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SIMULTANEOUS DETECTION OF ANEUPLOIDIES FOR CHROMOSOMES 4, 6, 10 AND 17 BY AUTOMATED FOUR COLOUR I--FISH IN HIGH HYPERDIPLOID ACUTE LYMPHOBLASTIC LEUKEMIA: DIAGNOSTIC ASSESSMENT, CLONAL HETEROGENEITY AND CHROMOSOMAL INSTABILITY IN ADULTS

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par

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pour Le Doyen
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Prof. Stefan Kunz

“Dedico questa tesi a mio marito Philippe e alle mie bambine Emmanuelle e Laetitia”

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Summary

Acute lymphoblastic leukemia (ALL) is a malignant hemopathy characterized by the accumulation of the immature lymphoid cells in the bone marrow and, most often, in the peripheral blood. ALL is a heterogeneous disease with distinct biological and prognostic entities. At diagnosis, cytogenetic and molecular findings constitute important and independent prognostic factors. High hyperdiploidy with 51-67 chromosomes (HeH), one of the largest cytogenetic subsets of ALL, in childhood particularly, is generally associated with a relatively favorable outcome. Chromosome gain is nonrandom, extracopies of some chromosome occurring more frequently than those of others. Concurrent presence of trisomy for chromosomes 4, 10 and 17 confers an especially good prognosis. The first aim of our work was to develop an automated four color interphase fluorescence in situ hybridization (I-FISH) methodology and to assess its ability to detect concurrent aneuploidies 4, 6, 10 and 17 in 10 ALL patients. Various combinations of aneuploidies were identified. All clones detected by conventional cytogenetics were also observed by I-FISH. However, in all patients, I-FISH revealed numerous additional abnormal clones, leading to a high level of clonal heterogeneity. Our second aim has been to investigate the nature and origin of this clonal heterogeneity and to test for the presence of chromosome instability (CIN) in HeH ALL at initial presentation. Ten HeH ALL and 10 non-HeH ALL patients were analysed by four colour I-FISH and numerical CIN values were determined for all four chromosomes together and for each chromosome and patient group, an original approach in ALL. CIN values in HeH ALL proved to be much higher than those in non-HeH ALL, suggesting that numerical CIN may be at the origin of the high level of clonal heterogeneity revealed by I-FISH. Our third aim has been to study the evolution of these cytogenetic features during the course of the disease in 10 HeH ALL patients. Clonal heterogeneity was also observed again during disease progression, particularly at relapse. Clones detected at initial presentation generally reappeared in relapse, in most cases with newly generated ones. A significant correlation between the number of abnormal clones and CIN suggested that the higher the instability, the larger the number of abnormal clones. Whereas clonal heterogeneity and its evolution most probably result from underlying chromosome instability, operating processes remain conjectural.

Résumé

La leucémie lymphoblastique aiguë (LLA) est une hémopathie maligne qui résulte de l'accumulation de cellules lymphoïdes immatures dans la moelle osseuse, et, le plus souvent, dans le sang périphérique également. La LLA est une affection hétérogène au sein de laquelle se distinguent plusieurs entités biologiques et pronostiques. Les données cytogénétiques et moléculaires font partie intégrante du diagnostic et jouent un rôle essentiel dans l'évaluation du pronostic. L'hyperdiploïdie élevée à 51-67 chromosomes (HeH), relativement fréquente, en particulier chez l'enfant, s'associe à un pronostic favorable. Le gain de chromosomes ne relève pas du hasard, certains chromosomes étant plus fréquemment impliqués que d'autres. La présence simultanée des trisomies 4, 6, et 17 s'associe à un pronostic particulièrement bon. Le premier but du travail a été de développer une méthode d'analyse automatique par hybridation in situ fluorescente interphasique (I-FISH) à 4 couleurs et de tester sa capacité à identifier la présence simultanée d'aneuploïdies 4, 6, 10 et 17 dans 10 cas de LLA. Différentes combinaisons d'aneuploïdies ont été identifiées. Tous les clones détectés par cytogénétique conventionnelle l'ont été par I-FISH. Or, chez tous les patients, l'I-FISH a révélé de nombreux clones anormaux additionnels générant un degré élevé d'hétérogénéité clonale. Notre deuxième but a été d'investiguer la nature et l'origine de cette hétérogénéité et de tester la présence d'instabilité chromosomique (CIN) chez les patients avec une LLA HeH en presentation initiale. Dix LLA HeH et 10 LLA non-HeH ont été analysées par I-FISH et les valeurs de CIN numérique ont été déterminées pour les 4 chromosomes ensemble et pour chaque chromosome et groupe de patients, approche originale dans la LLA. Ces valeurs étant beaucoup plus élevées dans la LLA HeH que dans la LLA non-HeH, elles favorisent l'hypothèse selon laquelle la CIN serait à l'origine de l'hétérogénéité clonale révélée par I-FISH. Le troisième but de notre travail a été d'étudier l'évolution de ces caractéristiques cytogénétiques au cours de la maladie dans 10 cas de LLA HeH. L'hétérogénéité clonale a été retrouvée lors de la progression de la maladie, en particulier en rechute, où les clones anormaux détectés en présentation initiale réapparaissent, généralement accompagnés de clones nouveaux. La corrélation existant entre nombre de clones anormaux et valeurs de CIN suggère que plus l'instabilité est élevée, plus le nombre de clones anormaux est grand. Bien que l'hétérogénéité clonale et son évolution résultent très probablement de l'instabilité chromosomique, les processus à l'œuvre ne sont pas entièrement élucidés.

Abbreviations

ALL, acute lymphoblastic leukemia

B-ALL, acute lymphoblastic leukemia of type B

BM, bone marrow

BMT, bone marrow transplantation

CC, conventional cytogenetics

CGH, comparative genomic hybridization

CIN, chromosomal instability

CNS, central nervous system

COG, Children's Oncology Group

CR, complete remission,

CT, chemotherapy

Cy3, cyanine dye 3

Cy3.5, cyanine dye 3.5

DAPI, 4',6-diamidino-2-phenylindole

DEAC, diethylaminocoumarin

FISH, fluorescence in situ hybridization

FITC, fluorescein isothiocyanate

GFCH, Groupe Français de Cytogénétique Hématologique

GVHD, graft versus host disease

HeH, high hyperdiploidy

I-FISH, interphase fluorescence in situ hybridization

NCI, National Cancer Institute

PB, peripheral blood

PCR, polymerase chain reaction

Ph+, Philadelphia positive

SCT, stem cell transplantation

SNP, single nucleotide polymorphism

T-ALL, acute lymphoblastic leukemia of type T

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1 Introduction

1.1 Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is characterized by the accumulation of malignant immature lymphoid cells in the bone marrow (BM) and, most often, in the peripheral blood (PB). ALL can be of type B (B-ALL) or T (T-ALL) depending on whether the leukemic cells, arrested at the lymphoblast stage, are committed to the B cell or T cell maturation pathway. In many clinical trials, the threshold for defining leukemia is 25% BM blasts (1). However, although uncommon, presentations with low BM blast counts can occur.

ALL is primarily a disease of children, 75% of the cases occurring in children under six years of age. The worldwide incidence is 1-4.75/100 000 persons per year (1). In B-ALL, the ratio male/female is 1.3 in children and 1.2 in adults. In adult T-ALL, the ratio is 2.2 (2, 3). As used in this paper, B-ALL does not include Burkitt leukemia/lymphoma.

Once leukemic cells enter the PB, they can be clinically detected in lymph nodes, spleen, thymus, liver, central nervous system and testis in male. Varying degrees of anemia, neutropenia and thrombocytopenia are observed as a consequence of BM failure. Lymphadenopathy, hepatomegaly and splenomegaly are common. In B-ALL, the leukocyte count can be decreased, normal or elevated. T-ALL is characterized by a high leukocyte count and patients often present a large mediastinal mass or another tissue mass. For a given number of leukocytes and tumour burden, T-ALL patients often show a relatively normal BM hematopoiesis compared to B-ALL.

The precise pathogenetic events at the origin of ALL development are unknown. An association with inherited predisposing genetic syndromes such as Down's syndrome, ataxia-telangiectasia, Xeroderma pigmentosum, Fanconi's anemia, Bloom's syndrome and Nijmegen breakage syndrome, or with ionizing radiation or exposure to specific chemotherapeutic drugs, has been observed in a few cases (< 5%) (4).

Retrospective identification of leukemia specific cytogenetic features or clonotypic immunoglobulin or T cell receptor loci on neonatal blood spots as well as studies of leukemia in monozygotic twins have indicated the prenatal origin of some childhood leukemia, including those with high hyperdiploidy (5-7).

As a whole, B-ALL has a relatively good prognosis in children, whereas it is less favorable in adults. Its cure rate is 80% in children, less than 50% in adults (1, 4, 8-10). T-ALL has a higher risk than B-ALL in children, whereas it may have a better prognosis than B-ALL in adults.

ALL is a heterogeneous disease with distinct biological and prognostic groups. At diagnosis, cytogenetic and molecular findings constitute important and independent prognostic factors (11-20).

Recurrent genetic abnormalities in association with morphology, cytochemistry, immunophenotype and clinical characteristics are used to identify distinct entities in the B-ALL disease group (1). In childhood ALL, the prognostic significance of cytogenetic features such as specific translocations or DNA ploidy is well defined. In adult ALL, the role of cytogenetics in patient management has been centered on the presence of t(9;22) which represents a high-risk factor but recent data brought evidence that other cytogenetic subgroups can also be used for risk stratification (19).

Even if specific chromosome abnormalities and gene mutations have also been observed in T-lineage ALL, they have not been used to define separate entities (21).

Chromosome changes include numerical and structural aberrations, sometimes associated. Clonal chromosome aberrations have been reported to occur in 80% of children and 70% of adult patients (22).

The frequency and prognostic significance of the major chromosome abnormalities in childhood and adult ALL are reported in Table 1 (23). The prevalence of various chromosome aberrations shows a marked difference between childhood and adult ALL. For instance, the t(9;22) translocation occurs in less than 5% of childhood ALL cases, whereas it accounts for nearly one fourth of adult cases (3, 19, 20).

Table1 : Frequency and prognostic significance of major chromosome abnormalities in pediatric and adult acute lymphoblastic leukemia (after Mrózek et al., 2009)

Chromosome Aberration	Children		Adults	
	Frequency	**Clinical Outcome**	**Frequency**	**Clinical Outcome**
High hyperdiploidy	23%-30%	Favorable	7%-8%	Favorable Intermediate
Hypodiploidy	6%	Intermediate (45 chromosomes) Adverse (<45 chromosomes) Intermediate (<46 chromosomes)	7%-8%	Adverse
Near-haploidy	0.4%-0.7%	Adverse	Rare	Not determined
t(12;21)(p13;q22)	22%-26%	Favorable	0%-4%	Not determined
t(9;22)(q34;q11.2)	1%-3%	Adverse	11%-29%	Adverse
t(4;11)(q21;q23)	1%-2%	Adverse	4%-9%	Adverse
t(1;19)(q23;p13.3)/ der(19)t(1;19)(q23;p13.3)	1%-6%	Favorable Intermediate	1%-3%	Favorable Intermediate Adverse
t(10;14)(q24;q11)	Rare	Not determined	0.6%-3%	Favorable Intermediate
del(6q)	6%-9%	Not prognostic	3%-7%	Intermediate
Abnormal 9p	7%-11%	Not prognostic Adverse	5%-15%	Favorable Relatively favorable Intermediate
Abnormal 12p	3%-9%	Not prognostic	4%-5%	Favorable
Normal karyotype (no aberration detected)	31%-42%	Relatively Favorable	15%-34%	Relatively favorable Intermediate

1.2 High hyperdiploidy in acute lymphoblastic leukemia

High hyperdiploidy (HeH) with 51-67 chromosomes as defined by Paulsson et al. (2), is one of the largest cytogenetic subsets of childhood ALL where it is observed in 25% to 30% of the cases. It is less frequent in adults (2-10%). Although improved compared to the other cytogenetic groups, outcome of adults with HeH is not comparable to the excellent outcome of HeH in children (23). The distribution of chromosome gain is non random (24). Extracopies of chromosomes X, 4, 6, 10, 17, 18 and 21 occur much more frequently than those of other chromosomes (25). Prognosis may be influenced by specific chromosome gains and the concurrent presence of trisomy for chromosomes 4, 10 and 17 (triple trisomy), as well as of chromosome 18, confer improved survival (26, 27). The presence of the triple trisomy was shown to confer an especially favourable prognosis to children with National Cancer Institute (NCI) standard risk features (age $<$ 10 years and white blood cell count $<$ 50'000/μl). Consequently, Sutcliffe et al. (26) concluded that fluorescence in situ hybridization (FISH) strategies involving these specific trisomies would be integrated in future Children's Oncology Group (COG) protocols and results used for risk assessment.

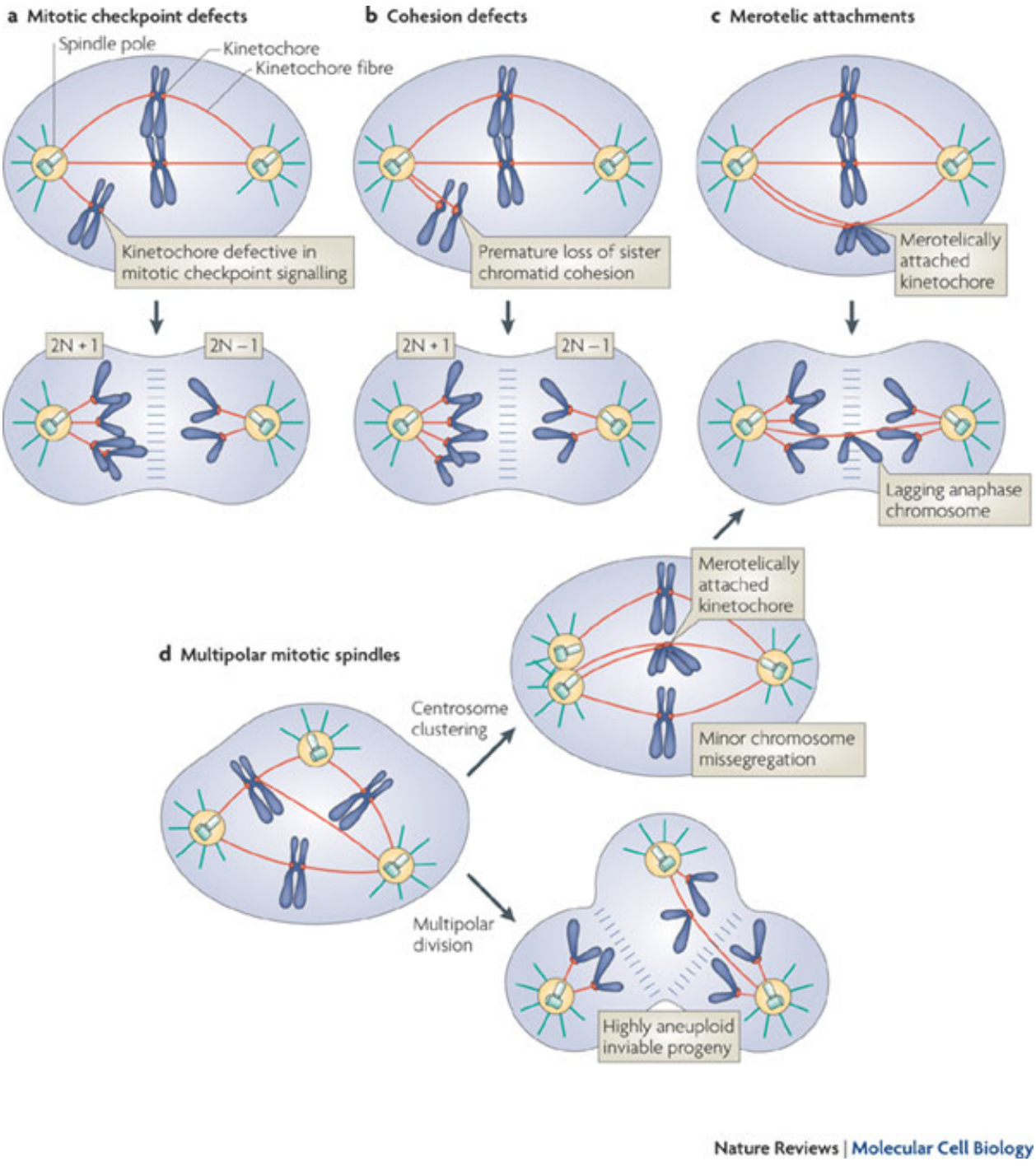
1.3 Aneuploidy

When a cell divides, it must accurately duplicate its genome and faithfully partition it into two daughter cells. Aneuploidy occurs when this process fails and two daughter cells inherit too many or too few chromosomes. One hundred years ago, the German zoologist and cytologist Theodor Boveri observed that sea urchin embryos undergoing abnormal mitotic divisions had an aberrant development. Based on this discovery and von Hansemann's former observations of abnormal mitotic figures in solid tumour cells, Boveri formulated the hypothesis that a malignant cell is a cell with a certain abnormal constitution, and that each process leading to this chromatin constitution would result in the origin of a malignant tumour (28, 29).

Aneuploidy is a remarkably common feature in human hematopoietic and solid tumours. Today the question whether aneuploidy is a cause or a consequence of malignant transformation remains a matter of debate (30). Recent evidence has shown that, although aneuploidy can have a causative role in tumour formation, it can also antagonize tumourigenesis in certain genetic contexts (31).

Aneuploidy is often due to errors in chromosome segregation and cells may gain or lose chromosomes by different mechanisms (31) (Fig.1). Cells with defects in mitotic checkpoint signalling can enter anaphase in the presence of unattached and misaligned chromosomes. Consequently, two copies of one chromosome may be inherited by a single daughter cell (Fig.1a). Chromosome missegregation may also be due to the premature loss or persistence during anaphase of sister chromatid cohesion (Fig.1b). In merotelic attachment, one kinetochore can attach to microtubules from both spindle poles (Fig.1c) and the persistence of these attachments into anaphase may lead to missegregation or exclusion of chromatid pairs from both daughter cells during cytokinesis. Cells with more than two centrosomes may form multiple spindle poles during mitosis (Fig.1d). In this case, a multipolar division occurs and the result can be the production of aneuploid daughter cells.

Figure 1: Pathways to the generation of aneuploidy (after Holland et al., 2009)



1.4 Chromosomal instability and clonal heterogeneity

Some tumours reveal a stable aneuploidy due to a chromosome missegregation occurring at some point during tumour development and leading to a stably propagating abnormal karyotype. However, more often, aneuploidy results from a chromosomal instability (CIN), characterized by an increase in the rate of loss or gain of whole chromosomes during mitosis leading to unstable karyotypes with cell-to-cell variation and multiple related and unrelated subclones and generating a state of clonal heterogeneity. It is important to precise that aneuploidy and CIN are not synonymous. Aneuploidy describes the state of an abnormal chromosome number while CIN refers to the rate of change in chromosome number (31, 32).

Although genetic instability is not proven to be necessary for tumour development, it affects the vast majority of cancers. It exists at two distinct levels, at the nucleotide level resulting in base substitution or in deletion or insertion of a few nucleotides, and at the chromosome level resulting in losses or gains of whole or part of chromosomes (32).

CIN was first studied in colorectal cancers and defined as the percentage of cells with a nonmodal chromosome number (33). Colorectal tumours without microsatellite instability were shown to present a persistent and striking defect in chromosome segregation, resulting in gains or losses in excess of 1% per chromosome per generation. CIN appeared to be a dominant trait, while microsatellite instability presented as a recessive trait (33).

