is not sufficient for leukemogenesis; additional genetic aberrations are needed for leukemia to develop.

What are these changes? Structural abnormalities are present in approximately half of all hyperdiploid childhood ALLs (Table 5). The most common of these are unbalanced, such as duplication of 1q, deletions of 6q, and isochromosome 17q, and although they may certainly be important in the leukemogenic process, none of them are present in more than a few percent of the cases.^{2,139} Within the hyperdiploid subgroup, structural aberrations have been reported to be associated with an increased age,¹⁴⁸ possibly suggesting a different etiology in these cases. However, cytogenetic as well as M-FISH and SKY analyses have failed to detect any primary fusion gene-forming rearrangements in the majority of hyperdiploid ALLs.^{139,140,142-144,IV} In contrast, mutations and deregulation of specific, leukemia-associated genes have been shown to occur with high frequencies in this cytogenetic subgroup. Thus, Taketani et al.²⁰⁰ and Armstrong et al.²⁰¹ demonstrated that activating point mutations in the *FLT3* gene are present in 20 – 25% of all cases with hyperdiploid childhood ALL, Tartaglia et al.²⁰² found an association between *PTPN11* mutations and this karyotypic feature, Wiemels et al.¹⁵⁸ detected mutations in the related *NRAS* and *KRAS* genes at a frequency of 30%, and Zheng et al.²⁰³ found that the promoter region of the putative tumor suppressor gene *FHIT* was hypermethylated, resulting in its inactivation, in more than half of the hyperdiploid cases. It

is noteworthy that the *FLT3*, *PTPN11*, *NRAS* and *KRAS* genes are all involved in the *RAS* signaling pathways, indicating that such deregulation may be a complementary event to hyperdiploidy in childhood ALL.¹⁵⁸ However, other genetic changes may also be required for leukemogenesis.

Pathogenetic impact – duplicated loci?

Some acquired trisomies in neoplasias have been shown to be associated with duplication of a mutated gene.⁴⁰⁻⁴³ However, this is unlikely to be the pathogenetic basis for hyperdiploidy, because mutated alleles would have to be present at each gained chromosome. Furthermore, the fact that the overwhelming majority of tetrasomies in hyperdiploid childhood ALL display EAD, i.e., duplication of both parental homologues,^{189,190,III,IV} strongly argues against duplication of a specific chromosome. Also, because hyperdiploidy generally arises prenatally,¹⁵³⁻¹⁵⁷ possible mutations would have to occur in utero, leaving little time for the, in this scenario, secondary gain of chromosomes. Hence, it seems highly unlikely that hyperdiploidy is generally associated with duplication of mutated genes.

Pathogenetic impact – imprinting?

That imprinting effects may be of importance in hyperdiploid childhood ALL was suggested by Haas⁴⁸, who speculated that gains of maternally or paternally derived chromosomes could result in deregulation of imprinted loci. This would be detectable as a skewed parental origin for the extra chromosomes.⁴⁸ To address this issue, ten hyperdiploid cases were investigated with polymorphic microsatellite markers for all commonly tri-/tetrasomic chromosomes in article III. None of the analyzed chromosomes displayed preferential duplication contributable to the parental origin, arguing against imprinting effects. However, trends towards significance were seen for chromosomes 8 and 14: 4/4 cases with +8 had gained the paternally inherited homologue ($P = 0.13$), whereas 7/8 cases with +14 had gained the maternally inherited homologue ($P = 0.07$). However, subsequent analyses of additional cases, reported by Wilson et al.⁴⁹ and in article IV, showed no such skewness. Thus, a total of 24 cases with trisomy 14 have been investigated; 14 of these had maternal origin of the duplicated chromosome ($P = 0.54$). Furthermore, of the eight analyzed cases with +8, six had paternal origin of the gained chromosome ($P = 0.29$). Hence, we can exclude that

imprinting effects related to the parental origin of the duplicated chromosomes are of importance in hyperdiploid childhood ALL.

UPDs may arise as a consequence of the mechanism behind the formation of hyperdiploidy, but they may also occur independently, with a possible pathogenetic impact. Because of the resulting allelic imbalance for imprinted loci, formation of UPDs could be the pathogenetic equivalent to duplicating a chromosome. Support for the presence of UPDs in hyperdiploid cases came from an LOH study of chromosome 6 in ALL; UPD for this chromosome was quite frequent and was found in at least one hyperdiploid case.²⁰⁴ To test if UPD is a common feature of hyperdiploid childhood ALL, all disomies for chromosomes X, 4, 6, 10, 14, 17, and 18 present in 27 cases, were analyzed in article IV. Only one of 29 such disomic chromosomes was a UPD, excluding the UPDs in a case that had originated via a near-haploid pathway. Furthermore, Irving et al.¹⁹¹ investigated all chromosomes in three cases and detected UPD for chromosomes 4 and 6 in one of these. However, this case also displayed UPD for chromosomes 12 and 20, and may have arisen via a tetraploid state. Hence, UPDs for the commonly trisomic chromosomes, not associated with a mechanism for hyperdiploidy that results in UPDs, are rare in hyperdiploid ALL.

