Analysis and detection of cryptic and complex chromosomal aberrations in acute leukemia

Dissertation

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This work is dedicated…

to my dear parents

to my family

to my lovely wife Boodor

Abbreviations

ABL1	v-abl Abelson murine leukemia viral oncogene homolog 1
AL	acute leukemia
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
Array-CGH	array comparative genomic hybridization
B-ALL	B - cell acute lymphoblastic leukemia
BAC	bacterial artificial chromosome
BCR	breakpoint cluster region
BM	bone marrow
bp	base pairs
CEP	centromere probe
CGAP	cancer genome anatomy project
CGH	comparative genomic hybridization
CLL	chronic lymphocytic leukemia
CML	chronic myelogenous leukemia
CN	cytogenetically normal
CN-AL	cytogenetically normal AL
CN-ALL	cytogenetically normal ALL
CN-AML	cytogenetically normal AML
CNAs	copy number alterations
CNVs	copy number variations
COBRA	combined ratio labeling
CR	complete remission
DGV	database of genomic variants
del	deletion
DNA	deoxyribonucleic acid
FAB	French-American-British
FISH	fluorescence in situ hybridization
GTG	Giemsa banding, G-bands by trypsin using Giemsa
HSCs	hematopoietic stem cells
HSCT	hematopoietic stem cell transplantation
ins	insertion
ISCN	international system for human cytogenetic nomenclature
ISH	in situ hybridization
ISIS	in situ imaging software (MetaSystems)
Kb	kilobasepairs
LSP	locus-specific probe
Mb	megabasepaires
MDS	myelodysplastic syndrome
m-FISH	multicolor FISH
M-FISH	multiplex-FISH using whole chromosome painting probes
MCB	multi-color-banding
MLPA	multiplex ligation dependent probe amplification
mMCB	multitude multicolor banding
MRD	minimal residual disease
NCBI	national center for biotechnology information
NGS	next generation sequencing

No.	number
PAC	P1-derived artificial chromosome
PCP	partial chromosome paint
PCR	polymerase chain reaction
PRINS	primed in situ labeling
RNA	ribonucleic acid
SKY	spectral karyotyping
SNP array-CGH	single nucleotide polymorphism based array comparative genomic hybridization
WBC	white blood cells
WCP	whole chromosome paint
WHG	whole human genome
WHO	world health organization
t	translocation
T-ALL	T -cell acute lymphoblastic leukemia
UCSC	university of California, Santa Cruz
UPD	uniparental disomy

Contents

Summary:

Acute leukemia (AL) is a heterogeneous and aggressive disease, with an incidence of approximately 5 cases per 100.000 individuals and per year. It consists of several subgroups with different specific cytogenetic and molecular genetic aberrations, clinical presentations and outcomes. Classification of AL is done (i) by clinical parameters and (ii) based on the bone marrow karyotype. Banding cytogenetics plays a pivotal role in the detection of recurrent chromosomal rearrangements and is the starting point of genetic analysis in AL, still. Nowadays, molecular (cyto)genetic tools provide substantially to identify previously non-detectable, so-called cryptic chromosomal aberrations in AL. However, AL according to banding cytogenetics with normal karyotype - in short cytogenetically normal AL (CN-AL) - represent up to ~50% of all new diagnosed AL cases and prognosis is unclear or denominated as intermediate. Thus, the overall goals of this thesis were (i) to identify and characterize the rate of cryptic alterations in CN-AL, (ii) to detect submicroscopic structural copy number alterations (CNAs) in AL and (iii) to identify yet unreported clonal acquired chromosomal rearrangements (therefore also 8 complex rearranged AL cases were studied) and align them with clinical outcome, as far as possible. This work included 103 AL cases and they were studies comprehensively using high resolution fluorescence in situ hybridization (FISH) based-banding technique, locus-specific probes (LSPs), array-based comparative genomic hybridization (aCGH), multiplex-ligation dependent probe amplification (MLPA) and analyses of the breakpoints by genomic browsers. DNA sequencing and single nucleotide polymorphism array-based comparative genomic hybridization (SNP array-CGH) have been used to detect mutations for a number of target genes that are known to key roles in lymphoid and myeloid development. Cryptic chromosomal aberrations were identified in 34% of cytogenetically normal acute lymphoblastic leukemia (CN-ALL) and in 28% of cytogenetically normal acute myeloid leukemia (CN-AML) cases respectively. Surprisingly, we detected high rates of CNAs in CN-ALL, whereas AML cases showed lower rates. Besides, we identified three new candidate genes; *CDK6* (7q12.2), *CDH2* (15q26.2) and *DCC* (18q21.2) that may play a key role in leukemogensis and progression.