The centrosomes, which nucleate and organize the cytoplasmic and spindle microtubules in interphase and mitotic cells, are actively involved in proper chromosome segregation during mitosis. Along with numerical chromosome aberrations, most solid tumours present centrosome amplification and, in breast tumour development, a significant correlation was observed between centrosome amplification, aneuploidy and CIN (34). In the same study, Lingle et al. observed that centrosome amplification occurred early in tumourigenesis, suggesting a driving role of CIN in breast tumour development.

In various clinical studies, involving solid tumours and recently myelodysplatic syndromes, CIN values correlated with poor outcome, bringing further support to the possible role of karyotypic instability in tumour progression (35, 36).

1.5 Automated analysis by interphase fluorescence in situ hybridization

Conventional cytogenetic analysis (CC) represents the standard method for the detection of chromosomal abnormalities. However, in ALL, this approach is limited by a frequently low proliferation rate of leukemic blasts *in vitro*, suboptimal spreading of mitotic chromosomes and poor banding pattern resolution. Moreover, since CC is usually based on the analysis of 20 to 25 metaphases, it may be a relatively insensitive assay in the presence of small abnormal clones.

To overcome these limitations, different methods have been developed such as FISH, flow cytometry and, more recently, integrative genomic approaches including genetic alteration profiling with oligonucleotide array-comparative genomic hybridization (CGH) or single nucleotide polymorphism (SNP) microarrays, and genome expression profiling (37-39).

Flow cytometry that enables DNA index determination is hampered by a relatively low sensitivity and is not precise enough for the diagnosis of hypo- or hyperdiploidy; moreover it cannot identify individual chromosomes and thereby detect specific aneuploidies of diagnostic and prognostic significance. Integrative genomic approaches have provided new insights into the genetics of ALL thanks to the identification of multiple novel genetic aberrations and may become an integral part of routine diagnostics allowing for identification of new clinically significant aberrations (39). Both flow cytometry and gene arrays technologies on bulk DNA or RNA from leukemic cells are not able to reveal clonal heterogeneity.

Owing to its sensitivity and its ability to detect aberrations of chromosome number in non-dividing cells, interphase FISH (I-FISH) represents a method of choice for ploidy assessment in ALL. Compared to other approaches, I-FISH has the additional advantage of being enumerative, that is, to provide information proper to each cell analysed and to reveal karyotype diversity.

The technique of FISH is based on the ability of single stranded DNA to anneal to complementary DNA. The target DNA is the nuclear DNA of interphase cells or DNA of metaphase chromosomes that

have been affixed to a glass slide after cell culture and chromosome preparation. FISH can also be performed with uncultivated BM or PB smears or fixed sectioned tissues. Different types of probes can be used, those that hybridize to unique sequences, those that hybridize to multiple sequences (chromosome painting) and those that recognize a specific chromosomal structure such as the centromere. Before hybridization, probe DNA is labelled by nick translation, random primed-labelling or PCR. Direct and indirect labelling are the two strategies commonly used. For indirect labelling, DNA probes are labelled by incorporation of a modified nucleotide containing a hapten. For direct labelling, DNA probes are labelled by incorporation of a modified nucleotide containing a fluorophore. Probe and target DNA are denatured to generate single stranded DNA and combined so as to allow the annealing of complementary sequences. If the probe has been indirectly labelled, an extra step based on an enzymatic or immunologic assay is required for signal visualization. Signals are observed by fluorescence microscopy (40).

Manual FISH analysis is limited by the difficulty in differentiating more than three fluorochromes by eye, and the use of ratio-mixing I-FISH that was proposed for the simultaneous detection of five different fluorochromes is hampered by the necessity to examine each signal in several planes of focus (41, 42). For these reasons one-colour and dual-colour I-FISH are the two main approaches currently used for aneuploidy detection in ALL (43, 44). Although aneuploidy for any chromosome can be detected individually by these methods, they cannot demonstrate more than two concurrent aneuploidies in a single nucleus. Recently however, Saez et al. (45) reported on the use of manual multicolour I-FISH with six different fluorochromes including counterstaining in the study of multiple myeloma.

Automated systems for FISH analysis have been used both in prenatal diagnosis (46) and cancer cytogenetics for the detection of aneuploidy (47), gene amplification (48, 49) and chromosomal abnormalities resulting in gene fusion (50-52). All such studies have shown that results derived from an automated method correlate closely with those obtained by manual scoring of signals. Compared to manual FISH analysis, automatic scanning presents several advantages, the most important of which is the simultaneous detection of several different fluorochromes. This gives the possibility of detecting an abnormal clone harbouring specific concurrent abnormalities which have a particular prognostic significance when observed together. Secondly, it allows the rapid analysis of much larger

numbers of nuclei than would be reasonably possible by manual scoring; in addition, the sensitivity can be improved by increasing the number of cells scored without a significant increase in the manual workload. The introduction of objective criteria avoids inter-observer variation and a possible reduction in efficiency due to fatigue.

1.6 Aims of our work

The present study was subdivided into three distinct parts:

Part 1. Development of an automated four colour I-FISH methodology for the simultaneous detection of specific aneuploidies of diagnostic and prognostic significance and its application to the diagnosis of high hyperdiploidy in acute lymphoblastic leukemia

Part 2. Investigation of clonal heterogeneity and chromosomal instability at disease presentation in high hyperdiploid acute lymphoblastic leukemia

Part 3. Study of evolution of clonal heterogeneity and chromosomal instability during the course of the disease in high hyperdiploid acute lymphoblastic leukemia

2 Automated analysis by four colour interphase FISH for the simultaneous detection of specific aneuploidies

2.1 Material and methods

2.1.1 Probe specificity

The choice of chromosomes 4, 6, 10, and 17 for this study was based on their most frequent overrepresentation in HeH ALL and on their diagnostic and prognostic significance (Groupe Français de Cytogénétique Hématologique" (GFCH). Centromeric probes were directly labelled by nick translation with four different fluorochromes (FITC,Cy3, Cy3.5, DEAC) as described in Blandin et al. (53). Probe specificity was tested by metaphase FISH on normal BM cells (Fig.1).

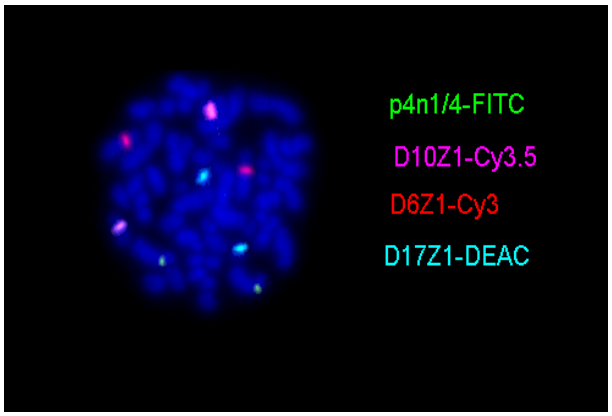


Fig 1: Metaphase chromosomes hybridized with 4 centromeric probes specific to chromosomes 4, 6, 10, and 17 respectively (bone marrow with a normal karyotype by conventional cytogenetics)

2.1.2 Automated analysis: detailed description

Automated analysis was performed using the microscopic scanning system Metafer4 (Metasystems Altlusseim, Germany). The system was based on a motorized epifluorescence microscope (AxioImager Z1; Zeiss, Feldbach, Germany) including a high resolution charge-coupled device (CCD) black and white camera (Zeiss, AxioCam, MRm), an eight slide motorized scanning stage (Marzhauser , Wetzlar, Germany), one 63x and one 40x objectives (Zeiss), and narrow band pass

filters specific for FITC, Cy3, Cy3.5, DEAC and DAPI. The Metacyte software for single cell analysis was integrated in the Metafer 4 scanning system.

The parameters of capture and exposure as well as those of image and cell processing were grouped in the Metacyte classifiers. They allowed to determine cell selection criteria and to analyse specific cell and signal characteristics such as cell area, aspect ratio, concavities or signal intensities (54) (Fig.2). Automated analysis consisted of two steps: nucleus selection and FISH signal detection.

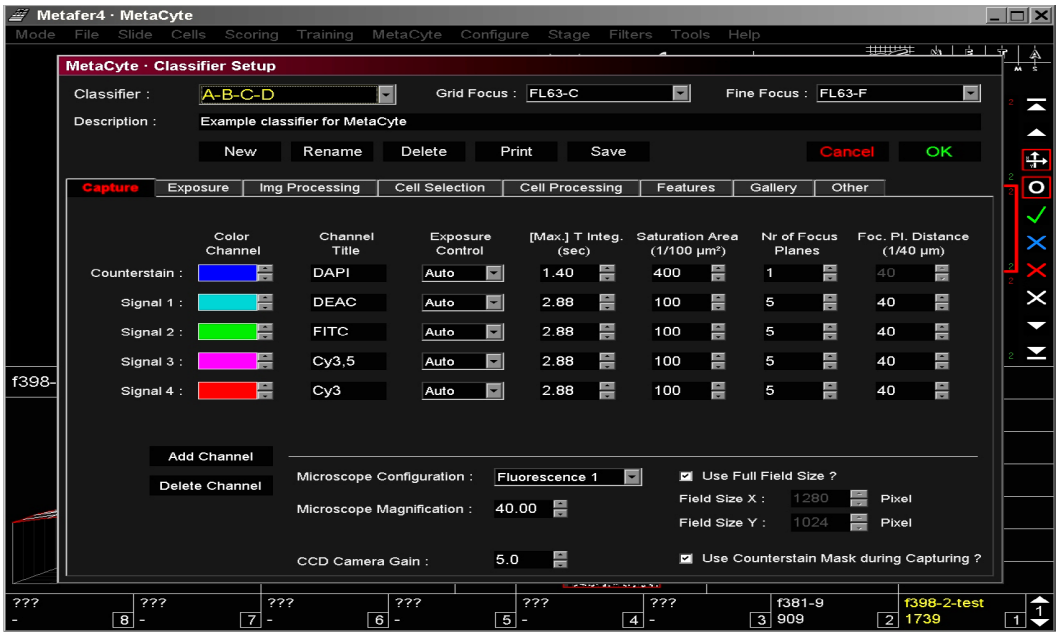


Fig.2: Illustration of a classifier exposing the parameters for capture, exposure, image processing, nucleus selection and FISH signal detection

Nucleus selection

During scanning, the DAPI channel was used to select nuclei suitable for FISH analysis. The search window was manually set. A virtual regular grid was laid over the window. Each square was called a field. The best focus plane was determined by automatic moving of the stage in the Z direction. Images were captured at 9 different focus planes with a Z spacing of 1.5 µm and the focus quality was automatically tested based on a local contrast criterion. The best focus position was established for all autofocus points. Image focus was further improved by repeating the operation at 7 focus planes at 0.5 µm apart in each image field (fine focusing). These two consecutive autofocus steps allowed for the detection of nuclei in a vertical range of 15 µm. After capture, the DAPI image was segmented using a fast contour algorithm. Minimum and maximum nuclear area, maximum concavity depth and

maximum aspect ratio were the parameters used to discriminate single nuclei from background irregularities and rubbish.

FISH signal detection

Once nuclei were selected, filter change occurred automatically and intensities of FITC, Cy3, Cy3.5 and DEAC were measured (50). Images were recorded at five different focus planes with a Z spacing of 0.2 µm. In each colour channel, signals were identified according to intensity, area, contrast and minimum distance; their number was counted automatically. For each nucleus, images were stored in a gallery and saved together with their coordinates, allowing eventual automatic relocation under the microscope (Fig.3).

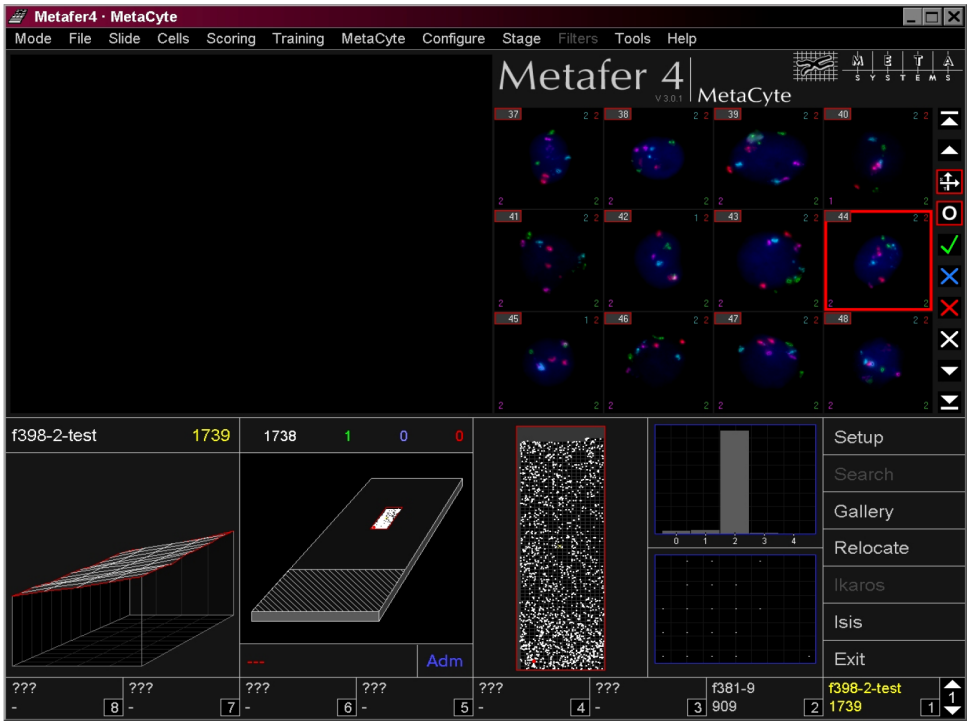


Fig.3: Illustration of a gallery. The bottom part of the figure provides data relating to focus quality (7-8), search window (5-6), nuclei distribution (4), and FISH signal counting (2-3). The top part illustrates a number of nuclei that have been selected, classified, stored and saved with their coordinates. The red point situated at the bottom left of the search window corresponds to nucleus 44 location on the slide

Setting of parameters for nucleus selection and FISH signal detection

An interactive training system enabled to establish the optimal set of parameters for nucleus selection and FISH signal detection. During training, images were captured from the different fields of the search window. Nuclei were selected and FISH signals scored manually by the operator. Then, using the same image fields, nuclei were selected and FISH signals scored automatically by the software. Errors of classification were displayed for every single image. Parameters were iteratively adjusted so as to optimize the process of nucleus selection and FISH signal detection.

2.2 Automated four-color interphase fluorescence in situ hybridization approach for the simultaneous detection of specific aneuploidies of diagnostic and prognostic significance in high hyperdiploid acute lymphoblastic leukemia

2.2.1 Summary

The aim of our work was first to develop a new system of cytogenetic analysis able to detect a combination of chromosome aneuploidies of diagnostic and prognostic significance in leukemic cells, then to apply this system to the identification of aneuploidy for chromosomes 4, 6,10 and 17 in ALL HeH.

This system consisted of an I-FISH strategy based on the simultaneous hybridization of four different centromeric probes, each probe being labeled by a distinct fluorochrome. Probes specific for chromosome 4, 6, 10 and 17 were labeled respectively in green, red, magenta and turquoise respectively. Signals were detected by automatic scanning using a commercial system (Metafer) that includes an image analysis program (Metacyte).

Parameters for nucleus selection and signal detection were established and evaluated.

Cut off values for chromosomes 4, 6, 10 and 17 aneuploidy were determined according to the model of Poisson. Combinations of aneuploidies were considered significant when each aneuploidy was individually significant. Various combinations of aneuploidies were identified.

The ability of this new I-FISH strategy to improve the yield of conventional cytogenetics was tested.

Results obtained by I-FISH in 10 patients with high hyperdiploid ALL were compared with those obtained by CC. All clones detected by CC were also observed by I-FISH but I-FISH revealed a number of additional abnormal clones in all patients, ranging from ≤ 1% to 31.6% of cells analyzed.

Automated four color I-FISH allowed for the analysis of a large number of cells and revealed a high level of chromosome variability at diagnosis and at relapse.

The cytogenetic picture provided by I-FISH was much more complex than that revealed by CC alone, paving the way for further research on chromosomal heterogeneity in ALL.

2.2.2 Personal contribution

- Set up of experimental conditions for performing FISH and automated analysis

- Carrying out of I-FISH analysis

- Determination of the cut off values according the Poisson model

- Assessment of the accuracy of the nucleus selection process and of the FISH signal detection

- Data formatting

- Design of the article and writing of the first draft

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Automated four-color interphase fluorescence in situ hybridization approach for the simultaneous detection of specific aneuploidies of diagnostic and prognostic significance in high hyperdiploid acute lymphoblastic leukemia

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Abstract In high hyperdiploid acute lymphoblastic leukemia (ALL), the concurrence of specific trisomies confers a more favorable outcome than hyperdiploidy alone. Interphase fluorescence in situ hybridization (FISH) complements conventional cytogenetics (CC) through its sensitivity and ability to detect chromosome aberrations in nondividing cells. To overcome the limits of manual I-FISH, we developed an automated four-color I-FISH approach and assessed its ability to detect concurrent aneuploidies in ALL. I-FISH was performed using centromeric probes for chromosomes 4, 6, 10, and 17. Parameters established for nucleus selection and signal detection were evaluated. Cutoff values were determined. Combinations of aneuploidies were considered relevant when each aneuploidy was individually significant. Results obtained in 10 patient samples were compared with those obtained with CC. Various combinations of aneuploidies were identified. All clones detected by CC were observed also by I-FISH, and I-FISH revealed numerous additional abnormal clones in all patients, ranging from $\leqslant$1% to 31.6% of cells analyzed. We conclude that four-color automated I-FISH permits the identification of concurrent aneuploidies of potential prognostic significance. Large numbers of cells can be analyzed rapidly. The large number of nuclei scored revealed a high level of chromosome variability both at diagnosis and relapse, the prognostic significance of which is of considerable clinical interest and merits further evaluation.

1. Introduction

Chromosomal aberrations have a major role in the diagnosis and risk assessment of acute lymphoblastic leukemia (ALL) [1]. Hyperdiploidy with >50 chromosomes (high hyperdiploidy) occurs in nearly 25% of pediatric ALL cases and is associated with a favorable prognosis. It is less frequent in adults (2–11%), for whom outcome is improved compared with the other ploidy groups but not as favorable as in children [2]. In hyperdiploid ALL, chromosome gain is frequently nonrandom, extra copies of some chromosomes occurring more frequently than those of others [3]: trisomy for chromosomes 4, 10, and 17 together confers a more favorable outcome than hyperdiploidy with $\geqslant$53 chromosomes in the absence of these trisomies [4].

Conventional cytogenetic analysis (CC) represents the standard method for the detection of chromosomal abnormalities. In ALL, however, this approach is limited by a frequently low proliferation rate of leukemic blasts in vitro, suboptimal spreading of mitotic chromosomes, and poor banding pattern resolution. Moreover, given that CC is usually based on the analysis of only 20–25 metaphases, it may be considered a relatively insensitive assay in the presence of small abnormal clones. Alternative methods such as

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flow cytometry, high-resolution comparative genomic hybridization (HR-CGH), and array-CGH, which can be used to determine different ploidy groups, are also hampered by their relatively low sensitivity [5–7]. Furthermore, cytometry cannot identify individual chromosomes and thus cannot detect specific aneuploidies of diagnostic and prognostic significance.

Owing to its sensitivity and its ability to detect aberrations of chromosome number in nondividing cells, interphase (I)-FISH represents the method of choice for ploidy assessment in ALL. Compared with other approaches such as HR- and array-CGH, I-FISH has the additional advantage of being enumerative; that is, I-FISH provides information proper to each cell analyzed.