Pathogenetic impact – gene dosage effects?

The first study of global gene expression in hyperdiploid ALL was performed by Yeoh et al.²⁰⁵, using a microarray platform containing 12,600 transcripts. Sixty-four of 327 investigated ALLs were hyperdiploid; these displayed a specific gene expression profile. Although Yeoh et al.²⁰⁵ did not report a general overexpression of genes on the gained chromosomes, 70% of the class-defining genes were localized to chromosomes 21 and X, which are frequently gained in hyperdiploid cases. Interestingly, the chromosome X genes were overexpressed also in cases without this trisomy, indicating upregulation by alternative mechanisms.²⁰⁵ In a further investigation of partly the same cases, Ross et al.²⁰⁶, using a microarray platform containing 39,000 transcripts, showed that hyperdiploid childhood ALL was characterized by overexpressed genes, upregulated by a factor of two. This was presumed to result from the gained chromosomes.²⁰⁶ In contrast, Qui et al.²⁰⁷ detected no overexpression of genes at the tri-/tetrasomic chromosomes in nine hyperdiploid cases, but a specific gene expression pattern was noted. Gruszka-Westwood et al.²⁹ used comparative expressed sequence hybridization to detect regions of up-regulation in hyperdiploid childhood ALL. They found that the gene

expression was correlated with copy number, with upregulation of genes at trisomic chromosomes and with genes at tetrasomies displaying higher expression than those at trisomies. Notably, not all regions in the gained chromosomes showed this pattern; instead, peaks of overexpression were seen. Furthermore, loci at disomic chromosomes were also downregulated.²⁰⁷

None of the above-mentioned studies addressed the biologic significance of the discriminating genes in the hyperdiploid subgroup, and data about this are therefore scarce. In a recent investigation, van Delft et al.²⁰⁸, analyzing 13 hyperdiploid childhood ALLs by gene expression microarrays, reported a common expression profile for such cases, those harboring the t(12;21)(p13;q22), and those with loss or gain of 12p or 21q, respectively. This may indicate that these cytogenetic subgroups affect the same pathways.²⁰⁸ Also, Kager et al.²⁰⁹ detected a specific expression pattern for genes involved in the folate pathway in hyperdiploid childhood ALL.

In conclusion, hyperdiploid cases display a unique gene expression signature compared to other cytogenetic subgroups of childhood ALL, reflecting, at least in part, upregulation of genes localized at the gained chromosomes. Little is known about the resulting effects on the biochemical pathways of the cell, and further studies are definitely warranted. However, the pathogenetic impact of hyperdiploidy in childhood ALL may well be associated with dosage effects resulting from the tri- and tetrasomies.

Conclusions and future directions

During the last decades, numerous investigations have addressed hyperdiploidy in pediatric ALL. Today, the clinical features have been described and the genetic patterns are being elucidated. We know that hyperdiploidy frequently – possibly always – arises before birth and that it usually does so by a simultaneous gain of chromosomes in a single abnormal mitosis. However, every new fact we learn about this cytogenetic subgroup seems to lead to more questions. What are the etiologic factors inducing hyperdiploidy already in utero? What are the cooperating genetic events needed for leukemogenesis? Do cases originating by different mechanisms have different biologic/clinical outcomes? Why are these specific chromosomes gained, especially if there is no gradual selection of chromosomes providing a proliferative advantage? Hopefully, future studies will address these questions and other enigmas concerning the hyperdiploid cytogenetic subtype in childhood ALL.

SVENSK SAMMANFATTNING (SUMMARY IN SWEDISH)

Hematologiska maligniteter är samlingsnamnet för ett stort antal cancersjukdomar som involverar blodet och den blodbildande benmärgen. De inkluderar bl a de *kroniska* och *akuta leukemierna*, som tillsammans årligen drabbar ca 1000 personer i Sverige. Kroniska leukemier förekommer huvudsakligen bland äldre personer, medan akut leukemi har en topp bland barn – där det är den vanligaste cancersjukdomen – och sedan uppvisar ökande incidens med åldern. Leukemier delas in i *myeloiska* och *lymfatiska*, beroende på vilka blodceller de involverar.

I princip alla celler i kroppen har 23 par *kromosomer*, som innehåller vår arvs massa – *generna*. Idag vet man att cancer beror på fel i cellens genetiska kontrollprogram. Detta innebär inte att all cancer är ärftlig – det är t ex mycket ovanligt med familjär förekomst av leukemi. Istället är det *förvärvade genetiska förändringar* som styr omvandlingen från en normal cell till en cancercell. Dessa avvikelser är inte medfödda, utan uppstår i en cell under individens levnad. Om en genetisk förändring innebär att cellen får en tillväxtfördel gentemot omkringliggande vävnad, så kommer dess dotterceller i ”nästa generation” att bli proportionerligt sett fler. Normalt är det immunförsvarets uppgift att ta hand om okontrollerad tillväxt, men detta kan förhindras genom att ytterligare förändringar uppstår, som osynliggör de genetiskt skadade cellerna. Så småningom har de tillförsäkrat sig så många genetiska avvikelser att de utvecklas till cancerceller. I akuta leukemier innebär detta i praktiken att ett stort antal omogna blodceller helt eller delvis tar över benmärgen och blodet, med infektionskänslighet, blödningar och trötthet som följd.