In conclusion, the present study highlights, that most likely all CN-AL cases hold cryptic genomic alterations and that complex AL still are a valuable source for detection of yet unrecognized chromosomal aberrations. Overall, the molecular cytogenetic approaches together with molecular methods are suited to identify cryptic chromosomal aberrations in AL and useful to define the genetic risk–based classification and correct determination of treatment protocols.

Zusammenfassung:

Die akute Leukämie (AL) ist eine heterogene und aggressive Erkrankung mit einer Inzidenz von etwa 5 Fällen pro 100.000 Individuen und Jahr. Sie besteht aus mehreren Untergruppen mit unterschiedlichen zyto- und molekular-genetischen Aberrationen, klinischen Bildern und Verläufen. Die Klassifizierung von AL basiert v.a. auf (i) klinischen Parametern und (ii) einer Karyotypisierung des Knochenmarks. Die Zytogenetik spielt eine zentrale Rolle beim Nachweis von wiederkehrenden Chromosomenaberrationen und ist immer noch der Ausgangspunkt für jedwede weiterführende genetische Analyse der AL. Heutzutage bieten moderne, molekular (zyto-)genetische Verfahren die Möglichkeit früher nicht nachweisbare, sog. kryptische Chromosomenaberrationen bei der AL zu identifizieren. Dennoch sind nach Bänderungszytogenetik heute immer noch bis zu ~50% der neu diagnostizierten ALs zytogenetisch unauffällig (abgekürzt CN-AL) und deren Prognose gilt als unklar oder intermediär. Ziele dieser Arbeit waren (i) den Anteil und die Art der vorhandenen kryptischen Veränderungen bei CN-AL Fällen zu bestimmen, (ii) submikroskopische Struktur- bzw. Kopienzahl-Veränderungen (CNAs) in ALs nachzuweisen, und (iii) bislang noch nicht beschriebene, erworbene klonale chromosomale Rearrangements in CN-AL sowie 8-komplexaberranten AL Fällen zu identifizieren und mit dem klinischen Verlauf zu korrelieren. In der vorliegenden Arbeit wurden 103 AL Fälle umfassend mittels hochauflösender Fluoreszenz in situ Hybridisierungs (FISH)-Bänderungs-Techniken, lokus-spezifischen Sonden, array-basierender vergleichender genomischer Hybridisierung (aCGH), MLPA (multiplex-ligation dependent probe amplification) und durch Bruchpunktanalysen mittels genomischer Browser untersucht. DNA-Sequenzierung und Single Nucleotide Polymorphismus basierte aCGH wurden verwendet, um Mutationen für eine Anzahl von Zielgenen, welche Schlüsselrollen bei der lymphoiden und myeloiden Entwicklung haben weiter zu untersuchen. Kryptische Chromosomenaberrationen wurden in 34% der zytogenetisch unauffälligen akuten lymphatischen Leukämiefälle (CN-ALL) und in 28% der zytogenetisch unauffälligen akuten myeloischen Leukämien (CN-AML) identifiziert. Es fanden sich mehr CNAs in CN-ALL als in CN-AML Fällen. Schließlich wurden 3 neue AL-assoziierte Kandidaten-Gene gefunden: *CDK6* (7q12.2), *CDH2* (15q26.2) und *DCC* (18q21.2), die eine wichtige Rolle in der Leukemogenese und Progression spielen könnten. Insgesamt ergab die vorliegende Arbeit, dass wohl alle CN-AL Fälle kryptische genomische Veränderungen tragen, und dass komplexe AL Fälle eine wertvolle Quelle für noch nicht erfasste Chromosomenaberrationen darstellen. Zusammenfassend konnte weiterhin gezeigt werden, dass molekularzytogenetische zusammen mit molekularen Methoden zur Klassifizierung kryptischer Chromosomenaberrationen in AL geeignet sind; diese Daten können künftig verwendet werden für eine korrekte Risikobestimmung und Auswahl geeigneter Behandlungsmethoden bei AL-Patienten.