Manual FISH analysis is limited by the difficulty in differentiating more than three fluorochromes by eye, and the use of ratio-mixing I-FISH (which has been proposed for the simultaneous detection of five different fluorochromes) is hampered by the necessity to examine each signal in several planes of focus [8,9]. For these reasons, single-color and dual-color I-FISH are the two main approaches currently used for aneuploidy detection in ALL [7,10]. Although aneuploidy for any chromosome can be detected individually, these methods cannot demonstrate more than two concurrent aneuploidies in a single nucleus. Recently however, Sáez et al. [11] reported on the use of manual multicolor I-FISH with six different fluorochromes, including counterstaining, in the study of multiple myeloma.

Automated systems for FISH analysis have been used both in prenatal diagnosis [12] and in cancer cytogenetics for the detection of aneuploidy [13], gene amplification [14,15], and chromosomal abnormalities resulting in gene fusion [16,17]. Compared with manual FISH analysis, automated scanning presents several advantages, the most important of which is the simultaneous detection of several fluorochromes.

Our objective was to develop an automated four-color FISH scanning system using centromeric probes and to evaluate its performance in the simultaneous screening for aneuploidy of chromosomes 4, 6, 10, and 17 in the bone marrow cells of ALL patients. We first assessed the accuracy of the automated system for cell nucleus selection and centromeric signal detection. We then determined cutoff values based on samples from control patients. Finally, we used the method to analyze samples from hyperdiploid ALL patients with aneuploidy for at least one of the four chromosomes studied, as previously determined by CC analysis. The results obtained by these two methods were compared.

2. Materials and methods

2.1. Patients

Interphase FISH analysis was performed on fixed cell suspensions of bone marrow obtained from 23 patients. Of 13 adult patients diagnosed with a malignant hemopathy associated with a normal karyotype (based on a minimum of 20 metaphases fully analyzed), 3 cases were tested to assay the power of the automated search and 10 cases were used as negative controls to determine cutoff values. Ten high hyperdiploid or paratetraploid ALL cases with at least one trisomy for chromosomes 4, 6, 10, or 17 at either disease presentation ($n = 6$) or relapse ($n = 4$) were used to assess the potential of the method for aneuploidy detection. These 10 patients were referred to our laboratory from the Hematology Departments of the University Hospitals of Zurich, Lausanne, and Basel and of the Mendrisio Regional Hospital between 1995 and 2002. Their karyotypes are described in Table 1. For the purposes of this study, no distinction was made for ALL subtype or for pediatric versus adult cases. Ethical approval for this project was obtained in accordance with the guidelines of the local Ethical Review Board.

2.2. Conventional cytogenetic analysis

Chromosome analysis was performed on bone marrow cells with or without isolation of mononuclear cells. Three different culture conditions were used in parallel: basic culture medium with 20% human AB serum only and basic culture medium with either 5 ng/mL phorbol-12,13-dibutyrate or 10% phytohemagglutinin tetradecanoyl phorbol acetate–leukocyte conditioned medium (PT-LCM) as previously described [18]. Cells were incubated at 37% without or with fluorodeoxyuridine (FdU) synchronization: 0.1 μmol/L FdU/4 μmol/L uridine added 4–72 hours after the initiation of cultures for 24 hours, followed by the addition of 10 μmol/L thymidine for a further 15 hours of incubation before harvesting. Colchicine was added for the final 30 minutes at a concentration of 0.2 μg/mL without FdU, or 0.05 μg/mL with FdU. Hypotonic shock, fixation, and G-banding were performed as previously described [19]. Karyotypes were written according to ISCN 2005 [20].

2.3. Fluorescence in situ hybridization

In eight cases, I-FISH was performed on cell suspensions obtained after incubation with 20% human AB serum only (but FdU synchronization in case 694/99), and in two cases on cell suspensions obtained after stimulation with phorbol (case 454/00) or PT-LCM (case 1527/97).

The choice of chromosomes 4, 6, 10, and 17 for this study was based on the recommendations of the Groupe Francophone de Cytogénétique Hématologique for aneuploidy screening in ALL patients [1].

Probes specific for α-satellite centromeric sequences were obtained from the American Type Cell Culture collection (ATCC, Manassas, VA) (D6Z1, D10Z1, D17Z1) or kindly provided by Professor Mariano Rocchi (University of Bari, Italy) (p4n1/4). The probes were directly labeled by nick translation with four different fluorochromes

Table 1
Clinical and cytogenetic findings in patients with acute lymphoblastic leukemia

Case	Sex/Age, yr	G-banding karyotype	Positive signals, Chr. 4 6 10 17[a]	Abnormal clones detected, % CC	I–FISH
1826/95	F/3	52,XX,+X,**+6,**+14,+14,+21,+21[16]/46,XX[12]	2322	57.1	45.9
694/99	M/18	55,XY,+X,**+4,+6,**+9,+14,**+17,**+18,+21,+21[2]/55,idem,add(19)(p13)[5]/46,XY[1]	3323	87.5	22.9
683/99	M/4	64,XY,+X,dup(1)(q21q32),+2,**+4,**+5,**+6,**+7,+?8,**+?10,**?der(11)(p?),+11,+12,+14, +14,**+17,**+18,+21,+21,+21,+22[3]/46,XY[14]	3333	17.6	4.1
454/00	M/2	54~55,XY,+X,**+6,**inc[10]/46,XY[12]	2322	45.5	24.7
192/01	M/3	55,XY,+X,**+4,+6,+10,**+14,**+17,**+18,+21,+mar[14]/46,XY[22]	3333	38.9	2.0
387/00	M/5	56,XY,+X,**+6,+10,+10,**+14,+18,+18,+21,+21,+mar[7]/46,XY[4]	2342	63.6	1.3
149/98[b,c]	F/23	56,XX,+X,add(1)(q42),add(2)(p1?3~1?5),+5,**+6,**der(7;9)(q10;q10),+9,**+10,+10,**+18, +21,+21,+22,+mar[12]/56,idem,t(20;22)(q13.3;q11)[3]/46,XX[5][c]	2342	75.0	4.2
403/01[b]	M/16	85~87,XY,+X,+1,+1,add(1)(q42),**+4,**+5,**+6,**add(6)(q21),+7,add(7)(q22),+8,+?8, +9,+9,**+10,+10,**+11,+11,add(11)(q23),add(12)(q15)x2,+13,+15,+?15,+16,**+17,** +18,+19,+20,+20,+21,+21,+22+22,+22,+mar1,+mar2,+mar3,+7~8mar,0~1min[cp5]	3343	100.0	1.5
1527/97[b]	M/28	57,XY,+X,+5,**+6,**add(7)(p13~p15),+9,**+10,**+11,−13,+18,+21,+21,+22, +22,+mar[5]/46,XY[15]	2332	25.0	13.3
601/02[b]	F/33	53~54,XX,+X,del(1)(q4?1),del(2)(p2?1),−2,del(3)(q?),+5,**+6,**+7,add(8)(q24), del(8)(q2?4),add(9)(q34),+10[3],der(11)t(1;11)(q21;q23)[3],+11,−13[3],−21,+mar1, +4mar[cp4]/46,XX[6]	2332	40.0	12.9

Abbreviations: CC, conventional cytogenetics; Chr., chromosome; I-FISH, interphase fluorescence in situ hybridization.

[a] Each of the four digits represents the number of positive signals detected in a single cell nucleus, for chromosomes 4, 6, 10, and 17, respectively. See Fig. 1 for detailed examples.

[b] Sample taken at relapse; all others at presentation.

[c] The marker chromosome is a probable add(4)(q21).

(FITC, Cy3, Cy3.5, and DEAC), a combination chosen to ensure that their respective emission wavelengths were sufficiently distinct from each other (Table 2). Samples were then precipitated with 50× sheared salmon sperm DNA and 50× yeast RNA.

Slides for FISH analysis were prepared using 10 μL fixed cell suspension applied to each slide in a cytogenetic drying chamber set to 22°C and 43% relative humidity (Thermotron, Holland, MI). The slides were incubated for 10 minutes, after which the hybridization mixture, consisting of 1 ng/mL of each of the four probes in 50% formamide−2× saline sodium citrate (SSC)−10% dextran sulfate, was added to the slides and both cells and probes denatured on a heating plate at 65°C for 10 minutes. After overnight incubation in a moist chamber at 37°C, slides were washed once in 0.3× SSC at 72°C for 5 minutes and twice (for 2 and 10 minutes, respectively) in 1× phosphate-buffered detergent (Oncor, Basel, Switzerland) at room temperature. DNA was counterstained with 200 ng/mL 4′,6-diamidino-2-phenylindole (DAPI) in 2× SSC and slides were mounted in an antifade solution (Vectashield; Vector Laboratories, Burlingame, CA).

2.4. Automated analysis

Automated FISH analysis was performed using a commercially available scanning system (MetaFer4/MetaCyte; MetaSystems, Altlussheim, Germany) and a motorized epifluorescence microscope (Axioplan 2; Zeiss, Feldbach, Germany) equipped with an automated scanning stage (Marzhauser, Wetzlar, Germany), a 63× objective (Zeiss), a high-resolution, charge-coupled device black-and-white camera (Zeiss AxioCam MRm), and narrow band pass fluorescence filters specific for FITC, Cy3, Cy3.5, DEAC, and DAPI (Table 2). Nuclei were imaged at five *Z*-levels with a spacing of 0.2 μm and were subsequently superimposed

Table 2
Probes, labeled nucleotides, and counterstain

Chromosome	Probe	Fluorochrome	λ_{ex}	λ_{em}	Color	Labeled nucleotide (source and catalog no.)
4	p4n1/4	FITC	490	520	green	fluorescein-12-dUTP (Roche; 1373 24 2)
6	D6Z1	Cy3	554	558	red	Cy3-dUTP (Amersham; PA 53022)
10	D10Z1	Cy3.5	581	588	magenta	CY3.5-dCTP (Amersham; PA 53521)
17	D17Z1	DEAC	426	480	cyan	DEAC-5-dUTP (PerkinElmer; NEL 455)
—	—	DAPI	350	456	blue	counterstain

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DEAC, diethylaminocoumarin; FITC, fluorescein isothiocyanate; λ_{ex}, excitation wavelength; λ_{em}, emission wavelength.

to create a composite image for each fluorochrome in turn. These composite images were then further superimposed to show all fluorochromes simultaneously and saved in the software image gallery. Images of nuclei were stored in a gallery and saved together with their coordinates, allowing automatic relocation under the microscope. Each nucleus was subsequently checked manually in the gallery and, if needed, under the microscope. Where appropriate, the number of signals was corrected or the nucleus was rejected outright. The optimal set of parameters was determined using an interactive training system, as previously described [16].

Given that there is often an unavoidable and unpredictable discrepancy in the quality of hybridization of cell suspensions obtained from different ALL patient samples, the three control samples used in this study were selected on the basis of differing hybridization quality (good, average, and poor), in order to determine the limitations of the system against this factor.

2.5. Statistics

Cutoff values were established according to the Poisson distribution, as previously described [21], by evaluating 1,000 nuclei for each control sample.

3. Results

3.1. Nucleus selection and FISH signal detection

The optimal values of the parameters for nucleus selection were defined in our laboratory as follows: 15% relative DAPI intensity threshold for segmenting nuclei, 15 μm^2 minimum and 300 μm^2 maximum object area, 0.1 maximum concavity depth, and 1.7 maximum aspect ratio. Based on these values, percentages of nuclei not recognized by the software (false negatives) and percentages of objects detected as cell nuclei (false positives) were 18.4% and 11.1%, 33.9% and 5.1%, 34.3% and 5.7% in the three control cases tested, respectively (global percentages: 31% and 6.2%).

The optimal values of the parameters for fluorescent signal detection were defined as follows: spot measurement area of 7 μm^2 for DEAC, FITC, and Cy3.5 and 4 μm^2 for Cy3 channels; minimum spot distance of 14 μm for DEAC, FITC, and Cy3.5, and 19 μm for Cy3; minimum relative spot intensity of 49% for DEAC and FITC, 52% for Cy3.5, and 39% for Cy3; maximum spot area of 60 μm^2 for DEAC and Cy3, 200 μm^2 for FITC, and 50 μm^2 for Cy3.5; minimum spot contrast of 80% for DEAC and Cy3, 100% for FITC, and 60% for Cy3.5. Based on these values, the percentages of correctly counted cells ranged between 78.8% and 69.6% (global percentage: 75%) for DEAC, 76.5% and 66.7% (71%) for Cy3.5, 66.1% and 58% (61%) for FITC, and 67.5% and 48.6% (56%) for Cy3.

3.2. Determination of significant aneuploidies

Based on cutoff values given in Table 3, significant aneuploidies for chromosomes 4, 6, 10, and 17 were determined in all 10 ALL patients (1,500 nuclei scored) and the results were compared with those obtained by CC (Table 4). In all patients, aneuploidies detected by CC were also detected by I-FISH. Proportions of abnormal cells were sometimes higher by I-FISH than by CC and sometimes lower, but no systematic trend could be established. In all patients, I-FISH revealed additional aneuploidies not detected by CC. The proportion of abnormal cells for aneuploidies not detected by CC varied between 0.3% and 44.4%. The CC approach revealed few tetrasomies, identifying only three cases of tetrasomy 10 and failing to detect any tetrasomy for chromosomes 4, 6, or 17, even though all four tetrasomies were detected by I-FISH in most patients, sometimes in elevated proportions.

3.3. Determination of relevant combinations of aneuploidies

The simultaneous detection of four different centromeric probes enabled the identification of clones with different combinations of aneuploidies (Fig. 1; Table 5). Combinations of relevant aneuploidies were identified based on the presence of individually significant aneuploidies.

The 10 clones detected by CC were also detected by I-FISH (Table 1). In all cases, the proportion of abnormal cells observed by I-FISH was lower than that observed by CC.

I-FISH revealed additional abnormal clones in all patients (Table 5). Very small abnormal clones of $<1\%$ were observed in all patients, totaling from 0.9% and 16.8% of

Table 3
Cutoff values for the detection of monosomy, trisomy, or tetrasomy for chromosomes 4, 6, 10, and 17 in bone marrow samples for selected numbers of scored nuclei according to the Poisson model

Nuclei scored, no.	Chr 4, no. (%)			Chr 6, no. (%)			Chr 10, no. (%)			Chr 17, no. (%)		
	−4	+4	+4,+4	−6	+6	+6,+6	−10	+10	+10,+10	−17	+17	+17,+17
200	13 (6.5)	8 (4.0)	3 (1.5)	11 (5.5)	7 (3.5)	2 (1.0)	11 (5.5)	7 (3.5)	3 (1.5)	19 (9.5)	8 (4.0)	3 (1.5)
500	23 (4.6)	13 (2.6)	4 (0.8)	19 (3.8)	11 (2.2)	3 (0.6)	19 (3.8)	11 (2.2)	3 (0.6)	35 (7.0)	14 (2.8)	4 (0.8)
1,000	38 (3.8)	19 (1.9)	5 (0.5)	30 (3.0)	16 (1.6)	4 (0.4)	30 (3.0)	17 (1.7)	4 (0.4)	60 (6.0)	21 (2.1)	5 (0.5)
1,500	53 (3.5)	26 (1.7)	5 (0.3)	41 (2.7)	21 (1.4)	4 (0.3)	40 (2.7)	22 (1.5)	4 (0.3)	84 (5.6)	28 (1.9)	5 (0.3)

Patients were deemed positive if the observed numbers of abnormal nuclei are greater than or equal to the tabulated values. Significance level: 0.001%.

Table 4
Findings in acute lymphoblastic leukemia patients obtained by conventional cytogenetics (CC) and interphase fluorescence in situ hybridization (I-FISH) for chromosomes 4, 6, 10, and 17

Patient	Methods	Metaphases or nuclei, no.	Percentage of significant individual aneuploidies							
			+4	+4,+4	+6	+6,+6	+10	+10,+10	+17	+17,+17
1826/95	CC	28	0.0	0.0	***57.1***	0.0	0.0	0.0	0.0	0.0
	FISH	1,500	0.0	*0.4*	***52.4***	*0.7*	0.0	0.0	0.0	0.0
694/99	CC	8	***87.5***	0.0	***87.5***	0.0	0.0	0.0	***87.5***	0.0
	FISH	1,500	***73.5***	*0.5*	***77.4***	*0.5*	*5.0*	0.0	***36.1***	*0.5*
683/99	CC	17	***17.6***	0.0	***17.6***	0.0	***17.6***	0.0	***17.6***	0.0
	FISH	1,500	***21.5***	*1.1*	***21.1***	*0.5*	***20.0***	*1.0*	***12.3***	*0.5*
454/00	CC	22	0.0	0.0	***45.5***	0.0	0.0	0.0	0.0	0.0
	FISH	1,500	*13.6*	*1.5*	***61.7***	*3.1*	*16.3*	*1.1*	*30.8*	*3.3*
192/01	CC	36	***38.9***	0.0	***38.9***	0.0	***38.9***	0.0	***38.9***	0.0
	FISH	1,500	***24.5***	*17.3*	***24.0***	*1.2*	***9.0***	*0.5*	***19.3***	*2.3*
387/00	CC	11	0.0	0.0	***63.6***	0.0	0.0	***63.6***	0.0	0.0
	FISH	1,500	*17.6*	*1.1*	***65.9***	*5.4*	*27.5*	***3.1***	*11.2*	*2.2*
149/98[a]	CC	20	0.0	0.0	***75.0***	0.0	0.0	***75.0***	0.0	0.0
	FISH	1,500	*44.4*	*1.7*	***37.3***	*2.1*	*27.9*	***25.3***	0.0	0.0
403/01[a]	CC	5	***100.0***	0.0	***100.0***	0.0	0.0	***100.0***	***100.0***	0.0
	FISH	1,500	***29.0***	*21.5*	***14.7***	*31.7*	*13.3*	***32.8***	***23.9***	*15.3*
1527/97[a]	CC	20	0.0	0.0	***25.0***	0.0	***25.0***	0.0	0.0	0.0
	FISH	1,500	*40.0*	*8.3*	***47.6***	*6.5*	***43.4***	*8.5*	*4.5*	*0.3*
601/02[a]	CC	10	0.0	0.0	***40.0***	0.0	***30.0***	0.0	0.0	0.0
	FISH	1,500	*12.9*	*3.5*	***32.7***	*4.0*	***27.0***	*5.4*	*5.7*	*0.3*

Bold italic highlight indicates clones detected by both CC and I-FISH; plain italic highlight indicates clones detected by I-FISH alone.
[a] Sample taken at relapse; all others at presentation.

all cell nuclei (not displayed in detail, but included under the Others category in Table 5). Abnormal clones with an incidence ranging from 1% to 31.6% were detected in all but one patient.

4. Discussion

The main advantage of interphase FISH is the detection of aneuploidy in nondividing cells, thus overcoming the limitations stemming from the often low proliferative index of leukemic cells in vitro and the poor resolution of G-banded chromosomes frequently encountered in ALL. However, the sensitivity of manual FISH is hampered by the incapacity of the human eye to recognize more than three fluorochromes simultaneously, and results may depend on the experience of the investigator and interobserver variation. In addition, a large number of nuclei need to be observed to ensure the accurate detection of small abnormal clones; this is time-consuming and tiring, and so increases the risk of errors.

In the present study, we developed an automated I-FISH approach allowing the simultaneous detection of four different centromeric probes and reliable assessment of a large number of cells. We then validated the method in 10 hyperdiploid ALL patients, to evaluate its usefulness in the detection of aneuploidy of chromosomes 4, 6, 10, and 17 at presentation or during the course of the disease.