Genetiska förändringar som syns på kromosomnivå hittas i mer än hälften av alla akuta leukemier. Identifieringen av dem är kliniskt mycket viktig, eftersom olika avvikelser ger information om vilken sjukdomstyp det handlar om samt påverkar prognosen och valet av behandlingsform. De genetiska förändringarna inkluderar bland annat omflyttningar av kromosomdelar – *translokationer* – som påverkar cellens kontrollgener. Sådana avvikelser är relativt välundersökta och forskningen kring dem har till och med lett till nya läkemedel. Dock är det lika vanligt att leukemicellerna har fått tillskott av kromosomer, s k *trisomier*. I motsats till translokationerna så vet man väldigt lite om hur trisomierna uppstår, hur de påverkar cellen och vad kopplingen till

leukemiutveckling är. Syftet med den här avhandlingen var att försöka finna svaren på de frågorna.

I de första två studierna undersöktes fall av akut myeloisk leukemi (AML) och myelodysplastiska syndrom (MDS) – en närbesläktad sjukdom som ibland övergår i AML – med trisomi för kromosom 8 (*trisomi 8* eller +8) som enda kromosomavvikelse. Detta är en av de vanligaste genetiska förändringarna i AML och MDS, men trots det har man ingen bra förklaring till hur den påverkar cellen. En teori är att +8 i sig själv inte är tillräcklig för leukemiuppkomsten, utan att andra avvikelser är nödvändiga. Eftersom trisomi 8 ofta ses som den enda förändringen på kromosomnivå, så skulle dessa eventuella extra genfel involvera så små bitar av kromosomerna att de inte syns med vanliga undersökningsmetoder. I studie I analyserades därför 13 fall av +8-positiv AML/MDS med fluorescent in situ hybridisering (FISH); en teknik där man använder sig av fluorescerande s k prober för att färga in olika kromosomdelar, som sedan kan studeras i speciella mikroskop. Med FISH kan man t ex se om små bitar av kromosomerna har bytt plats eller försvunnit. Med denna metod upptäcktes dock inte några ytterligare genetiska förändringar i de 13 fallen.

I studie II användes en annan teknik: ”array-baserad CGH”, som möjliggör upptäckt av tillskott eller förlust av små kromosombitar. Totalt studerades tio AML- och MDS-fall med synbarligen enbart trisomi 8. Undersökningen resulterade i identifiering av tolv små genetiska förändringar som inte hade upptäckts med sedvanlig kromosomanalys. Dessa fanns i totalt fem AML/MDS-fall, d v s i hälften av de undersökta fallen, och var troligtvis av betydelse för leukemiuppkomsten. Upp-täckten visar att det kan finnas ytterligare genetiska skador, som inte syns med vanlig kromosomanalys, i leukemiceller och stöder uppfattningen att enbart trisomi 8 inte är tillräcklig för leukemiutveckling.

I studie III och IV undersöktes s k *hyperdiploid* akut lymfatisk leukemi (ALL) hos barn. Hyperdiploidi, som innebär att cellerna innehåller många extra kromosomer, d v s många trisomier, i ett specifikt mönster, är den vanligaste leukemitypen i den här åldersgruppen och är förknippad med en god prognos. Resultaten från en tidigare studie antydde att de extra kromosomerna troligtvis tillkom under en enda felaktig celldelning, men andra uppkomstmekanismer kunde inte säkert uteslutas. Vi undersökte detta med molekyärgenetiska metoder i tio fall i studie III, och kunde bekräfta att en enda felaktig celldelning var det troligaste alternativet. För att helt kunna utesluta övriga mekanismer studerades i studie IV ytterligare 27 fall. Utifrån våra resultat kunde vi

dra slutsatsen att tidigare nämnda mekanism förekommer i den stora majoriteten av hyperdiploid barn-ALL, men att de extra kromosomerna kan tillkomma på andra sätt i en del av fallen.

Vi studerade också den biologiska bakgrunden till att tillskott av kromosomer kan leda till leukemi. I varje kromosompar har de två kromosomerna ärvts från varsin förälder och vissa gener är olika aktiva beroende på om de nedärvt från modern eller fadern. En möjlighet var därför att det hade betydelse från vilken förälder de extra kromosomerna ursprungligen kom i hyperdiploid ALL. För att undersöka detta samlade vi in blodprov från föräldrar till barn med denna sjukdom i studie III och IV och gjorde med hjälp av dessa föräldraskapstest på trisomierna. Resultaten visade att teorin inte stämde: tillkomst av kromosomer sker oberoende av från vilken förälder de ursprungligen nedärvt. Sammanfattningsvis så har undersökningarna i den här avhandlingen lett till en större förståelse av sambanden mellan trisomier och leukemi och av hur sådana kromosomavvikelser uppstår i cancerceller.

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