1. Introduction

Hematological malignancies are the most common cancer disease worldwide, particularly acute leukemia (AL). AL is the severest life threatening acquired disorder, studies are required for better understanding of underlying disease biology. The latter is primarily based on identification and characterization of acquired genetic alterations in AL. This chapter, first covers the molecular cyto(genetic) techniques nowadays used to identify acquired cryptic alterations in AL as well as to characterize complex chromosomal rearrangements (chapter 1.1). Afterwards an overview on AL is provided, including definition, classification, cytogenetics and molecular genetics (chapters 1.2 to 1.5). These data will lead to the questions treated in this work (chapter 1.6). The present work is cumulative and based on ten own papers; thus, after showing them (chapter 2) they are discussed (chapter 3) and a finally conclusion and outlook on further possible developments based on presented data is given (chapter 4).

1.1. Cytogenetic and molecular (cyto)genetics

The beginning of human cytogenetics is ascribed to the end of 19th century. Tjio and Levan reported in 1956, based on their study of human embryonic lung tissues from several individuals, that the human diploid chromosome number is 46 (2n = 46) (Tjio and Levan 1956). Continued developments of cell culture and harvesting techniques allowed for the identification of chromosomal abnormalities correlated with specific disorders and diseases. Thus, in 1959, Lejeune and colleagues described an extra chromosome in patients with Down syndrome (Lejeune et al. 1959). The first and most important finding of tumor cytogenetics in these early years was attributed to Peter Nowell and David Hungerford in 1960. They found a small acrocentric chromosome in the white blood cells (WBCs) of patients with chronic myelogenous leukemia (CML). This abnormal chromosome appeared to be terminally deleted, and was denominated as “Philadelphia chromosome” (Nowell and Hungerford 1960). The development of chromosome banding techniques started in the end of the 1960s. They allowed the chromosomes to be individually identified and specifically addressed in inherited diseases, and in case of acquired alterations in human malignancies (Caspersson et al. 1968). Therefore, the reciprocal translocation t(8;21)(q22;q22) was the first by means of banding approaches characterized alteration in acute myeloid leukemia (AML) in 1972 (Caspersson et al. 1972, Rowley 1973a). Shortly afterwards also the “Philadelphia chromosome” was identified to be part of a balanced translocation between the long arms of chromosomes 9 and chromosome 22; specifically a t(9;22)(q34;q11) (Rowley 1973b).

1.1.1. Chromosome banding

A number of banding and staining techniques directed towards metaphase chromosomes have been developed between 1968 and 1980s. Thus, since the 1970s chromosome analysis became an essential tool in diagnosis of leukemia and lymphoma, as many recurrent numerical and structural aberrations were recognized (Lawler 1977). G-banding still known as a gold standard of banding techniques; it is considered as the most commonly used method in routine clinical and tumor cytogenetic diagnostic worldwide. G-bands are obtained, when the chromosomes are pretreated with a proteolytic enzyme, like trypsin and then stained with Giemsa, to produce reproducibly dark and light bands along the human chromosomes, which can be seen and analyzed by standard light microscopy. G-banding enables to detect both numerical (gain or loss of a chromosome) and structural aberrations (e.g., translocation, deletion, inversion, etc.). This method has, however, several weaknesses. The resolution of this technique is still limited, with a count of approximately 400-550 bands per haploid tumor cytogenetic genome; due to this many important chromosomal alterations can be missed and complex aberrations are too difficult to be interpreted (Wang and Fedoroff 1972, Yunis 1976, Othman et al. 2014). The designation of the regions, bands and sub-bands for each chromosome are describe in the International System for Human Cytogenetic Nomenclature (ISCN) (Shaffer et al. 2013).