The first issue was to determine the accuracy of cell nucleus selection and FISH signal detection on bone marrow control samples. For each sample tested, the result of automated cell nucleus scoring was evaluated and compared with manual classification. The global proportion of false negatives (nuclei not detected by automated analysis) was 31%; that of false positives was 6.2%. Various reasons might account for the false positive and negative rates observed: first, nuclei might not have been recognized because of DAPI counterstaining that was either too weak or too bright; second, given the parameters used, there might have been difficulty in distinguishing between single nuclei and clusters of nuclei. A further reason could be that incomplete nuclei located at the edges of the image field were included in the selection. The reason for our percentage of false negatives being considerably higher than the 11% observed in peripheral blood leukocytes by Kajtár et al. [16], using a similar approach, could be due to the greater heterogeneity both of bone marrow cells and of the clinical status of the control patients in our study. Our rate of false positives was somewhat lower than the 10.4% found by Kajtár et al. [16]. Although the proportion of undetected cells seems to be high, this may not affect the result in abnormal cases. Indeed, provided the criteria used for nucleus selection do not result in a particular cell population being missed, cell loss will be random, and thus comparable in the normal and abnormal populations.

Efficient signal detection and evaluation is an essential requirement of an automated system; it is a major difficulty, as signals may show great variation in size, intensity, shape, and localization, depending on the probes and fluorochromes used and the quality of the cytogenetic preparations. The evaluation of automatically scored FISH signals showed that they were counted correctly in 75% of nuclei for DEAC, 61% for FITC, 71% for Cy3.5, and

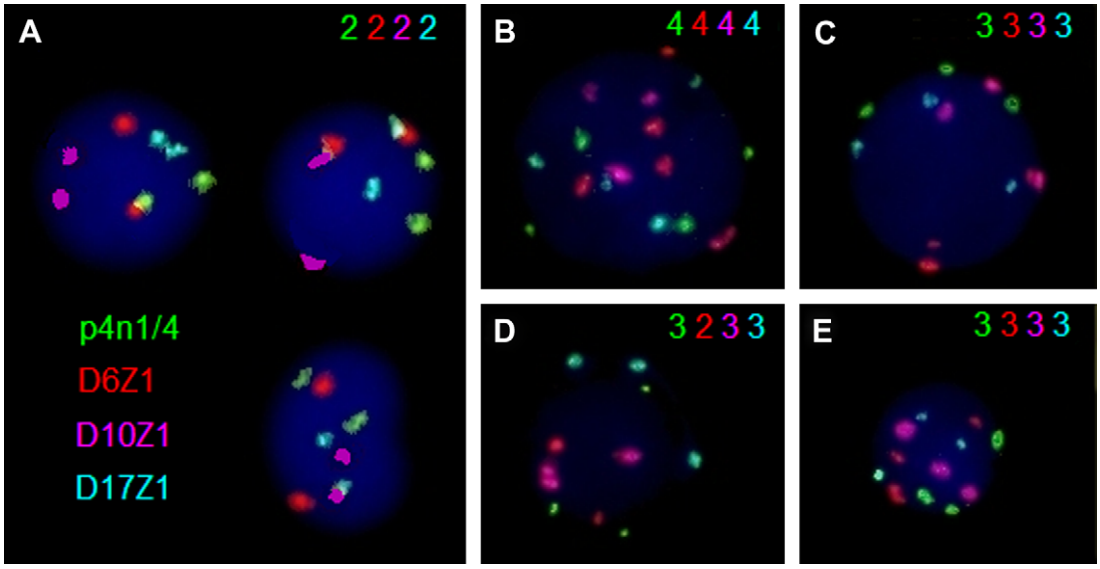

Fig. 1. Illustration of four-color FISH using centromeric probes specific for chromosomes 4, 6, 10, and 17 in normal bone marrow cells of negative controls (A) and in abnormal bone marrow cells (B–E) from ALL patients. The numbers of positive signals detected in a single cell nucleus are reported in the order green, red, magenta, and cyan. For example, the combination 3233 represents, respectively, three green signals for chromosome 4, two red signals for chromosome 6, three magenta signals for chromosome 10, and three cyan signals for chromosome 17, as seen in a pathological cell (D).

56% for Cy3. Incorrect signal scoring was due to misinterpretation of signals because of ambiguous morphology and errors in nucleus selection (false positive nuclei). The reduced accuracy observed with the FITC-labeled probe is most likely caused by a frequently diffuse signal due to centromeric heteromorphism of chromosome 4 [22,23]. The level of accuracy obtained with the Cy3-labeled probe relates to the Cy3 filter used. Although a filter with the narrowest band pass available was chosen, it is nonetheless not specific enough to make an unambiguous distinction between Cy3 and very bright Cy3.5 signals. A custom filter made to our specification should resolve this problem.

Nonetheless, even if a number of nuclei have to be reassessed by the observer, the method still offers numerous advantages. It allows the simultaneous detection of four different probes and, unlike the alternative methods we have mentioned, it is enumerative. It allows the rapid and consecutive screening of large numbers of nuclei, thus minimizing human errors due to fatigue or to interobserver variation. If required, the number of nuclei scored can be easily and rapidly increased. Moreover, as images of each nucleus scored are saved automatically together with their coordinates, they can be easily located in the image gallery.

In interphase FISH, an important issue is the determination of the sensitivity and specificity of the method, and it is therefore necessary to have reliable cutoff values. This is especially relevant for the detection of small abnormal clones, not only at diagnosis but also for the assessment of early relapse or residual disease. Due to the high number of color patterns generated by the use of four different fluorochromes, critical values for each of these combinations are difficult to define. Therefore, aneuploidies in ALL patients were identified individually for each chromosome, based on the cutoff values determined in the control samples. Combinations of aneuploidies were considered relevant only when each aneuploidy was significant on its own.

Our study corroborates the widely reported discrepancies between CC and I-FISH with respect to the proportions of cells with different levels of aneuploidy. Although all aneuploidies detected by CC were also identified by I-FISH, not all aneuploidies detected by I-FISH were observed by CC. Trisomy 6 was the one trisomy detected in all patients by both I-FISH and CC. Tetrasomies were detected by I-FISH alone, with the exception of tetrasomy 10, which was observed by both methods in three patients. Tetrasomy demonstrated by I-FISH escaped detection by CC, even when the proportion of tetrasomic cells was relatively high. These observations may be accounted for by the difference in the proliferative rates in vitro of trisomic and tetrasomic cells and by the relatively small number of metaphases available for CC analysis in some cases. In a study including 2,339 ALL patients with high hyperdiploidy identified by CC [3], the frequencies of tetrasomy were reported to be relatively low for chromosomes 4, 6, 10, and 17, compared with trisomy for these chromosomes, which suggests the existence of a proliferative or survival advantage for only certain combinations of additional chromosomes. Nonetheless, chromosome aneuploidy in ALL seems to differ from chromosome 8 aneuploidy in myeloid malignancies, where cells with a higher level of aneuploidy appear to have a greater proliferative advantage [24].

In all patients, a number of small abnormal clones occurred in addition to well-represented abnormal clones; this might reflect a high level of chromosome instability and, consequently, karyotype variability in leukemic cells. Our

Table 5
Clones in patients with acute lymphoblastic leukemia, expressed as a percentage

Clones, chr	Patient									
4 6 10 17[a]	1826/95	694/99	683/99	454/00	192/01	387/00	149/98[b]	403/01[b]	1527/97[b]	601/02[b]
2222	***39.6***	***6.0***	***55.0***	***17.3***	***47.3***	***14.9***	***27.7***	*31.0*	***18.5***	***39.2***
2322	***45.9***	*9.4*	*3.7*	***24.7***	*6.6*	*29.0*	*2.7*	*1.0*	*4.7*	*8.3*
3342	—	—	—	—	—	—	*10.1*	—	*2.2*	—
3332	—	*2.0*	*5.0*	—	*1.7*	*3.4*	*9.1*	—	*12.9*	*1.8*
3232	—	—	*1.8*	—	—	—	*6.7*	—	*3.5*	*1.4*
3222	—	*7.5*	*3.1*	*2.0*	*7.0*	*2.4*	*6.2*	*7.7*	*10.5*	*4.6*
3242	—	—	—	—	—	—	*5.1*	—	—	—
2232	—	—	*2.8*	*1.9*	*1.5*	*2.3*	*4.6*	*1.1*	*2.7*	*5.7*
2332	—	—	*2.1*	*5.1*	—	*14.1*	*4.5*	—	***13.3***	***12.9***
2342	—	—	—	—	—	***1.3***	***4.2***	—	*1.9*	*2.5*
3322	—	*31.6*	*2.0*	*3.6*	*4.4*	*5.8*	*3.5*	—	*3.0*	*1.7*
2242	—	—	—	—	—	—	*2.9*	—	—	—
3333	—	*1.1*	***4.1***	*1.1*	***2.0***	—	—	—	—	—
2223	—	*1.1*	*2.5*	*4.7*	*6.8*	*2.2*	—	*1.3*	—	*1.9*
3223	—	*4.6*	*1.3*	—	*2.8*	—	—	—	—	—
3233	—	—	*1.0*	—	—	—	—	—	—	—
3323	—	***22.9***	*1.0*	*2.7*	*3.2*	—	—	—	—	—
2323	—	*4.9*	—	*13.5*	*1.4*	*3.1*	—	—	—	—
2333	—	—	—	*3.4*	—	*2.2*	—	—	—	—
2422	—	—	—	*1.1*	—	*1.9*	—	—	—	—
2432	—	—	—	—	—	*1.3*	—	—	*1.6*	—
3443	—	—	—	—	—	—	—	*5.5*	—	—
4444	—	—	—	—	—	—	—	*5.1*	—	—
4443	—	—	—	—	—	—	—	*4.3*	—	—
3444	—	—	—	—	—	—	—	*2.8*	—	—
4222	—	—	—	—	—	—	—	*2.3*	*1.5*	*1.3*
2443	—	—	—	—	—	—	—	*1.8*	—	—
3343	—	—	—	—	—	—	—	***1.5***	—	—
4442	—	—	—	—	—	—	—	*1.5*	—	—
3433	—	—	—	—	—	—	—	*1.3*	—	—
4343	—	—	—	—	—	—	—	*1.3*	—	—
4434	—	—	—	—	—	—	—	*1.2*	—	—
3442	—	—	—	—	—	—	—	*1.2*	—	—
4344	—	—	—	—	—	—	—	*1.1*	—	—
4433	—	—	—	—	—	—	—	*1.1*	—	—
2442	—	—	—	—	—	—	—	*1.0*	—	—
4332	—	—	—	—	—	—	—	—	*2.7*	—
3432	—	—	—	—	—	—	—	—	*1.2*	—
2324	—	—	—	*1.2*	—	—	—	—	—	—
2233	—	—	—	*1.3*	—	—	—	—	—	—
Others[c]	13.6	5.8	10.1	8.4	8.3	8.0	9.4	8.1	9.1	9.9
Ignored[d]	*0.9*	*3.1*	*4.5*	*8.0*	*7.0*	*8.1*	*3.3*	*16.8*	*10.7*	*8.8*

Clones detected by both I-FISH and CC (Table 1) are indicated in bold and those identified by I-FISH alone are in italics.

[a] Each of the four digits represents the number of positive signals detected in a single cell nucleus, for chromosomes 4, 6, 10, and 17, respectively. See Fig. 1 for detailed examples.

[b] Sample taken at relapse; all others at presentation.

[c] Cumulated percentage of very small clones (<1% each).

[d] Combinations with at least one nonsignificant aneuploidy were disregarded.

data also suggest that the frequency and number of these small aneuploid clones are greater at relapse than at disease presentation. Nonetheless, the differences observed between patients and their clinical status may be due to variation in cell type or in the quality of both the cytogenetic preparation and the hybridization. The diagnostic and prognostic significance of these numerous additional clones is of considerable clinical interest, however, and should be investigated in a large cohort of ALL patients.

Although CC will remain the method of choice for the genetic analysis of ALL at disease presentation for the global overview it provides, I-FISH constitutes a valuable complement to CC analysis both at diagnosis and during the course of the disease. Most important, the power of the method can be considerably increased by the use of an automated multicolor approach. The method reported here allows the simultaneous detection of four colors and, consequently, permits the identification of different

concurrent abnormalities of specific prognostic significance. It makes possible the rapid analysis of large numbers of nuclei while avoiding interobserver variability. It offers an optimal means of detecting small abnormal clones and consequently allows the evaluation of minimal residual disease in the presence of cytogenetic abnormalities that cannot be assessed by molecular techniques such as RT-PCR. Through its detection of several clones not identified by CC, the I-FISH approach provides a detailed cytogenetic picture that is much more complex than that revealed by CC alone, thus paving the way for further research on chromosomal heterogeneity in ALL and its significance in terms of pathogenesis and clinical management.

This particularly flexible automated system offers various possibilities for future methodological developments. Once optimized for a specific type of probe, the parameters can be adapted for use with other probe types, and the number of fluorochromes used in parallel can be increased by the use of additional filters, thus allowing the detection of other concurrent specific aneuploidies, such as in hypodiploidy/near-triploidy in ALL, or a combination of aneuploidies and structural rearrangements in both lymphoid and myeloid malignant hemopathies.

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2.3 Technical improvements and results

After completion of our first publication, our scope has been to bring further improvements to image analysis methodology.

For efficient FISH signal detection, the captured image has to be of high quality. In order to diminish the risk of the presence of a hallo around the nuclei (prejudiciable to image quality) due to the use of immersion oil and difficult slide clean out process, the 63x oil objective was replaced by a 40x dry objective. This change had no effect on the accuracy of FISH signal detection as the size of centromeric FISH signals was large enough to allow the use of a less powerful objective. Parameters had to be adapted and a new set of parameters specific to the 40x objective was established.

Another scope was to create a unique classifier able to select both BM and PB cells and not only BM cells as previously reported. For this purpose we used BM and PB cells from 3 patients with ALL and an aneuploidy for at least one of the chromosomes tested. A total of 3136 nuclei were classified in the training file, compared to 1381 BM cells in the first part of our study (53).

Based on the optimal set of parameters established for the use of a 40x objective, the software estimated the rates of false positives and false negatives for nucleus selection and the percentages of nuclei correctly counted (i.e. nuclei for which FISH signals were correctly counted for each fluorochrome taken separately). In order to verify the reliability of the error estimates made by the software, automatic search was performed on another 10 ALL patients (5 cases with a normal karyotype by CC and 5 patients with an aneuploidy for at least one of the chromosomes tested). For every single patient the machine scored 500 nuclei and for each patient the operator checked the results of the machine in a gallery displaying the 500 scored nuclei. Doing so we were able to evaluate the actual rate of false positive nuclei as well as the percentage of cells correctly counted for each colour channel. We challenged the accuracy of the nucleus selection process and of the FISH signal detection mechanism by comparing the values found by the technician and those provided by the software.

The time needed by the operator for checking and correction of a gallery finally including 500 correctly classified nuclei was measured.

Parameters for nucleus selection and FISH signal detection

The optimal set of parameters adapted for a 40x objective is reported in Table 1.

Table 1: Parameters for nucleus selection and FISH signal detection

Nucleus selection		FISH signal detection	FITC	Cy3	Cy3.5	DEAC
Relative DAPI intensity threshold for segmenting nuclei (%)	10	Spot measurement area (μm^2)	46	46	43	10
Minimum object area (μm^2)	37	Minimum spot distance (µm)	15	32	30	19
Maximum object area (μm^2)	600	Minimum relative spot intensity (%)	40	39	37	47
Maximum concavity depth	0.1	Maximum spot area (μm^2)	50	50	46	20
Maximum aspect ratio	1.8	Minimum spot contrast (‰)	10	10	10	10
Number of focal planes	1	Number of focal planes	5 (spacing of 0.2 µm)			

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole (blue, counterstain); FITC, fluorescein isothiocyanate (green); Cy3, cyanine dye 3(55); Cy3.5, cyanine dye 3.5 (magenta); DEAC, diethylaminocoumarin ($C_{27}H_{33}N_4O_{16}P_3$) (turquoise)

Accuracy of nucleus selection and FISH signal detection

The rate of false positives and false negatives for nucleus selection and the percentages of nuclei correctly counted for each color channel, as estimated by both software and operator, are reported in Table 2.

Table 2: Percentages of false negatives and false positives for nucleus selection and percentages of nuclei correctly counted for each fluorochrome

	Nucleus selection		**FISH signal detection**				**Number of nuclei classified**
	False positives (%)	False negatives (%)	% of nuclei correctly counted				
Fluorochromes	DAPI		FITC	Cy3	Cy 3.5	DEAC	
Software	7.0	19.6	68.0	67.0	72.1	63.8	3136
Operator	2.1	-	79.6	73,6	77,1	75,2	5000

Time analysis

The average time needed by the operator for checking and correction of a gallery finally including 500 correctly classified nuclei was of 1h and 16 minutes.

3 Chromosomal Instability and clonal heterogeneity at disease presentation in high hyperdiploid acute lymphoblastic leukemia

3.1 Summary

In the first part of our study, automated four colour I-FISH revealed, in all hyperdiploid ALL patients analyzed, the presence of a number of small abnormal clones in addition to well represented abnormal ones. This observation let surmise a high level of chromosomal instability and, consequently, karyotype variability in hyperdiploid ALL. Aneuploidy is a remarkable cytogenetic feature in hematopoietic malignancies and in solid tumours. Whether it is a cause or a consequence of malignant transformation remains an open question. In some tumours, aneuploidy may be stable, due to a chromosome missegregation occurring at some point during tumour development and leading to a stably propagating abnormal karyotype. More often, aneuploidy is the result of a chromosomal instability, characterized by an increase in the rate of gain or loss of whole chromosomes during cell division, leading to an unstable karyotype with cell-to-cell variation and multiple subclones and clonal heterogeneity. CIN was first studied and defined in colorectal cancers, then in other types of solid tumours and recently in myeloid malignant hemopathies but not in ALL.

To test for the presence of CIN in HeH ALL at disease presentation, 20 patients (10 HeH patients and 10 non HeH patients considered as negative controls) were analyzed by automated four colour I-FISH using centromeric probes for chromosomes 4, 6, 10 and 17.

In HeH ALL patients the proportion of abnormal cells ranged between 36.3% and 92.4% and various aneuploid populations were identified. The size of abnormal clones varied between < 1% and 33.4%. As a whole, the largest clones observed harbored a trisomy for both chromosomes 4 and 6 (33.4%) and for both chromosomes 6 and 10 (31.2%) respectively. In the majority of HeH ALL patients, clones with two or three concurrent aneuploidies were more frequent than those with a single trisomy suggesting a proliferative advantage of cells with two or more aneuploidies compared to those with a single one. Out of the larger clones identified both by CC and I-FISH, our observations revealed a number of additional clones of various size, some of them being very small. Very small abnormal clones consisted of abnormal clones whose size was inferior to 1%; they represented 2.2% to 8.6% of

the total. These observations reflected a high level of clonal chromosome heterogeneity in HeH ALL at initial presentation.

To investigate the nature and origin of this clonal heterogeneity, we determined average numerical CIN values for all 4 chromosomes together and for each chromosome and patient group. CIN values in HeH ALL were relatively high (range: 22.2 % to 44.7%) compared to those in controls (3.2% to 6.4%) accounting for numerical CIN and karyotypic instability. We concluded that numerical CIN may be at the origin of the high level of clonal heterogeneity monitored by I-FISH in HeH ALL at presentation, which brings further support to the potential role of CIN in tumour pathogenesis.

3.2 Personal contribution

- Carrying out of I-FISH analysis

- Determination of numerical CIN

- Data formatting

- Design of the article and writing of the first draft

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Clonal heterogeneity and chromosomal instability at disease presentation in high hyperdiploid acute lymphoblastic leukemia

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Abstract Although aneuploidy has many possible causes, it often results from underlying chromosomal instability (CIN) leading to an unstable karyotype with cell-to-cell variation and multiple subclones. To test for the presence of CIN in high hyperdiploid acute lymphoblastic leukemia (HeH ALL) at diagnosis, we investigated 20 patients (10 HeH ALL and 10 non-HeH ALL), using automated four-color interphase fluorescence in situ hybridization (I-FISH) with centromeric probes for chromosomes 4, 6, 10, and 17. In HeH ALL, the proportion of abnormal cells ranged from 36.3% to 92.4%, and a variety of aneuploid populations were identified. Compared with conventional cytogenetics, I-FISH revealed numerous additional clones, some of them very small. To investigate the nature and origin of this clonal heterogeneity, we determined average numerical CIN values for all four chromosomes together and for each chromosome and patient group. The CIN values in HeH ALL were relatively high (range, 22.2−44.7%), compared with those in non-HeH ALL (3.2−6.4%), thus accounting for the presence of numerical CIN in HeH ALL at diagnosis. We conclude that numerical CIN may be at the origin of the high level of clonal heterogeneity revealed by I-FISH in HeH ALL at presentation, which would corroborate the potential role of CIN in tumor pathogenesis.

1. Introduction

Although aneuploidy is a remarkably common cytogenetic feature in human cancers, whether it is a cause or a consequence of malignant transformation remains a matter of debate [1]. Some tumors reveal a stable aneuploidy, due to a chromosome missegregation occurring at some point during tumor development and leading to a stably propagating abnormal karyotype. More often, however, aneuploidy results from chromosomal instability (CIN), which is characterized by an increase in the rate of loss or gain of whole chromosomes during mitosis, leading to unstable karyotypes with cell-to-cell variation and multiple related and unrelated subclones [2,3]. Although the two are sometimes equated with one another, aneuploidy and CIN are not synonymous. Aneuploidy describes the state of an abnormal chromosome number, whereas CIN refers to the rate of change in chromosome number.

Chromosomal instability defined as the percentage of cells with a nonmodal chromosome number was first studied in colorectal cancers [4]. It has since been further investigated in other types of solid tumors [1,4−7], and recently also in myeloid malignant hemopathies [8], but it has not previously been studied in acute lymphoblastic leukemia (ALL).

High hyperdiploidy (HeH) with 51−67 chromosomes occurs in nearly 25% of pediatric B-cell precursor ALL cases [9]; it is less frequent in adult cases, and is rarely found in T-cell or mature B-cell ALL. In HeH ALL, the pattern of chromosome gains is clearly nonrandom, with extra copies of chromosomes X, 4, 6, 10, 14, 17, 18, and 21 occurring much more frequently than extra copies of other chromosomes [10].

The origin of aneuploidy in HeH ALL, along with the question of whether HeH ALL is karyotypically stable, has been a matter of conjecture [9]. Based on conventional cytogenetics (CC), 15−20% of childhood HeH ALL cases present subclones differing from the stemline by additional

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Table 1
Demographic and cytogenetic findings at disease presentation for 10 study patients and 10 negative control subjects with acute lymphoblastic leukemia

Case	Age, yr/Sex	G-banding karyotype
		Study patients: abnormal I-FISH findings
2385/95	26/M	52~57,XY,+X,+5,+6,add(7)(p13~15),+9,+10,+11,−13,+18,+21,+21,+22,+22,+der(?)t(1;?)(q12~21;?)[cp6]/46,XY[10]
683/99[a]	4/M	64,XY,+X,dup(1)(q21q32),+2,+4,+5,+6,+7,+?8,+?10,?der(11)(p?),+11,+12,+14,+14,+17,+18,+21,+21,+21,+22[3]/46,XY[14]
694/99[a,b]	18/M	55,XY,+X,+4,+6,+9,+14,+17,+18,+21,+21[2]/55,idem,add(19)(p13)[5]/46,XY[1]
387/00[a]	5/M	56,XY,+X,+6,+10,+10,+14,+18,+18,+21,+21,+mar[7]/46,XY[4]
454/00[a]	2/M	54~55,XY,+X,+6,inc[10]/46,XY[12]
192/01[a]	3/M	55,XY,+X,+4,+6,+10,+14,+17,+18,+21,+mar[14]/46,XY[22]
131/05	21/F	52,XX,+X,+4,+10,+11,+14,?der(16)t(1;16)(p22;q22),del(17)(p11.2),−21,+mar1,+mar2[4]/46,XX[36]
241/05	51/M	54,XY,+X,+2,+4,+4,+6,inv(9)(p11q13)c,t(9;22)(q34;q11.2),+21,+21,+der(22)t(9;22)[3]/55,idem,?del(2)(q3?2q3?5),add(2)(q35~37),+18[4]/46,XY,inv(9)c[12]
88/06	18/M	55,XY,+X,dup(1)(q25q32),+4,+6,9p?,+10,?10,+14,+17,+18,+21,+21[25]/46,XY[5]
363/09	66/F	55,XX,+X,+6,+10,+14,+17,+18,+18,+21,+21[7]/46,XX[3]
		Negative control subjects: normal I-FISH findings
199/07[c]	60/F	46,XX,der(9)idic(9)(p?13)t(9;22)(q34;q11.2),der(22)t(9;22)[15]/46,XX[3]
446/07[c,d]	55/F	47~48,XX,+X,inv(9)(p11q13),+21[cp4]/46,XX,inv(9)(p11q13)[16]
619/07[c]	42/F	47,XX,t(4;11)(q21;q23),+21[20]
1085/07[c]	44/F	46,XX,t(4;11)(q21;q23)[10]/46,XX[5]
1159/07[c,e]	21/F	46,XX[8]
600/08[c]	39/F	46,XX,der(19)t(1;19)(q23;p13.3)[8]/46,idem,dup(1)(q21q32)[2]/46,XX[2]
990/08[c,e]	46/M	46,XY[7]
1198/08[c]	34/M	44,XY,+X,−3,−7,−9,−16,+22[4]/46,XY[6]
1538/08[c]	32/F	46,XX,t(4;11)(q21;q23)[10]
284/09[c,f]	27/M	46,XY,t(11;19)(q23;p13.3)[1]/46,XY[15]

Abbreviations: I-FISH, interphase fluorescence in situ hybridization.

Study patients had high hyperdiploid acute lymphoblastic leukemia with trisomy for at least one of chromosomes 4, 6, 10, and 17 according to conventional cytogenetic analysis. The negative control subjects had acute lymphoblastic leukemia with pseudodiploidy, low hyperdiploidy. or a few normal metaphases without evidence of extra copies of chromosomes 4, 6, 10, and 17 by conventional cytogenetics.

[a] Patients reported in part by Blandin et al., 2008 [11].

[b] Patient included in the LALA-94 study [13].

[c] Patients included in the GRAALL 2005 study (NCT00327678 at http://www.clinicaltrials.gov).

[d] The inv(9)(p11q13) most probably is constitutional, given that it is present in aneuploid as well as in nonaneuploid cells. However a "c" was not added to the description, because the constitutional karyotype of peripheral blood T lymphocytes was not studied in this respect.

[e] Would be considered a failure according to GRAALL 2005 cytogenetic guidelines.

[f] Only one abnormal metaphase was observed; however, the result was considered meaningful because of an *MLL* rearrangement detected by FISH.

chromosomes or structural defects. Given the presence of a single subclone in most cases, Paulsson and Johansson [9] suggested that clonal evolution may be more frequent than CIN in these cases. In contrast with previously published data suggesting a cell-to-cell variation in HeH ALL at diagnosis [11,12], however, their own interphase fluorescence in situ hybridization (I-FISH) results did not reveal significant variation in the cases studied—evidence of the need for further studies. Here we report on recent data obtained by four-color I-FISH that bring further evidence of a high level of instability of chromosomes 4, 6, 10, and 17 in HeH ALL patients at initial presentation.

2. Materials and methods

2.1. Patients

Ten patients with HeH ALL established by CC and with trisomy for at least one of chromosomes 4, 6, 10, and 17 were included in the study group. Five cases were reported in part in a previous methodological article [11]. Ten ALL patients with pseudodiploidy, low hyperdiploidy, or a few normal metaphases without evidence of extra copies of chromosomes 4, 6, 10, or 17 by CC served as negative controls. Cytogenetic findings are given in Table 1. Because of the small number of patients in this study, no distinction was made for ALL subtype or for pediatric versus adult cases (4 children and 16 adults).

Patients were referred between 1995 and 2009 to our laboratory from the hematology departments of the University Hospitals of Lausanne, Basel, Bern, Geneva, and Zurich and of the cantonal and regional hospitals of St. Gallen, Lucerne, Aarau, Sion, and Bellinzona. All patients received induction and consolidation chemotherapy and some also received eventual stem cell transplantation. One patient was enrolled in the LALA-94 study [13], and 10 patients (serving as negative controls) were enrolled in the GRAALL 2005 study (NCT00327678 at http://www.clinicaltrials.gov). The Group for Research on Adult Acute Lymphoblastic Leukemia (GRAALL) includes the former France–Belgium Group for

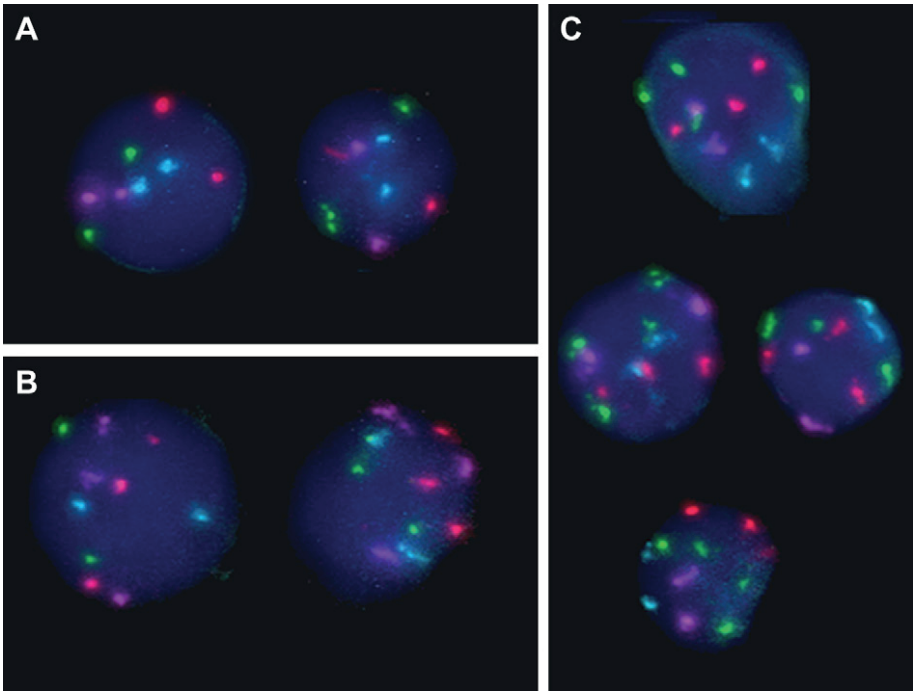

Fig. 1. Interphase nuclei were hybridized with labeled centromeric probes specific for chromosomes 4 (green), 6 (red), 10 (magenta), and 17 (turquoise): pretreatment bone marrow from negative control (A) and high hyperdiploid acute lymphoblastic leukemia (B,C) patients.

Lymphoblastic Acute Leukemia in Adults (LALA), the French Western–Eastern Group for Lymphoblastic Acute Leukemia (GOELAM), and the Swiss Group for Clinical Cancer Research (SAKK).

Ethical approval for this project was obtained in accordance with the guidelines of the local Ethical Review Board.

2.2. Conventional cytogenetics and four-color I-FISH

Conventional cytogenetics (G-banding) and I-FISH analyses using centromeric probes specific for chromosome 4 (p-4n1/4; kindly provided by Prof. Mariano Rocchi, University of Bari, Italy) and for chromosomes 6, 10, and 17 (D6Z1, D10Z1, and D17Z1, respectively; American Type Culture Collection–ATCC, Manassas, VA) were performed on pretreatment bone marrow samples (Fig. 1). Probes were directly labeled by nick translation with four different fluorochromes (FITC, Cy3, Cy3.5, and DEAC) that have emission wavelengths sufficiently distinct from each other. The conventional and FISH methods were used as previously described [11].

2.3. Automated analysis

Automated four-color I-FISH analysis was performed with the scanning system Metafer 4/MetaCyte (MetaSystems, Altlussheim, Germany) according to a modification of our previously reported method that includes use of a motorized epifluorescence microscope (AxioImager Z1; Zeiss, Feldbach, Germany) equipped with a 40× objective (Zeiss) [11,14–16]. Optimal values of the parameters for nucleus selection and fluorescent signal detection were adapted in this respect (Tables 2 and 3).

2.4. Chromosomal instability

We determined the modal chromosome number for each chromosome tested and calculated the percentage of cells whose number differs from the modal value (CIN), according to Lengauer et al. [4]. Average CIN was first determined for all four chromosomes together and then for each selected chromosome, according to Lingle et al. [6] and Miyoshi et al. [7].

2.5. Statistical analysis

In each sample, a minimum of 500 nuclei was scored. Significant aneuploidies were determined based on cutoff values established according to the Poisson distribution, as previously defined [11]. For all patients, combinations of aneuploidies were considered relevant when at least one aneuploidy was determined to be significant.

Table 2
Parameters for nucleus selection

Parameter	Value
Relative DAPI intensity threshold for segmenting nuclei, %	10
Minimum object area, μm^2	37
Maximum object area, μm^2	600
Maximum concavity depth	0.1
Maximum aspect ratio	1.8
Number of focal planes	1

Abbreviation: DAPI,4',6-diamidino-2-phenylindole (blue, counterstain).

Table 3
Parameters for FISH signal detection

Parameter	FITC	Cy3	Cy3.5	DEAC
Spot measurement area, μm^2	46	46	43	10
Minimum spot distance, μm	15	32	30	19
Minimum relative spot intensity, %	40	39	37	47
Maximum spot area, μm^2	50	50	46	20
Minimum spot contrast, ‰	10	10	10	10
Number of focal planes	5 (at a spacing of 0.2 μm)			

Abbreviations: Cy3, cyanine dye 3 (red signal); Cy3.5, cyanine dye 3.5 (magenta signal); DEAC, diethylaminocoumarin ($C_{27}H_{33}N_4O_{16}P_3$) (turquoise signal); FITC, fluorescein isothiocyanate (green signal).

3. Results

For several of our patients (and as is generally the case in ALL), the number of abnormal metaphases available for CC analysis was relatively small, because of the low proliferation rate of abnormal cells in vitro, poor chromosome quality, or both factors. All abnormal metaphases available were karyotyped, but sometimes only partially, in which case composite or incomplete karyotypes are reported. Despite the limited number of abnormal metaphases analyzed in some cases, all abnormal clones reported here fulfill ISCN 2009 clonality criteria [17] and should be considered significant. Nonetheless, abnormal clones observed in these cases may account for only a small fraction of the tumor karyotype diversity.

In the negative control group of patients, no significant aneuploidy for chromosomes 4, 6, 10, and 17 was detected by I-FISH, which confirms results obtained by CC.

In the study patients, the number of abnormal cells karyotyped ranged between 3 and 25, and I-FISH revealed a proportion of total abnormal cells ranging from 36.3% to 92.4% of scored nuclei. Various combinations of aneuploidies were identified (Table 4). All clones detected by CC were also observed by I-FISH, but I-FISH revealed numerous additional clones in all patients, indicative of a high level of heterogeneity at disease presentation in HeH ALL patients. The size of abnormal clones varied between <1% and 33.4%. Very small abnormal clones (<1%) represented 2.2–8.6% of the total (reported as Others in Table 4).

Overall, the largest clones observed harbored both trisomies 4 and 6 (33.4%) and both trisomies 6 and 10 (31.2%). The proportion of cells with a relevant tetrasomy generally was very small (<1–3.0%), except that in the case of the Philadelphia-positive patient (case 241/05) the clone with tetrasomy 4 and trisomy 6 amounted to 13.6%.

Average CIN values determined for all four chromosomes together ranged from 22.2% to 44.7% in study patients and from 3.2% to 6.4% in the negative control group (Table 5).

Table 4
Clones involving chromosomes 4, 6, 10, and 17 in patients with high hyperdiploid acute lymphoblastic leukemia

	Clones at disease presentation, %									
	2385/95	683/99	694/99	387/00	454/00	192/01	131/05	241/05	88/06	363/09
Normal	24.20	63.73	7.60	17.47	20.55	53.07	18.40	53.80	51.00	9.80
+4	1.20	3.53	7.80	2.80	2.40	7.40	8.60	9.00	3.80	—
+6	17.20	3.93	10.60	30.87	***27.02***	7.13	—	4.00	2.60	6.20
+10	15.00	3.07	—	2.67	2.13	1.80	15.80	—	2.20	8.60
+17	—	2.53	1.13	2.27	5.34	7.33	5.40	—	2.60	7.60
+6,+17	—	—	5.07	3.27	14.08	1.53	—	—	—	7.40
+6,+10	***31.20***	2.27	—	15.07	5.34	—	—	—	4.00	20.20
+4,+17	—	1.33	4.60	—	—	2.87	4.80	—	1.20	—
+4,+6	—	2.07	33.40	6.47	3.80	4.53	—	8.80	2.60	—
+4,+10	—	1.87	—	—	—	—	***16.80***	—	3.60	—
+10,+17	—	—	—	—	1.33	—	9.20	—	1.20	10.80
+4,+10,+17	—	1.00	—	—	—	—	14.00	—	2.40	—
+4,+6,+10	—	***5.07***	2.13	3.53	—	1.67	—	—	5.20	—
+6,+10,+17	1.00	—	—	2.20	3.40	—	—	—	1.80	***23.20***
+4,+6,+17	—	1.00	***23.07***	—	2.74	3.20	—	—	1.60	—
+4,+6,+10,+17	—	4.13	1.07	—	1.07	***2.00***	—	—	***7.00***	1.00
+4,+4	—	—	—	—	—	—	—	8.60	1.20	—
+4,+4,+6	—	—	—	—	—	—	—	***13.60***	—	—
+4,+4,+6,+10	—	—	—	—	—	—	—	—	1.40	—
+6,+6	1.40	—	—	2.20	1.20	—	—	—	—	—
+6,+6,+10	3.00	—	—	1.27	—	—	—	—	—	—
+6,+10,+10	—	—	—	***1.33***	—	—	—	—	—	—
+6,+10,+17,+17	—	—	—	—	—	—	—	—	—	1.20
+4,+10,+17,+17	—	—	—	—	—	—	1.20	—	—	—
+6,+17,+17	—	—	—	—	1.20	—	—	—	—	—
Others[a]	5.80	4.47	3.53	8.60	8.40	7.47	5.80	2.20	4.60	4.00

The 10 study patients are further detailed in Table 1.
Clones detected by both interphase fluorescence in situ hybridization and conventional cytogenetics (Table 1) are highlighted in bold italic type.
[a] Cumulative percentage for multiple very small clones (<1% each).