1.1.2. Molecular cytogenetics

The term molecular cytogenetics refers to the study of DNA or genes visualised at chromosome or cell-level (Speicher and Carter 2005). In 1986, the first successful fluorescence in situ hybridization (FISH) experiments was carried out by the group of Dan Pinkel using chromosome-specific probe sets and to recognize the numerical and structural chromosomal abnormalities (Pinkel et al. 1986). Indeed, introducing of molecular cytogenetics, namely the FISH approach (see 1.1.2.1) is to overcame the lower resolution of banding techniques (>5-10 Mb). Nowadays, one of the best ways to characterize chromosomal breakpoints, particularly in leukemia, is application of the FISH-technique. Currently, major advances in molecular technology and bioinformatics, precisely comparative genomic hybridization (CGH), array-CGH and single nucleotide polymorphism array-based comparative genomic hybridization (SNP array-CGH), are powerful tools used to study copy number alterations (CNAs) across the genome. The goal of such studies is to improve the understanding of leukemia/cancer genesis, the identification of new biomarkers and potential therapeutic targets (Glassman and Hayes 2005, Le Scouarnec and Gribble 2012).

1.1.2.1. The technique of fluorescence in situ hybridization (FISH)

The principle of FISH technique is based on the ability of a single-stranded DNA sequence to hybridize to its complementary target DNA sequence. The targets DNA are metaphase chromosomes, interphase nuclei, or tissue sections fixed to a glass slide (Fig. 1.1). The potential of all FISH-technologies is their ability to detect also submicroscopic deletions, duplications or rearrangements of single genes. Additionally, cryptic aberrations and complex chromosomal rearrangements can be fully characterized by FISH. Furthermore, interphase directed FISH is possible in case of low mitosic yield in leukemia (Liehr 2009, Bishop 2010).