Table 5
Chromosomal instability at disease presentation in 10 study patients with high hyperdiploid acute lymphoblastic leukemia and in 10 negative control subjects

	Modal number (cells differing from modal number, %)				
Case	Chr 4	Chr 6	Chr 10	Chr 17	Average
	Study patients				
2385/95	2 (5.80)	3 (48.00)	3 (47.60)	2 (9.20)	2.5 (27.65)
683/99	2 (24.87)	2 (22.93)	2 (22.80)	2 (18.33)	2 (22.23)
694/99	3 (26.47)	3 (22.60)	2 (6.60)	2 (39.67)	2.5 (23.84)
387/00	2 (21.27)	3 (34.13)	2 (32.33)	2 (15.80)	2.25 (25.88)
454/00	2 (16.88)	3 (38.29)	2 (19.61)	2 (38.29)	2.25 (28.27)
192/01	2 (29.33)	2 (26.00)	2 (12.00)	2 (24.27)	2 (22.90)
131/05	3 (51.00)	2 (NA)	3 (41.40)	2 (41.80)	2.5 (44.73)
241/05	2 (44.00)	2 (29.80)	2 (NA)	2 (NA)	2 (36.90)
88/06	2 (35.20)	2 (33.80)	2 (35.00)	2 (25.60)	2 (32.40)
363/09	2 (NA)	3 (38.80)	3 (33.40)	3 (49.00)	2.75 (40.40)
	Negative control subjects				
199/07	2 (6.20)	2 (4.20)	2 (4.80)	2 (7.00)	2 (5.55)
446/07	2 (6.00)	2 (5.00)	2 (4.80)	2 (9.60)	2 (6.35)
619/07	2 (4.90)	2 (2.20)	2 (2.50)	2 (7.10)	2 (4.18)
1085/07	2 (5.50)	2 (1.80)	2 (3.80)	2 (7.00)	2 (4.53)
1159/07	2 (5.40)	2 (3.80)	2 (4.80)	2 (9.60)	2 (5.90)
600/08	2 (3.80)	2 (4.20)	2 (4.20)	2 (7.60)	2 (4.95)
990/08	2 (6.80)	2 (5.20)	2 (3.40)	2 (9.00)	2 (6.10)
1198/08	2 (5.60)	2 (5.00)	2 (4.40)	2 (6.40)	2 (5.35)
1538/08	2 (3.40)	2 (2.40)	2 (1.40)	2 (5.60)	2 (3.20)
284/09	2 (5.80)	2 (4.40)	2 (3.20)	2 (5.20)	2 (4.65)

Abbreviations: NA, not aneuploid.
Study patients and control subjects are further detailed in Table 1.

The distribution of CIN values by chromosome and patient group is presented in Figure 2. Based on data obtained, samples were subdivided into two distinct subgroups, one being the HeH ALL patients with relatively high CIN values (range, 27.9–32.7%) and the other the negative control subjects with much lower values (range, 3.7–7.4%).

4. Discussion

Most FISH studies indicate that using two different probes is sufficient to distinguish diploid from aneuploid clones. Nonetheless, using more than two probes has the advantage of allowing recognition of additional clonal populations and identification of high clonal heterogeneity [6,18]. In this respect, the two notable qualities of automated four-color I-FISH are that a large number of nuclei can be observed and a variety of clonal aneuploid combinations identified, even if present in a small number of cells. Along with the larger clones identified both by CC and I-FISH, the I-FISH observations revealed a number of additional clones of various size, some of them being very small. The present findings corroborate our previous results and the widely reported discrepancies between CC and I-FISH, mainly due to the differences in the sensitivity of both approaches [11], and also demonstrate a high level of clonal chromosome heterogeneity in HeH ALL at initial presentation.

Because high clonal heterogeneity is likely due to an aneuploidy resulting from a chromosomal instability, we calculated the CIN values for both groups of patients. Initially calculated to test the rate of change in chromosome number of different colorectal cell lines through a number of generations, CIN values have since been used to test for chromosomal instability in cell lines, solid tumors, and myelodysplastic syndromes at first presentation [6–8,19]. Our data revealed genuine differences in the rates of chromosome gain or loss in patients with HeH ALL, compared with negative controls. In study patients, CIN values were much higher than those in the negative control group. The control group percentages were comparable to the background numbers observed by Lengauer et al. [4] in near-diploid cell lines and in normal lymphocytes, as well as to the numerical CIN levels detected by Heilig et al. [8] in their control patients (Table 5). It thus appears that HeH ALL has numerical CIN at disease presentation. Our present findings are consistent with karyotypic and FISH observations, suggesting that HeH ALL may be genetically unstable [12].

This chromosomal instability is probably responsible for the karyotypic heterogeneity detected by I-FISH and for the simultaneous presence of numerous related and unrelated clones, some of them undetected in CC investigation because of their small size or low proliferation rate. In 7 of the 10 study patients, clones with two or three concomitant aneuploidies were more frequent than those with a single trisomy, illustrating a possible proliferative advantage of cells with two or more aneuploidies, relative to those with a single aneuploidy (Table 4). Considering the nonrandom pattern of additional chromosomes, certain chromosome combinations may confer a proliferative advantage to leukemic cells and thus lead to an increased capacity of clonal expansion and clonal evolution.

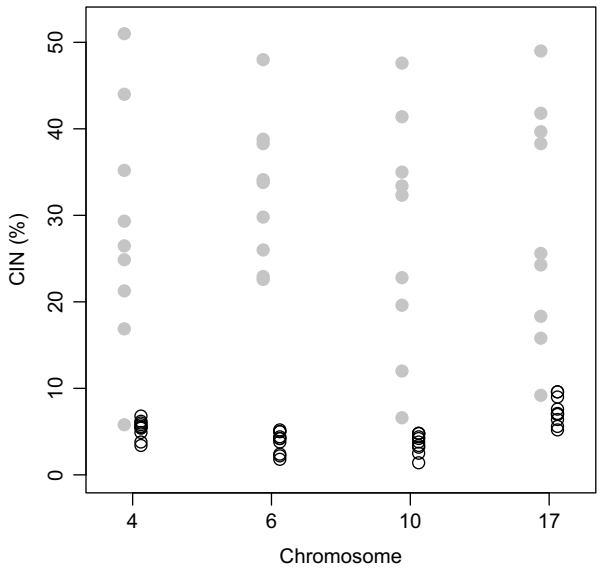

Fig. 2. Distribution of chromosomal instability (CIN) values for chromosomes 4, 6, 10, and 17 in high hyperdiploid acute lymphoblastic leukemia patients (solid symbols) and in negative control patients (open symbols) at disease presentation.

Further studies are needed to determine whether CIN is a general feature of HeH ALL, how it behaves during disease evolution, and to what extent it may affect outcome and so constitute additional useful information for prognostic assessment and therapy decision making.

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4 Chromosomal instability and clonal heterogeneity during the course of disease in high hyperdiploid acute lymphoblastic leukemia

4.1 Summary

In the second part of our work we suggested that numerical chromosome instability may be at the origin of the high level of clonal chromosomal aneuploidy observed in high hyperdiploid ALL at initial presentation. The next step of our study has been to investigate the evolution of this clonal heterogeneity and chromosomal instability during the course of the disease in a cohort of 10 HeH ALL patients whose age ranged from 15 and 54 years. Thirty-four BM samples were analyzed at diagnosis, hematological complete remission and at relapse by automated four color I-FISH using centromeric probes for chromosomes 4, 6, 10 and 17. Out of these 34 samples, 33 were also investigated by CC. At initial presentation, the largest abnormal clone observed harbored trisomy 10 alone (45.8%) and was detected in the single patient with T-ALL. In patients with B ALL the largest clones observed at diagnosis presented both trisomies 4 and 6 (34.3%) and both trisomies 6 and 10 (31.2%) respectively. High levels of clonal heterogeneity were observed during the course of the disease, at relapse more particularly. Clones detected at initial diagnosis generally reappeared at relapse, some of them being larger due to proliferative advantage, others smaller because of selective pressure; in most cases these formerly detected clones were accompanied by newly generated ones. In our previous study we demonstrated that, at initial presentation, CIN values were higher in HeH ALL than in non HeH ALL. Despite the small number of our study patients, present data reveal a significant correlation between the number of abnormal clones and CIN values suggesting that the higher the number of abnormal clones, the larger the instability. Different mechanisms have been evoked to account for HeH in ALL. Our previous and present I-FISH data revealed that HeH is accompanied by a high level of clonal heterogeneity both at diagnosis and during the course of the disease, thus raising the question of the origin of this precocious heterogeneity and its evolution. Although karyotypic heterogeneity most probably result from chromosome instability, the biological operating processes remain a matter of debate.

4.2 Personal contribution

- Carrying out of I-FISH analysis

- Determination of numerical CIN

- Data formatting

- Design of the article and writing of the first draft

4.3 Article (submitted)

Evolution of clonal heterogeneity and chromosomal instability during the course of the disease in high hyperdiploid acute lymphoblastic leukemia.

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Abstract

High hyperdiploid acute lymphoblastic leukemia (HeH ALL) at diagnosis reveals high levels of clonal aneuploidy heterogeneity likely due to numerical chromosome instability (CIN). To study the evolution of these cytogenetic features during disease course, more particularly at relapse, we investigated 10 adult patients with HeH ALL by four colour I-FISH. Thirty-four samples were analysed (presentation: 7, hematologic remission: 19, relapse: 8), status of heterogeneity and CIN level were determined. Four colour I-FISH further demonstrated its capacity to detect very small clones and abnormal clone diversity, proving useful for clinical assessment. High levels of clonal heterogeneity were also observed during the course of the disease, at relapse in particular. Clones detected at presentation generally reappeared at relapse, mostly accompanied by newly generated ones. Whereas the mean total number of abnormal clones did not clearly differ between diagnostic and relapse samples, the range of their variation did, being much larger at relapse. Despite the small number of patients, data reveal a significant correlation between number of abnormal clones and CIN, suggesting that the higher the instability, the larger the number of abnormal clones. Whereas clonal heterogeneity and its evolution most probably result from chromosome instability, operating processes remain conjectural.

1. Introduction

Chromosome aberrations play a major role in the diagnosis and risk assessment of acute lymphoblastic leukemia (1-3). High hyperdiploidy with 51-67 chromosomes (HeH) is one of the largest cytogenetic subsets of childhood ALL where it occurs in 25% to 30% of B cell precursor ALL and is associated with low risk. It is less common in adult B cell precursor ALL for whom outcome is improved compared to the other cytogenetic groups, but not as favorable as in children (4). It is rarely found in T cell or mature B cell ALL.

In HeH ALL, chromosome gain is frequently nonrandom, extracopies of chromosomes X, 4, 6, 10, 14, 17, 18 and 21 occurring much more frequently than extracopies of other chromosomes (5-7). HeH including concurrent trisomies for chromosomes 4, 10 and 17 with no associated poor risk clinical features was shown to be associated with superior outcome and the presence of this triple trisomy is currently used for risk stratification by the Children's Oncology Group (8).

Despite the generally good prognosis of HeH ALL, a proportion of cases relapse and most of them experience very poor outcome. Although recent studies using single nucleotide polymorphism array and mutation analysis demonstrated that, in childhood HeH ALL, structural changes were significantly more common at relapse than at initial presentation, they were not able to identify single and recurrent genetic changes that may be specifically associated with an increased risk of eventual relapse (9-11).

Automated four color interphase FISH (I-FISH) revealed a high level of clonal chromosome aneuploidy heterogeneity in HeH ALL compared with non-HeH ALL, at initial presentation(12). Numerical CIN was supposed to be at the origin of this high level of clonal heterogeneity, which would corroborate the potential role of CIN in both malignant transformation and tumour progression. Clinically, numerical CIN proved to be associated with poor prognosis in lung cancer (13) and, as reported recently, also in myelodysplastic syndromes (MDS)(14).

We investigated a series of HeH ALL adult patients by four colour I-FISH with the aim, first to define the status of clonal chromosome aneuploidy heterogeneity and the CIN level of chromosomes 4, 6, 10, and 17 in paired diagnosis and follow up samples, then to study the evolution of these cytogenetic features during the course of the disease and more particularly at relapse, and finally to test for a possible relationship between the number of abnormal clones and CIN level.

2. Materials and methods

2.1. Patients

Ten adult patients with HeH ALL (51-67chromosomes) established by conventional cytogenetics (CC) were investigated at presentation and/or during the course of the disease (remission and/or relapse). Patients were referred between 1995 and 2009 to our laboratory from the hematology departments of the University Hospitals of Basel, Zurich, Bern, Lausanne, of the cantonal and regional Hospitals of Sankt-Gallen, Aarau, Sion, Mendrisio, Bellinzona and of the Clinique of Genolier. One patient was enrolled in the Swiss Group for Clinical Cancer Research (SAKK) ALL 33-86/90 studies (15) (M. Wernli, personal communication) and one patient in the GRAALL 2005 study (http://www.clinicaltrials.gov/: NCT00327678). GRAALL represents the Group for Research on Adult Acute Lymphoblastic Leukemia (GRAALL). GRAALL includes the former France-Belgium Group for

Lymphoblastic Acute Leukemia in Adults (LALA), the French Western-Eastern Group for Lymphoblastic Acute Leukemia (GOELAM), and the SAKK.

Ethical approval for this project was obtained in accordance with the guidelines of the local Ethical Review Board.

2.2. Conventional cytogenetics, automated four color I-FISH and chromosome instability

Conventional cytogenetics (G-banding) was performed on bone marrow (35 samples) or peripheral blood (2 samples) cells.

Four color I-FISH using centromeric probes specific for chromosome 4 (p-4n1/4, kindly provided by Prof. Mariano Rocchi, University of Bari, Italy), chromosomes 6, 10 and 17 (D6Z1, D10Z1 and D17Z1 respectively; American Type Culture Collection - ATCC, Manassas, VA) was performed on bone marrow cells (34 samples). Chromosomes 4, 6, 10, and 17 were chosen based on their high frequency in HeH ALL and the recommendations of the Groupe Francophone de Cytogénétique Hématologique for aneuploidy screening in ALL. Probes were directly labeled by nick translation with four different fluorochromes (Cy3, Cy3.5, DEAC and FITC). Automated four colour I-FISH analysis was realized with the scanning system Metafer 4/MetaCyte (MetaSystems, Altlussheim, Germany) using a motorized epifluorescence microscope (AxioImager Z1; Zeiss, Feldbach, Germany) equipped with a 40x objective (Zeiss). In each sample, a minimum of 500 interphase nuclei was scored, except in one case (331/06) in which only 350 nuclei could be classified.

Significant aneuploidies were determined based on cutoff values previously defined according to the Poisson distribution. For all the patients, combinations of aneuploidies were considered relevant when at least one aneuploidy was determined to be significant. Average CIN was determined for all four chromosomes together and then for each selected chromosome.

Conventional cytogenetics, FISH analysis and CIN determination were performed as previously defined (12, 16).

The correlation between the number of abnormal clones and the level of CIN at different stages of the disease was measured using the Spearman correlation coefficient.

3. Results

3.1. Cytogenetic and clinical findings (Table1)

There were 4 women and 6 men ranging in age from 15 to 54 years at initial diagnosis. Five patients had a precursor B ALL, 2 had a Philadelphia-positive B ALL and one patient had a T ALL. Two patients presented a B ALL whose phenotype was not further determined.

Patients were subdivided into 2 groups. The first group comprised 5 patients (patients 1 to 5) with HeH at diagnosis and no HeH as demonstrated by CC and I-FISH during follow up (complete hematological remission). The second group consisted of 5 patients (patients 6 to 10) with HeH or no result at initial diagnosis and HeH residual clones or HeH cytogenetic relapses as demonstrated by CC and I-FISH during the course of the disease (bone marrow hematological remission or relapse).

As a whole, 37 analyses were performed, either by CC alone (3), by I-FISH alone (1) or by CC and I-FISH simultaneously (33). An additional analysis was performed by CC at initial presentation in another laboratory (Brasil) with no detailed result available (patient 7).

3.2. Percentage of abnormal cells detected by I-FISH and clonal chromosome aneuploidy heterogeneity

Disease presentation:

Out of the 10 analyses performed by CC, 8 revealed an abnormal karyotype and 2 did not reveal evidence of a chromosome abnormality (patient 6: 9 metaphases analysed and patient 8: 20 metaphases analysed from peripheral blood). In these 2 cases no material was available for I-FISH analysis (Table 1).

In the 7 cases analysed by I-FISH, the percentages of abnormal cells ranged between 46.2% and 90.2% (Table 2). The number of abnormal clones varied from 8 (including 5 clones whose size was <1%) to 29 (13) and abnormal clones <1% represented a total percentage varying between 2.2% and 5.8% (Table 2: Others).

Hematologic (bone marrow) complete remission:

One patient (patient 6) with an isolated central nervous system relapse and no evidence of relapse in the bone marrow was included in this group (sample 1001/03) (Table 1).

Out of the 19 analyses performed by CC, 18 revealed a normal karyotype and 1 presented an isolated t(2;11)(p10;p10) not observed at initial presentation (Table 1). Out of the 19 analyses performed by I-FISH, 3 revealed aneuploid clones whose proportions represented a total of 13% (19 abnormal clones including 16 clones < 1%), 1.4% and 0.6% of scored nuclei respectively (patient 8)(Table 2: Others). In these two last cases, the number of abnormal clones varied from 3 (540/09) to 7 (29/09) and only tetrasomies were monitored (tetrasomy for chromosomes 6, 10 and 17 in sample 29/09 and tetrasomy10 in sample 540/09).

Hematologic relapse:

Out of the 9 analyses performed by CC, 6 revealed a HeH, 2 a normal karyotype (20 metaphases analysed) and 1 failed (Table 1). In the 8 analyses performed by I-FISH, the percentages of abnormal cells ranged between 3.2% and 78.3% (Table 2). The number of abnormal clones varied from 2 (including 1 clone whose size is <1%) to 56 (43). Abnormal clones <1% represented a total percentage varying between 0.6% (158/98) and 11.5 % (1527/97) (Table 2: Others).

3.3. Evolution of clonal heterogeneity in patients with hematologic and cytogenetic relapse (Fig. 1)

The mean total numbers of abnormal clones at initial diagnosis (19, median: 20) and at relapse (22, median: 12) were rather close to each other, as were those of clones <1% (12, median 13, versus 16, median 9 respectively). However their ranges showed a clear difference, extending at presentation from 8 to 29 (clones <1%: 5 to 15) and at relapse from 2 to 56 (clones <1%: 1 to 43) (Table 2).

The evolution of the number of abnormal clones from presentation to relapse could be monitored in 2 patients (patients 9 and 10) (Fig. 1). For patient 9, the clone number was 20 at presentation, it increased to 56 at first relapse, decreased to 12 at second relapse and then to 2 at third relapse (very few abnormal cells). For patient 10, the number of abnormal clones did not show an appreciable change between presentation and relapse.

The evolution of the number of abnormal clones during the course of the disease (no material for I-FISH analysis at diagnosis) could be monitored in 2 other patients (patients 7 and 8). For patient 7, the

numbers of abnormal clones were not clearly different between the two analyses performed at 2 weeks interval. For patient 8 (Fig.1), the number of abnormal clones increased from 19 (13% abnormal nuclei, bone marrow complete hematological remission) to 40 at relapse.

The dynamics of individual abnormal clones from presentation through disease progression was investigated in 3 patients, in two cases from presentation to relapse (patients 9 and 10), in another one from complete hematological remission to relapse (patient 8). The study focused on abnormal clones which represented $\geq$1% of scored nuclei at least once during the course of the disease (Fig.1). For patient 9, all clones >1% at first relapse already occurred at presentation, some of them <1% at that time. Two clones >1% at diagnosis regressed to <1% at first relapse and 3 newly generated clones were observed. At second relapse, no newly generated clone appeared and a number of formerly observed clones disappeared. At third relapse, only one abnormal clone was detected, representing a very small proportion of scored nuclei. For patient 10, all three abnormal clones >1% detected at presentation were found again at relapse, in the absence of newly generated ones, thus showing a high level of stability. For patient 8, all clones >1% at relapse already occurred at presentation, some of them <1% at that time. Eight newly generated clones were observed. None of the combinations detected at presentation and at relapse were found in eventual complete remission.

3.4. Chromosome instability

Disease presentation:

Average CIN for all four chromosomes together varied from 17.1% (patient 10, 388/02) to 34.6% (patient 1, 131/05) (Table 3). CIN percentages of chromosomes 4, 6, 10 and 17 ranged respectively between 5.2% (patient 10, 388/02) and 51.0% (patient 1, 131/05), 4.0% (patient 1, 131/05) and 48.0% (patient 9, 2385/95), 3.8% (patient 4, 241/05 and patient 5,1086/07) and 50.6% (patient 10, 388/02), and 7.0% (patient 4, 241/05) and 49.0% (patient 3, 363/09) (Table 3).