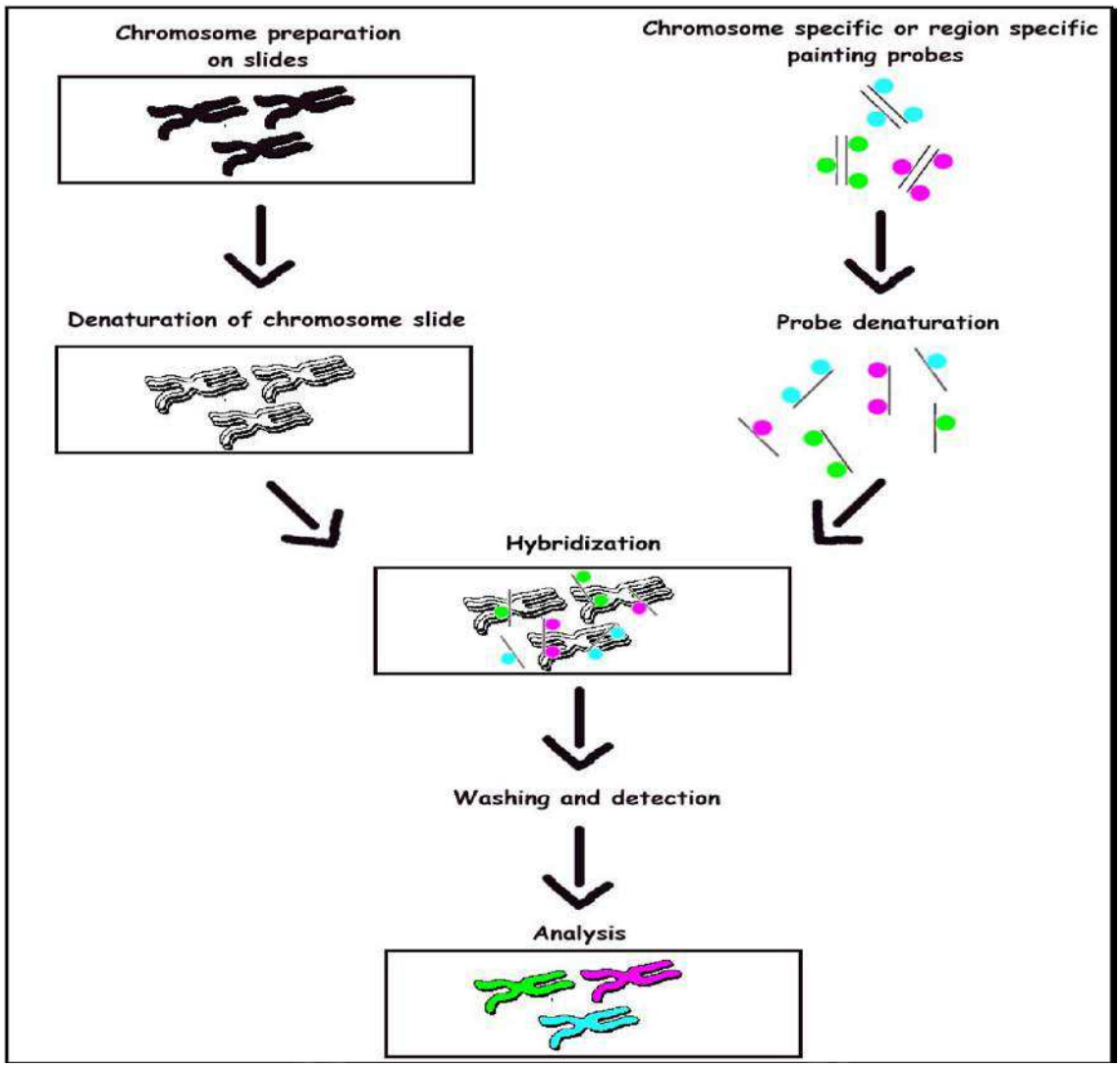

Figure 1.1. Principle of a FISH experiment performed on metaphase chromosomes. Fluorescent-labeled DNA probe complementary to a chromosomal region of interest is used together with the target DNA which is fixed onto the slide surface. DNA probes and target DNA are denatured and hybridized together. Not shown in the figure, the Cot-1 DNA is necessary to cohybridized with the probe to reduce the binding of repetitive sequences. After washing the slides they can be visualized under a fluorescence microscope. If the DNA complementary to the probe is present a signal with the color of the emission wavelength of the fluorochrome of the probe is seen [figure adapted from the Department of Medical Genetics, Université de Sherbrooke, Sherbrooke, Quebec, Canada].

1.1.2.2. Probes used for FISH

For FISH many different DNA probes can be applied, which can be grouped as outlined below.

1.1.2.2.1. Locus-specific probes (LSP)

LSP cover chromosomal regions or loci of 0.1 to several megabase pairs (Mb) in size. In leukemia diagnostics and research LSP are applied to identify amplified oncogenes, deletion of tumor suppressor genes, or fusion genes or fissions (Liehr et al. 2015).

1.1.2.2.2. Chromosome painting probes

Whole chromosome painting (WCP) probes are generated by flow sorting or whole chromosome microdissection. The short and long arm of a particular chromosome can be painted by so-called partial chromosome painting (PCP) probe; PCPs can only be generated by microdissection. PCPs and WCPs have been particularly valuable in leukemia where specific chromosome rearrangements (numerical or structural) correlate with the severity of disease and may influence the plan of therapy (Cremer et al. 1988, Pinkel et al. 1988, Guan et al. 1994).

1.1.2.2.3. Centromeric probes

Chromosome-specific centromeric probe (CEP) hybridize to centromeric regions of one (in case of D13/21Z1 and D14/22Z1 to two and in case of D1/5/19Z1 to three) specific human chromosome(s). They are commercially available and used to detect aneuploidy in both interphase and metaphase. In clinical diagnosis, for example, CEP are useful to confirm a trisomy of chromosome 21 in Down syndrome, while in AL typically monosomy 7 and/or trisomy 8 need to be checked, as they implicate in the prognosis of AML during therapy (Liehr et al. 2015).

1.1.2.2.4. Multicolor FISH probe (mFISH)

Several methods have been developed to paint each of the 24 human chromosomes in a specific color combination: spectral karyotyping (SKY) (Schröck et al. 1996), multiplex FISH (M-FISH) (Speicher et al. 1996), m-FISH (Senger et al. 1998), COmbined Binary Ratio labelling-FISH (COBRA-FISH) (Tanke et al., 1999) and 24-color-FISH (Azofeifa et al., 2000). These approaches use four to seven different fluorochromes in a combinatorial labeling and/or ratio-labeling (Riegel 2014, Liehr et al. 2004, Liehr 2009). Nowadays, SKY, M-FISH,

and COBRA-FISH are the most advanced WCP-based FISH approaches, and allow the simultaneous visualization of all 24 human chromosomes, in a single hybridization, and in one metaphase spread. It is useful in defining complex translocations and marker chromosomes with unknown origin (Liehr 2015).