Hematologic (bone marrow) complete remission:

Average CIN for all four chromosomes together varied from 3.3% (patient 3, 877/09) to 6.9% (patient 8, 1282/07) (Table 3). CIN percentages of chromosomes 4, 6, 10 and 17 ranged respectively between 1.4% (patient 2, 178/09) and 8.2% (patient 8, 1282/07), 2.0% (patient 6, 688/02) and 7.1% (patient 8, 1282/07), 1.60% (patient 6, 688/02) and 5.2% (patient 8, 29/09), and 3.2% (patient 3, 877/09) and 8.6% (patient 6, 1001/03) (Table 3).

Hematologic relapse:

Average CIN for all four chromosomes together varied from 5.5% (patient 9, 158/98) to 41.8% (patient 9, 1527/97) (Table 3). CIN percentages of chromosomes 4, 6, 10 and 17 ranged respectively between 4.6% (patient 10, 95/03) and 51.1% (patient 9, 1527/97), 2.6% (patient 7, 261/06) and 52.4% (patient 9, 1527/97), 5.2% (patient 9, 158/98) and 54.1% (patient 9, 1527/97), and 5.2% (patient 7, 261/06) and 32.3% (patient 8, 1181/08) (Table 3).

3.5. Correlation between the number of abnormal clones and CIN

Numbers of abnormal clones and the associated values of CIN (%) were correlated in 18 analyses performed at different stages of disease (7 at initial presentation, 3 in hematological complete remission and 8 at relapse) (Fig. 2). There is an increasing curvilinear trend, as suggested by the broken line (a nonparametric smooth). The Spearman correlation coefficient (0.89) was highly significant ($p<10^{-6}$).

4. Discussion

Our present data bring further evidence to the advantage of automated four colour I-FISH for the detection of aneuploidy in HeH ALL. As previously reported, automated four color I-FISH not only detected all clones identified by conventional cytogenetics, but also a number of additional clones of variable size that escaped conventional cytogenetic analysis due to their small extent or incapacity to divide under in vitro conditions (12, 16). Four colour I-FISH has the advantage to give a comprehensive picture of the bone marrow in terms of presence of abnormal clones, abnormal clone diversity and abnormal clone relative size, both at diagnosis and during the course of the disease.

At initial presentation, the largest abnormal clone observed (45.8%) presented trisomy 10 alone and was detected in the single patient with T ALL. In patients with B ALL, the largest abnormal clones observed harboured simultaneous trisomies 4 and 6 (34.3%) and trisomies 6 and 10 (31.2%) respectively (patients 5 and 9). Simultaneous trisomies 4, 10 and 17, whose favorable prognostic significance was questioned recently (9), were detected in 2 patients only and occurred, along with other abnormal clones, in 14% and 2.4% nuclei respectively (patients 1 and 2). Interestingly, for patient 2, the triple trisomy was observed in all abnormal metaphases karyotyped (25 out of 30 metaphases analyzed), suggesting a proliferative advantage of this chromosome combination under in vitro conditions.

Out of 19 analyses performed in hematologic complete remission, all with a normal result by conventional cytogenetics, three revealed the presence of HeH by I-FISH in a total of 13%, 1.4% and 0.6% cells respectively (patient 8). The presence of 13% abnormal cells spoke in favor of a cytogenetic relapse, which was actually followed by hematologic relapse 10 months later. The percentages of abnormal nuclei detected in the subsequent analyses were difficult to interpret. Although very small, they were superior to the cut-off values defined according to the Poisson model and therefore could not be dismissed. As some of them were already present at former relapse, they might be susceptible to proliferate and do require attentive hematologic follow up. Thus, beside its capacity to reveal clonal chromosome heterogeneity, four color I-FISH also proved to be useful for follow up monitoring and constitutes a good tool to detect changes that may precede hematologic and clinical manifestations.

At relapse, the largest abnormal clones observed in B and precursor B ALL presented trisomy for chromosomes 4 and 10 (24.3%) and trisomy for chromosomes 6 and 10 (14.3%) respectively. In the T ALL patient, the largest clone revealed trisomy 10 alone (19.8%).

In a previous study on HeH ALL at presentation, four color I-FISH revealed the presence of a bunch of abnormal clones giving rise to a high level of clonal chromosome heterogeneity (12). In the present study, clonal heterogeneity was met again during the course of the disease, more particularly at hematological and cytogenetic relapse. Clones detected at initial diagnosis generally reappeared at relapse, some of them being larger, likely due to a proliferative advantage, other ones smaller due to selective negative pressure. However, in most cases, remaining initial clones were accompanied by newly generated ones.

Conventional cytogenetic studies have shown an increased number and complexity of chromosome abnormalities in acute leukemia, including HeH ALL, at relapse (17-22). An intriguing question was whether, along with the persistent clonal heterogeneity observed at relapse, the number of abnormal clones would be larger at relapse than at initial presentation. Whereas the mean number of abnormal clones $\geq$ 1% did not differ significantly, the range of their variation was different, much larger at relapse than at presentation. The same was true for very small clones <1%.

In a previous paper, we demonstrated that, at initial presentation, CIN values were much higher in HeH ALL than in non-HeH ALL, suggesting that HeH ALL may be genetically unstable. Despite the small size of our study cohort, our present findings show a significant correlation between the number of abnormal clones and CIN values, suggesting that the higher the instability, the larger the number of abnormal clones.

The origin of HeH in ALL has been a matter of reflection and, based on molecular data, four possible mechanisms have been proposed to account for its formation (5). Out of HeH arising through a near-haploid state, ALL hyperdiploidy most commonly result from tetraploidy with subsequent chromosome loss (30% of the cases) or simultaneous gains (70%). An origin by sequential gain is not excluded and may happen in a minority of cases.

Our previous and present four colour I-FISH findings have revealed that HeH was accompanied by a high level of chromosome heterogeneity both at diagnosis and during the course of the disease which raises the question of the origin of this precocious heterogeneity and its evolution.

Whereas karyotypic heterogeneity is the probable result of chromosome instability, its genesis remains an object of conjecture. Theoretically, heterogeneity may arise through two different mechanisms, sequential or simultaneous chromosome gains or losses. The diversity and interweaving of the different combinations occurring at presentation and during the course of the disease in patients 8 and 9 do not allow concluding to a sequential process, even if most combinations are closely related. Despite their limited size, our present data rather suggest that clonal heterogeneity and its variation may derive from simultaneous gains or losses in different cell divisions. However additional patients should be studied to determine if this a general feature of HeH ALL. The case of T ALL needs actually to be handled with caution, as one mechanism may be as probable as the other.

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Legends to figures 1 and 2

Fig.1: Evolution of individual abnormal clones that represented ≥1% of scored nuclei at least once during the course of the disease, from initial presentation to relapse in patients 9 and 10 and from hematological complete remission to relapse in patient 8. Clones whose size was ≥1% of scored nuclei were colored in yellow, clones whose size was <1% at that stage of disease in white, newly generated clones in green, clones which totally disappeared in red.

Fig. 2: Correlation between the number of abnormal clones and CIN values (%) in 18 bone marrow samples from 10 patients with high hyperdiploidy acute lymphoblastic leukemia at disease presentation (black points), hematological complete remission (white points) and relapse (grey points).

Fig. 1

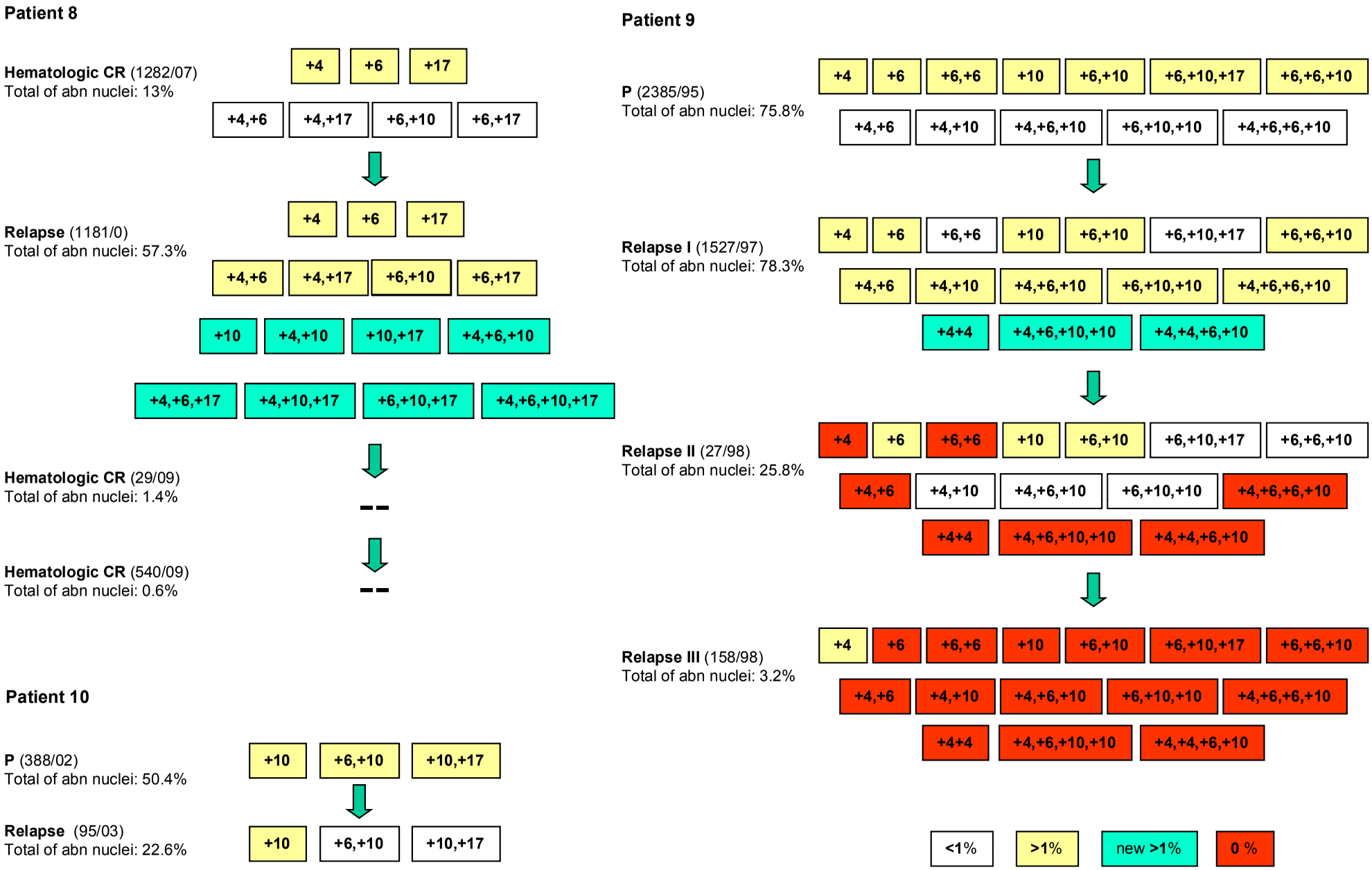
Patient 8
Hematologic CR (1282/07)
Total of abn nuclei: 13%
+4
+6
+17
+4,+6
+4,+17
+6,+10
+6,+17
Relapse (1181/0)
Total of abn nuclei: 57.3%
+4
+6
+17
+4,+6
+4,+17
+6,+10
+6,+17
+10
+4,+10
+10,+17
+4,+6,+10
+4,+6,+17
+4,+10,+17
+6,+10,+17
+4,+6,+10,+17
Hematologic CR (29/09)
Total of abn nuclei: 1.4%
Hematologic CR (540/09)
Total of abn nuclei: 0.6%
Patient 9
P (2385/95)
Total of abn nuclei: 75.8%
+4
+6
+6,+6
+10
+6,+10
+6,+10,+17
+6,+6,+10
+4,+6
+4,+10
+4,+6,+10
+6,+10,+10
+4,+6,+6,+10
Relapse I (1527/97)
Total of abn nuclei: 78.3%
+4
+6
+6,+6
+10
+6,+10
+6,+10,+17
+6,+6,+10
+4,+6
+4,+10
+4,+6,+10
+6,+10,+10
+4,+6,+6,+10
+4+4
+4,+6,+10,+10
+4,+4,+6,+10
Relapse II (27/98)
Total of abn nuclei: 25.8%
+4
+6
+6,+6
+10
+6,+10
+6,+10,+17
+6,+6,+10
+4,+6
+4,+10
+4,+6,+10
+6,+10,+10
+4,+6,+6,+10
+4+4
+4,+6,+10,+10
+4,+4,+6,+10
Relapse III (158/98)
Total of abn nuclei: 3.2%
+4
+6
+6,+6
+10
+6,+10
+6,+10,+17
+6,+6,+10
+4,+6
+4,+10
+4,+6,+10
+6,+10,+10
+4,+6,+6,+10
+4+4
+4,+6,+10,+10
+4,+4,+6,+10
Patient 10
P (388/02)
Total of abn nuclei: 50.4%
+10
+6,+10
+10,+17
Relapse (95/03)
Total of abn nuclei: 22.6%
+10
+6,+10
+10,+17
<1%
>1%
new >1%
0 %

Fig. 2

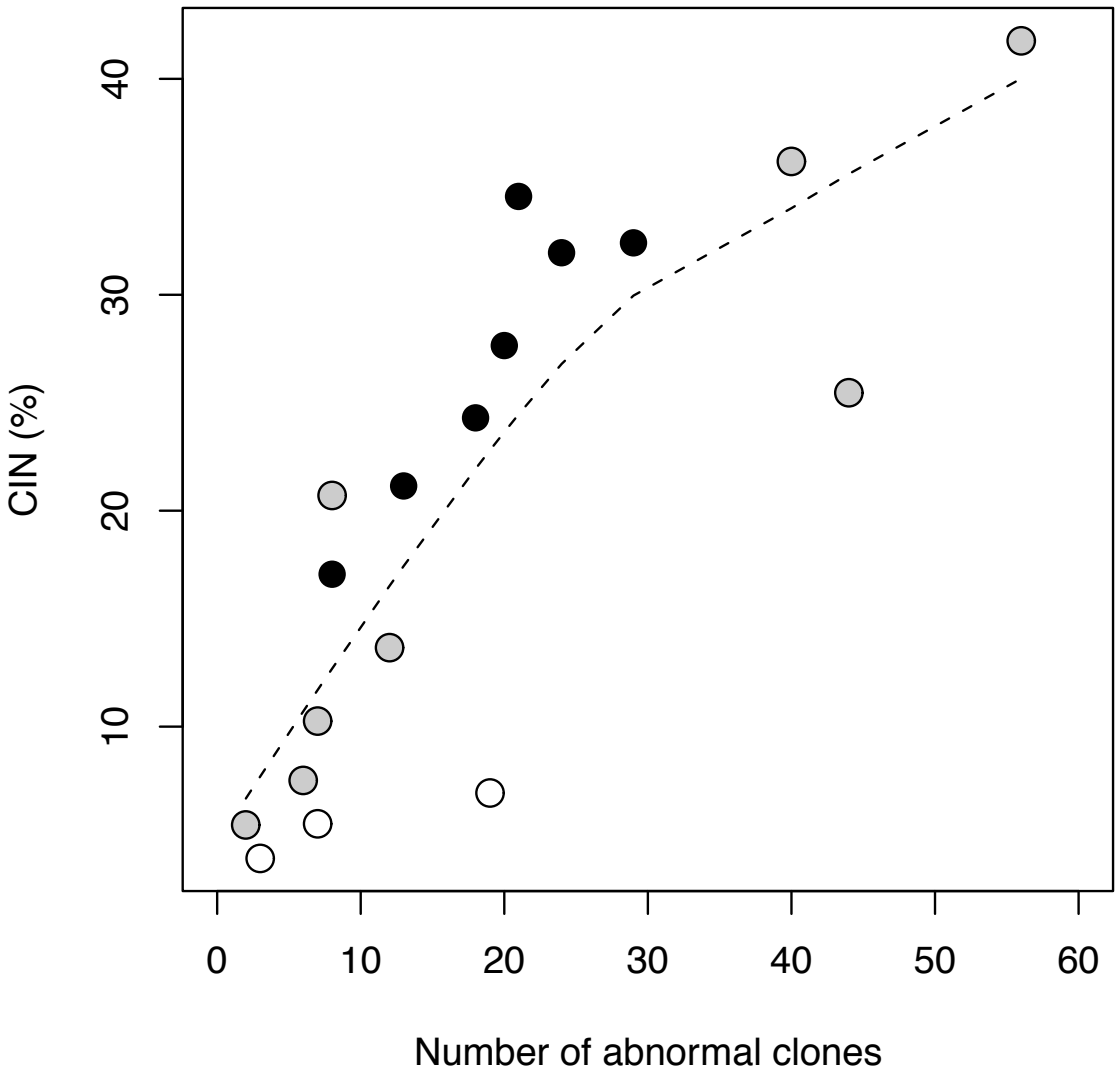

Table 1: Clinical and cytogenetic findings at presentation and /or during the course of disease for 10 patients with high hyperdiploidy acute lymphoblastic leukemia