1.1.2.2.5. FISH-banding approaches

Many different FISH-banding approaches were introduced in the end of last century. Multitude multicolor banding (mMCB) is a FISH-banding technique which provides the possibility to characterize simultaneously subregions in each chromosome, using overlapping microdissection derived libraries, that are differentially labeled, and produce reproducible multicolored bands and unique patterns of fluorescence ratios along all chromosomes. These fluorescence ratios can be transformed into pseudocolour banding by specific software. This approach allows the differentiation of chromosome region specific areas at the band and subband level, with resolutions between 400-800 bands per haploid karyotype, and provides the possibility to analyses chromosomes irrespective of their condensation grades (Weise et al. 2003, Liehr et al. 2002a). mMCB is applied to characterize inter-and intra-chromosomal rearrangements of the whole human karyotype in one single experiment, to describe marker and/or derivative chromosomes in clinical and tumor cytogenetics (Liehr et al. 2002b, Liehr 2009). Besides, this approach is also available, and was first introduced as a single-chromosome directed application, called multicolor banding (MCB) (Liehr et al. 2002a).

1.1.2.3. Array comparative genomic hybridization (array-CGH)

Array-CGH was developed based on the same principles as CGH on chromosome level. The latter was already introduced in 1992 and enabled the characterization of genetic imbalances in tumors, which could not be karyotyped (Kallioniemi et al. 1992). The development of array-CGH technology for `molecular karyotyping' with a much higher resolution than CGH (i.e. ~50-100 kilobases (kb)) is an example of the tremendous technical advances in cytogenetics. It offers higher resolution for genome-wide detection of chromosomal alterations and enables diagnostic and research to analyze hundreds to thousands of genes in one experiment. This lead to massive changes in clinical diagnostics and tumor research approaches (Le Scouarnec and Gribble 2012). In array-CGH the target-DNA are large numbers of mapped genomic clones, initially BAC or PAC (bacterial/P1-derived artificial chromosomes), which are spotted onto a standard glass slide (Fig.1.2) (Pinkel et al. 1998). The resolution of the different platforms is dependent on the size, number, and uniformity of

the genomic distribution of the probes. Array-CGH has been widely used to identify chromosomal imbalances through the detection of CNAs especially in leukemia and lymphoma, to distinguish the candidate genes that involved in the pathogenesis of cancer, and leading to cancer classification proposals. Indeed, array-CGH is not suitable technique to detect the recurrent balanced translocations, inversions or insertions but only to identify submicroscopic imbalances (Riegel 2014, Le Scouarnec and Gribble 2012).

Besides, SNP array-CGH based approaches greatly improved the resolution of this approach down to ~1kb and enables the detection of stretches of homozygosity, which may be hints on deletions or uniparental disomy (UPD) (Le Scouarnec and Gribble 2012).

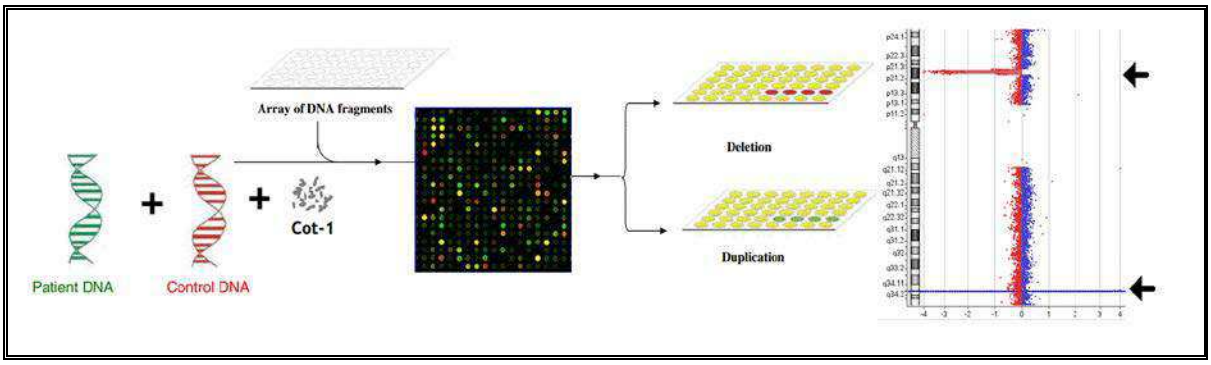

Figure 1.2. Principle of array-CGH. Test DNA and control DNA are differentially labeled. Here shown examples of a T-ALL case with deletion in 9p21.3 to 9p21.3 and duplication in 9q34.12 to 9q34.13 were identified [adapted from Othman et al. 2015].