Patient	Time of cytogenetic analysis (identification number) and/or clinical data	Age/sex	Diagnosis	Follow up (months)	Status	Conventional cytogenetics	I-FISH 4/6/10/17
	09.02.2005 (131/05)	20/F	precursor B		presentation	52,XX,+X,+4,+10,+11,+14,?der(16)t(1;16)(p22;q22),del(17)(p11.2), -21,+mar1,+mar2[4]/46,XX[36]	A
1	07.09.2005 (886/05)			+7	CR	46,XX,t(2;11)(p10;p10)[2]/46,XX[38]	N
	30.11.2005			+10	allo BMT		
	30.08.2010			+67	CR		
	23.01.2006 (88/06)	18/M	precursor B		presentation	55,XY,+X,dup(1)(q25q32),+4,+6,9p?,+10,10?,+14,+17,+18,+21,+21[25]/46,XY[5]	A
2	20.05.2008 (729/08)			+28	CR	46,XY[25]	N
	22.09.2008 (1320/08)			+32	CR	46,XY[25]	N
	03.02.2009 (178/09)			+36	CR	46,XY[25]	N
	16.03.2009 (363/09)	46/F	precursor B		presentation	55,XX,+X,+6,+10,+14,+17,+18,+18,+21,+21[7]/46,XX[3]	A
3	23.04.2009 (565/09)			+1	CR	46,XX[25]	N
	29.06.2009 (877/09)			+3	CR	46,XX[50]	N
	19.07.2010			+16	CR		
	14.03.2005 (241/05)	51/M	Ph+B		presentation	54,XY,+X,+2,+4,+4,+6,inv(9)(p11q13)c,t(9;22)(q34;q11.2),+21,+21, +der(22)t(9;22)[3]/55,idem,?del(2)(q3?2q3?5),add(2)(q35-37),+18[4]/ 46,XY,inv(9)(p11q13)c[12]	A
4	28.06.2005 (604/05)			+3	CR	46,XY,inv(9)(p11q13)c[20]	N
	07.07.2005			+4	allo SCT (sister)		
	09.07.2009			+52	CR		
	05.09.2007 (1086/07)	54/M	Ph+B		presentation	53,XY,+X,+2,+4,+6,t(9;22)(q34;q11.2),+21,+21,+der(22)t(9;22)[14]/46,XY[1]	A
5[a]	11.10.2007 (1243/07)			+1	CR	46,XY[25]	N
	05.11.2007 (1358/07)			+2	CR	46,XY[25]	N
	23.07.1994 (1410/94)	15/F	precursor B		presentation	46,XX[9] ,failure	NA
	15.03.1995 (529/95)			+8	CR	46,XX[29]	N
	20.06.1996			+23	allo BMT (sister) after 1st relapse		
	17.07.2002 (601/02)			+96	2d relapse	53-54,XX,+X,del(1)(q4?1),del(2)(p2?1),-2,del(3)(q?),+5,+6,+7,add(8)(q24), del(8)(q2?4),add(9)(q34),+10[3],+11,der(11)t(1;11)(q21;q23)[3],-13[3],-21, +mar1,+4mar[cp4]/46,XX[6]	A
6[b]	15.08.2002 (688/02)			+97	no evidence of residual blasts in BM after CT	46,XX[20]	N
	06.11.2003 (1001/03)			+112	isolated CNS relapse (BM CR)	46,XX[20]	N
	28.04.2004 (357/04)			+117	CR	46,XX[20]	N
	21.02.2005			+127	relapse and death		
	01.09.2004	20/F	B		presentation (Brasil)	HeH (Brasil)	NA
	30.11.2005 (Zürich)			+14	relapse	54,XY,+X,dup(1)(q12q25),add(2)(p11.2),+6,add(6)(q2?),+14, add(14)(q32),+18,+21,+21,+3~4mar[cp8]/46,XY[2], PB	NA
	07.03.2006 (261/06)			+17	relapse	53-56,dup(1)(q?),+21,+21,inc[7]/46,XY[30]	A
7	23.03.2006 (331/06)			+17.5	aplastic BM, isolated blasts	no result	A
	03.04.2006			+18	allo SCT (unrelated)		
	08.05.2006 (540/06)			+19	CR	46,XY[25]	N
	12.07.2006 (761/06)			+21	CR	46,XY[25]	N
	30.08.2006			+22	death (GVHD)		
	31.08.2006 (Zürich)	18/M	precursor B		presentation	46,XY[20][c], PB	NA
	18.10.2007 (1282/07)			+14	CR	46,XY[20]	A
	22.08.2008 (1181/08)			+24	relapse	55-56,XY,+X,dup(1)(q?21q?32),+4,?add(4)(q?21),+6,+10, ?del(12)(p11.2p13)[2],?del(13)(q12q14),+14, -16,+17,+18,+21,+21,+1-2mar[cp8]/46,XY[8]	A
8	08.01.2009 (29/09)			+28	CR	46,XY[20]	A
	22.01.2009			+29	allo SCT (unrelated)		
	18.02.2009 (259/09)			+30	CR	46,XY[25]	N
	20.04.2009 (540/09)			+32	CR	46,XY[25]	A
	26.01.2010			+41	CR		
	17.12.1995 (2385/95)	26/M	B		presentation	52-57,XY,+X,+5,+6,add(7)(p13-15),+9,+10,+11,-13,+18,+21,+21,+22,+22, +der(?)t(1;?)(q12-21;?)[cp6]/46,XY[10]	A
9	07.08.1997 (1527/97)			+20	1strelapse	57,XY,+X,+5,+6,add(7)(p13-15),+9,+10,+11,-13,+18,+21,+21,+22,+22, +der(?)t(1;?)(q12-21;?)[5]/46XY[15]	A
	08.01.1998 (27/98)			+25	2drelapse	46,XY[20]	A
	02.03.1998 (158/98)			+27	3drelapse	46,XY[20]	A
	28.04.1998				death		
	08.05.2002 (388/02)	45/M	T		presentation	54,XY,?del(4)(p?),-7,+8,+10,+11,+13,+13,+14,+19,+mar1,+mar2[4]	A
10	30.01.2003 (95/03)			+8	relapse	54,XY,add(7)(q32),+8,+?10,+?11,+13,+13,+14,18?,+19,+mar1[2]/46,XY[18]	A
	May 2003			+11	death		

Abbreviations : A, abnormal; BM, bone marrow; BMT, bone marrow transplantation; CNS, central nervous system; CR, hematologic (BM) complete remission; CT, chemotherapy; GVHD, graft versus host disease; N, normal; NA, not available; PB, peripheral blood; Ph+, Philadelphia positive; SCT, stem cell transplantation

[a] : patient included in the GRAAL 2005 study (http://www.clinicaltrials.gov/: NCT00327678)

[b] : patient included in the SAKK ALL 33-86/90 studies

[c] : +21q22 (15 %) by FISH

Table 2: Clones involving chromosomes 4, 6, 10 and 17 at presentation and /or during the course of disease in 10 patients with high hyperdiploid acute lymphoblastic leukemia , expressed as percentages

	Clones expressed as %																	
Patients	**1**	**2**	**3**	**4**	**5**	**6**	**7**		**8**				**9**				**10**	
Analysis	131/05[b]	88/06[b]	363/09[b]	241/05[b]	1086/07[b]	601/02[c]	261/06[c]	331/06[c]	1282/07[d]	1181/08[c]	29/09[d]	540/09[d]	2385/95[b]	1527/97[c]	27/98[c]	158/98[c]	388/02[b]	95/03[c]
Normal	18.4	51.0	9.8	53.8	20.7	44.2	86.4	54.6	87.0	42.7	98.6	99.4	24.2	21.7	74.2	96.8	49.6	77.4
+4	8.6	3.8	-	9.0	13.0	5.3	4.8	14.3	3.5	3.3	-	-	1.2	11.4	-	2.6	-	-
+6	-	2.6	6.2	4	28.3	9.3	-	-	3.7	4.4	-	-	17.2	5.4	4.4	-	-	-
+10	15.8	2.2	8.6	-	-	6.4	2.0	4.6	-	3.4	-	-	15.0	3.0	9.4	-	45.8	19.8
+17	5.4	2.6	7.6	-	-	2.2	-	-	3.5	2.6	-	-	-	-	-	-	-	-
+6,+17	-	-	7.4	-	-	-	-	-	-	**2.6**	-	-	-	-	-	-	-	-
+6,+10	-	4.0	20.2	-	-	13.9	-	-	-	**7.8**	-	-	31.2	14.3	9.2	-	1.2	-
+4,+17	4.8	1.2	-	-	-	-	-	-	-	**1.1**	-	-	-	-	-	-	-	-
+4,+6	-	2.6	-	8.8	34.3	1.7	-	-	-	**1.6**	-	-	-	**3.3**	-	-	-	-
+4,+10	16.8	3.6	-	-	-	1.4	5.6	24.3	-	2.8	-	-	-	**3.7**	-	-	-	-
+10,+17	9.2	1.2	10.8	-	-	-	-	-	-	2.0	-	-	-	-	-	-	1.2	-
+4,+10,+17	14.0	2.4	-	-	-	-	-	-	-	2.1	-	-	-	-	-	-	-	-
+4,+6,+10	-	5.2	-	-	-	2.1	-	-	-	5.4	-	-	-	**13.4**	-	-	-	-
+6,+10,+17	-	1.8	23.2	-	-	-	-	-	-	6.4	-	-	1.0	-	-	-	-	-
+4,+6,+17	-	1.6	-	-	-	-	-	-	-	1.9	-	-	-	-	-	-	-	-
+4,+6,+10,+17	-	7.0	1.0	-	-	-	-	-	-	6.0	-	-	-	-	-	-	-	-
+4,+4	-	1.2	-	8.6	-	1.5	-	-	-	-	-	-	-	1.8	-	-	-	-
+4,+4,+6	-	-	-	13.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-
+4,+4,+6,+10	-	1.4	-	-	-	-	-	-	-	-	-	-	-	2.8	-	-	-	-
+6,+6	-	-	-	-	-	-	-	-	-	-	-	-	1.4	-	-	-	-	-
+6,+6,+10	-	-	-	-	-	-	-	-	-	-	-	-	3.0	1.6	-	-	-	-
+4,+6,+6,+10	-	-	-	-	-	-	-	-	-	-	-	-	-	**1.4**	-	-	-	-
+4,+6,+10,+10	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5	-	-	-	-
+6,+10,+10	-	-	-	-	-	2.7	-	-	-	-	-	-	-	**2.1**	-	-	-	-
+6,+10,+17,+17	-	-	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
+4,+10,+17,+17	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
+6,+17,+17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Others[a]	5.8	4.6	4.0	2.2	3.7	9.4	1.2	2.3	2.3	3.9	1.4	0.6	5.8	11.5	2.8	0.6	2.2	2.8
Total of abnormal clones	21(13)	29(13)	24(15)	13(8)	18(15)	44(34)	6(3)	8(5)	19(16)	40(25)	7(7)	3(3)	20(13)	56(43)	12(9)	2(1)	8(5)	7(6)

[a] Cumulated percentage of very small clones (< 1%); [b] Presentation; [c]Relapse; [d] Hematologic (bone marrow) complete remission; ( ) Number of very small abnormal clones (<1%)

Clones already present in the previous analysis (<1%) are highlighted in bold type

Study patients are further detailed in Table 1

Table 3: Chromosomal instability at presentation and/or during the course of disease in 10 patients with high hyperdiploid acute lymphoblastic leukemia

Patient	Identification number	Follow up (months)	Status	Modal number (cells differing from modal number,%)				Average
				Chr 4	Chr 6	Chr 10	Chr 17	
1	131/05		presentation	3 (51,00)	2 (4,00)	3 (41,40)	2 (41,80)	2,50 (34,55)
	886/05	+7	CR	2 (4,80)	2 (4,60)	2 (4,60)	2 (6,60)	2 (3,95)
2	88/06		presentation	2 (35,20)	2 (33,80)	2 (35,00)	2 (25,60)	2 (32,40)
	729/08	+28	CR	2 (3,20)	2 (3,80)	2 (3,80)	2 (7,40)	2 (4,55)
	1320/08	+32	CR	2 (4,60)	2 (5,40)	2 (3,40)	2 (5,80)	2 (4,80)
	178/09	+36	CR	2 (1,40)	2 (2,80)	2 (3,40)	2 (8,20)	2 (3,95)
3	363/09		presentation	2 (6,60)	3 (38,80)	3 (33,40)	3 (49,00)	2,75 (31,95)
	565/09	+1	CR	2 (4,40)	2 (4,00)	2 (3,60)	2 (6,80)	2 (4,70)
	877/09	+3	CR	2 (5,40)	2 (2,60)	2 (2,00)	2 (3,20)	2 (3,30)
4	241/05		presentation	2 (44,00)	2 (29,80)	2 (3,80)	2 (7,00)	2 (21,15)
	604/05	+3	CR	2 (4,00)	2 (4,40)	2 (2,60)	2 (6,40)	2 (4,35)
5	1086/07		presentation	3 (50,80)	3 (35,20)	2 (3,80)	2 (7,40)	2,50 (24,30)
	1243/07	+1	CR	2 (4,20)	2 (2,60)	2 (3,70)	2 (7,30)	2 (4,45)
	1358/07	+2	CR	2 (4,80)	2 (3,30)	2 (3,70)	2 (7,90)	2 (4,93)
6	529/95	+8	CR	2 (4,80)	2 (3,00)	2 (2,60)	2 (4,40)	2 (3,70)
	601/02	+96	2^{d}relapse	2 (18,67)	2 (38,40)	2 (33,93)	2 (10,87)	2 (25,47)
	688/02	+97	no evidence of residual blasts	2 (6,60)	2 (2,00)	2 (1,60)	2 (5,80)	2 (4,00)
	1001/03	+112	isolated CNS relapse (BM CR)	2 (3,40)	2 (4,80)	2 (2,60)	2 (8,60)	2 (4,85)
	357/04	+117	CR	2 (6,60)	2 (4,60)	2 (4,80)	2 (6,40)	2 (5,60)
7	261/06	+17	relapse	2 (12,40)	2 (2,60)	2 (9,80)	2 (5,20)	2 (7,50)
	331/06	+17,5	aplastic BM, isolated blasts	2 (41,71)	2 (4,00)	2 (31,71)	2 (5,43)	2 (20,71)
	504/06	+19	CR	2 (5,00)	2 (5,00)	2 (2,20)	2 (4,60)	2 (4,20)
	761/06	+21	CR	2 (5,20)	2 (3,60)	2 (4,20)	2 (5,80)	2 (4,70)
8	1282/07	+14	CR	2 (8,20)	2 (7,10)	2 (4,50)	2 (7,90)	2 (6,93)
	1181/08	+24	relapse	2 (29,70)	2 (41,40)	2 (41,30)	2 (32,30)	2 (36,18)
	29/09	+28	CR	2 (6,80)	2 (5,20)	2 (5,20)	2 (4,80)	2 (5,50)
	259/09	+30	CR	2 (6,00)	2 (3,20)	2 (4,40)	2 (7,00)	2 (5,15)
	540/09	+32	CR	2 (3,40)	2 (3,20)	2 (3,20)	2 (5,80)	2 (3,90)
9	2385/95		presentation	2 (5,80)	3 (48,00)	3 (47,60)	2 (9,20)	2,50 (27,65)
	1527/97	+20	1^{st}relapse	2 (51,07)	3 (52,40)	2 (54,07)	2 (9,53)	2,25 (41,77)
	27/98	+25	2^{d}relapse	2 (6,40)	2 (18,60)	2 (22,40)	2 (7,20)	2 (13,65)
	158/98	+27	3^{d}relapse	2 (5,60)	2 (5,00)	2 (5,20)	2 (6,00)	2 (5,45)
10	388/02		presentation	2 (5,20)	2 (5,20)	3 (50,60)	2 (7,20)	2,25 (17,05)
	95/03	+8	relapse	2 (4,60)	2 (3,00)	2 (25,00)	2 (8,40)	2 (10,25)

Abbreviations : BM, bone marrow; CNS, central nervous system; CR, Hematologic (bone marrow) complete remission; CT, chemotherapy

Study patients are further detailed in Table 1

5 Conclusion and perspectives

We have shown that automated four colour I-FISH is a valuable tool for the detection of concurrent aneuploidies in hematopoietic malignancies. It overcomes the human eye limitations when more than three fluorochromes are used and is of special interest in the diagnosis of cytogenetic subsets of diagnostic and prognostic significance such as HeH in acute lymphoblastic leukemia.

This automated analysis system allows for the rapid scoring of a large number of nuclei making it possible to detect very small abnormal clones (less than 1%). This approach is therefore very useful for follow up monitoring and is a good mean for detecting precocious relapses.

It has the advantage of being able to run on its own, even out of business hours, saving time, labour and money.

It requires less fixed cell suspension than needed by single- or dual-colour I-FISH assays.

After the initial setup phase, we tuned the machine so as to optimize the automated four colour I-FISH process.

We improved the efficiency of automatic nucleus selection and of FISH signal detection by replacing the 63x objective with a 40x one.

The rate of false negative nuclei as estimated by the software with optimized parameters proved to be inferior with the 40x objective and, with this new optics, the percentage of nuclei correctly counted was increased for every colour channel. Two main reasons accounted for these observations. First, with the 40x optics there was no need to use oil, which greatly reduced the background. Second, this new optical geometry allowed further optimization of the parameters accounting for cell morphology assessment.

In order to validate the percentage of false positive nuclei estimated by the software we compared its value to that found by an operator who checked a total of 5000 nuclei taken from 10 ALL patients. The software estimates proved to be much larger than the true error.

We validated in a similar way the percentage of nuclei correctly counted for every colour channel. Values revealed by the software were lower than those found by the operator.

In four colour I-FISH, it is not possible to compare the time needed for a manual and an automated scoring because of the human eye limitations.

Despite the fact that the acquisition step is automated, our approach still requires an operator to check and correct the nuclei listed in the gallery. In order to avoid this manual correction phase, and thus gain time, it would be possible to develop statistical methods able to correct systematic errors (56).

For nucleus selection, we chose geometrical parameters able to detect objects with a regular, circular and polylobed form. We excluded from our analysis overlapping nuclei, grapes and irregular forms. In a recent study, Neumann et al (57) used time-lapse microscopy to automatically classify 1 918 544 775 nuclei from HeLa cells. These authors identified distinct morphological classes such as 'polylobed' (exhibiting multilobed nuclei), 'grape' (many micronuclei) and 'binuclear' (two nuclei) cells. These different mitotic phenotypes arise as a consequence of problems during mitosis including premature nuclear assembly, chromosome segregation errors and cytokinesis failure. In order to take these abnormal clones into account, we suggest, in a future study, to identify the abnormal mitotic phenotypes occurring in leukemic bone marrow cells and to combine signal scoring and morphological analysis.

Automated four colour I-FISH is a method of choice for studying clonal heterogeneity because several probes can be used simultaneously and information provided for every single cell (34, 58). Although allowing identification of genetic alterations at very high resolution, microarray-based analysis of DNA copy number alterations and loss of heterozygosity are not able to reveal cell-to-cell heterogeneity (39).

The automatic scanning method developed here could detect up to 5 concurrent aneuploidies, thus increasing its potential for aneuploidy and clonal heterogeneity assessment in ALL as well as in other hematologic malignancies, such as plasma cell neoplasms for instance, or in solid tumours.

Four colour I-FISH allowed the identification of a number of clones not detected by CC. These additional clones, of variable size, might have escaped conventional cytogenetic analysis due to their small extent or incapacity to divide under in vitro conditions. The presence of numerous abnormal clones at disease presentation suggested a high level of heterogeneity probably due to underlying CIN. This assumption was tested by the determination of average numerical CIN values for

chromosomes 4, 6, 10 and 17 together and for each chromosome and patient group (HeH ALL and nonHeH ALL). Values proved to be high in HeH ALL, compared with those observed in non HeH ALL. Numerous abnormal clones were also observed at relapse. Although the mean number of abnormal clones did not differ significantly between initial presentation and relapse, the range of number variation was different, much larger at relapse. A significant correlation between the number of abnormal clones and CIN values further suggested that the higher the instability, the larger the number of abnormal clones.

Our data suggests the existence of a proliferative advantage for some specific combinations of additional chromosomes confirming the observations of Heerema et al. (25). In our patients, trisomies were much more frequent than tetrasomies. In most HeH B-ALL patients, the largest abnormal clones at disease presentation and at relapse harbored two concurrent trisomies, i.e. trisomy for chromosomes 4 and 6 and trisomy for chromosomes 6 and 10 respectively. In the single T-ALL patient, the main abnormal clone presented trisomy 10 alone both at presentation and at relapse.

We could investigate the dynamics of individual abnormal clones from presentation through disease progression in 3 patients, in two cases from presentation to relapse, in the third one from complete hematological remission to relapse. Most clones detected at initial diagnosis reappeared at relapse, some of them being larger, likely due to a proliferative advantage, other ones becoming smaller due to a selective negative pressure. In contrast with the T-ALL patient, whose clinical course was associated with an apparent cytogenetic stability, the two B-ALL patients acquired a number of newly generated clones during disease progression, in accordance with former observations by conventional cytogenetic showing an increase in chromosome number and complexity of chromosome abnormalities in relapsed acute leukemia, including HeH ALL (59-64).

Despite our relatively small number of patients, analysis of paired diagnostic/relapse samples bring further evidence to the probable presence of multiple, genotypically distinct and independent leukemic cells at diagnosis and the ability of propagating leukemic cells to acquire additional genetic changes that may favor evolution and eventual selection according to a Darwinian process (7).

Despite impressive progress in therapy of ALL, especially in children ALL, relapse remains a substantial cause of poor evolution and leukemia-related death, even in entities with good prognosis.

Therefore it is of extreme importance to identify relapse associated factors which would allow to assign these patients to specific risk groups and proper therapy. Although recent studies using single nucleotide polymorphism array and mutation analysis revealed that, in childhood HeH ALL, additional structural alterations were more common at relapse than at disease presentation, they were not able to identify single and recurrent genetic changes that may be specifically associated with an increased risk of eventual relapse (65-67). Based on the correlation between the number of abnormal clones and CIN values observed in our patients and the poor outcome associated with CIN in solid tumours and myelodysplastic syndromes (35, 36), one may expect the nature and extent of clonal heterogeneity at the time of diagnosis to be of prognostic significance in HeH ALL. The small number of patients studied here did not permit to draw any conclusion for the present time, however the question is of interest and would merit to be investigated in a large and homogeneous cohort of HeH ALL. The same holds good for a possible difference between B- and T HeH ALL and between adult and children HeH ALL.

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