1.1.3. Molecular genetics

In the 1980s technical improvements led to the discovery of genes. Our understanding of the mechanisms and pathways involved in leukemogenesis became to be uncovered. In 1983 and 1984 Grosveld and colleagues cloned the genes involved in the CML-specific translocation t(9;22). They could show, that the 5'*ABL* gene which maps to chromosome 9q34 fused to the 3'*BCR* gene mapping to chromosome 22q11. Also they could show that a novel chimeric *BCR-ABL* gene was formed (Heisterkamp et al. 1983, Groffen et al. 1984, Rowley 1999). Nowadays there are countless molecular genetic approaches available (Murphy and Bustin 2009, Kohlmann et al. 2013). In the following emphases is given only to three selected approaches that are of special interest for this work.

1.1.3.1. Multiplex ligation-dependant probe amplification (MLPA)

MLPA is one of the many different polymerase chain (PCR) reaction based approaches invented during the last 2 decades. It was first described for the detection of exon deletions

and duplications for *BRCA1*, *MSH2* and *MLH1* genes and for detection of trisomies (Schouten et al. 2002). So far, several modifications of MLPA technique have been developed, that include expression profiling (RT-MLPA), detection of known point mutations (array-based MLPA), and determination of the methylation status for imprinted genes and promoter regions (MS-MLPA) (Hömig-Hölzel and Savola 2012). MLPA is a multiplex polymerase chain reaction (M-PCR)-based technique, used to detect small CNAs within DNA sequences in a quantitative way. It enables to detect an aberrant copy number of up to 50 genomic DNA sequences in a single experiment (Fig 1.3). Still it cannot differentiate between a point mutation hampering PCR itself from a loss of copy numbers. MLPA is relatively fast, easily interpreted, cost effective, and e.g. method of choice for routine diagnostic of chronic lymphocytic leukemia (CLL). MLPA has also limitation and not suitable for the detection of the balanced translocations, inversions, unknown point mutations and distinguish diploid from haploid sets (Hömig-Hölzel and Savola 2012, Alhourani et al. 2014).

1.1.3.2. New high throughput approaches

DNA sequencing is considered to be the gold standard tool for detection of point mutations associated with inherited and acquired genetic disease. Full sequencing of genes or genomes was not involved in routine cancer diagnostics until to date. Currently, next generation sequencing (NGS) technology (also known as “massively parallel” sequencing) allows to sequence the whole human genome (WHG), exome or transcriptome within a few days. It is based on sequencing of millions of DNA molecules simultaneously, after library preparation with production of sequence reads of 30-400 base pairs (bp) (Ilyas et al. 2015, Koboldt et al. 2013).

1.1.3.3. Quantitative Real-time polymerase chain reaction (qRT-PCR)

PCR provides a method for amplifying and studying alleles of specific genes or the mRNA transcribed from those genes. qRT-PCR is an in vitro method for reverse transcription of RNA followed by amplification of complementary DNA (cDNA). qRT-PCR is also very useful in detecting of the recurrent chromosomal translocations and rearrangements that generate oncogene fusion transcripts. For example, translocations t(4;11), t(8;21), t(9;22), t(12;21) and t/inv(16) can be simultaneously screened. Moreover, this technique is an efficient and highly sensitive in diagnostic that assist in selection of appropriate therapy and monitor the minimal residual disease (MRD) (Murphy and Bustin 2009, Olesen et al. 2004).

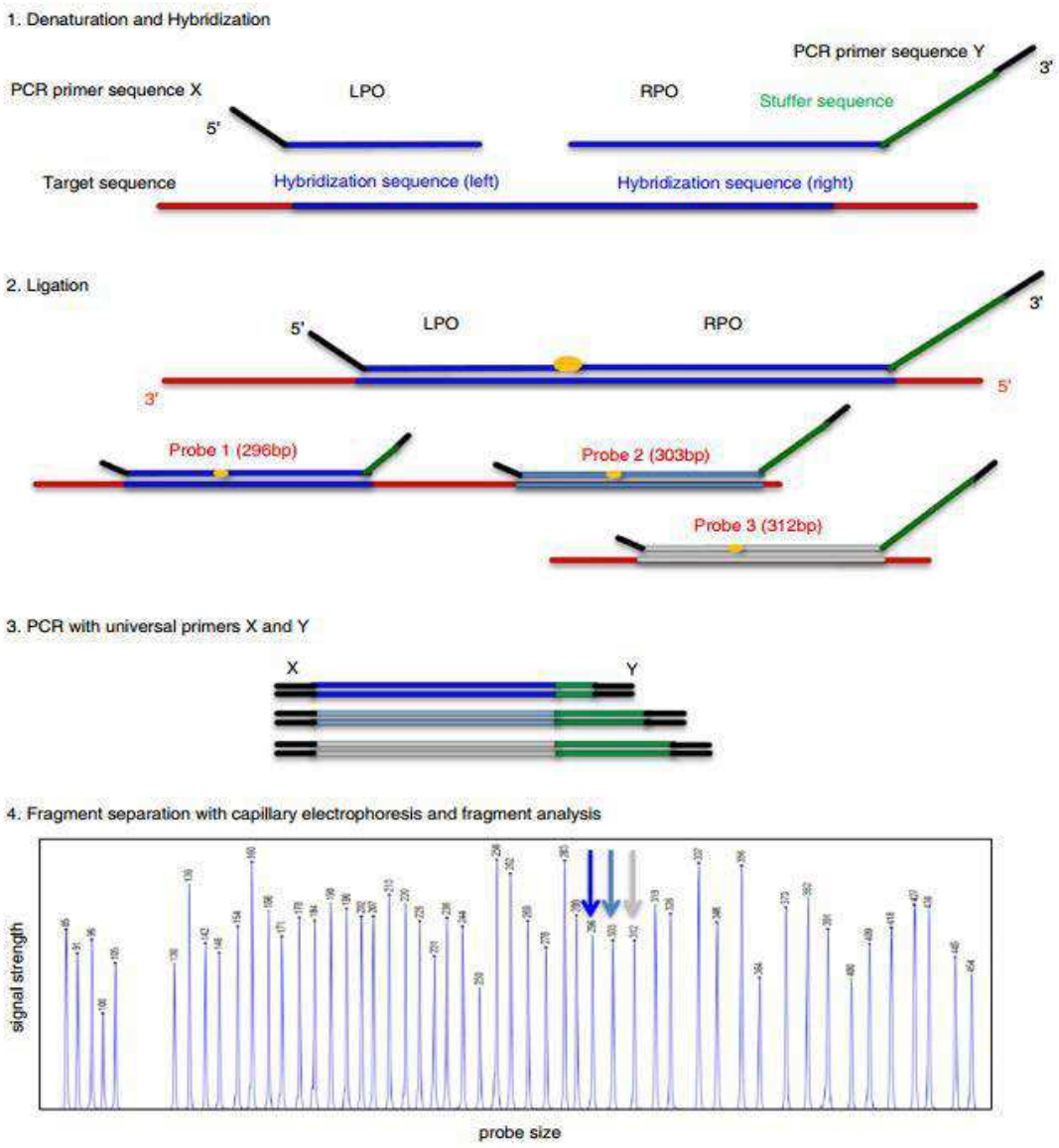

Figure 1.3. Principle of MLPA reaction including: 1) DNA denaturation and hybridization of MLPA probes; 2) ligation reaction; 3) PCR reaction; 4) separation of amplified products by electrophoresis and data analysis [adapted from Hömig-Hölzel and Savola 2012].

1.2. The biology of leukemia

All lineages of blood cells originate from a pool of self-renewing hematopoietic stem cells (HSCs) that resides in the bone marrow (BM). They can differentiate into two main lineages: lymphoid and myeloid progenitor cells (Longo 2013). Lymphoid progenitors can differentiate into B-lymphocytes, or T-lymphocytes. B-lymphocytes (or B-cells) differentiate in BM, while T-lymphocytes (or T-cells) proliferate and differentiate in the thymus (Hardy and Hayakawa 2001, Rothenberg et al. 2008). Mature B- and T-cells leave to peripheral lymphoid organs through the bloodstream. The myeloid progenitors can give rise to bipotent granulocyte-monocyte progenitors, whose final progeny are nucleated cells (monocytes and granulocytes);

besides they can differentiate into megakaryocyte-erythroid progenitors, which give rise to mature thrombocytes and erythrocytes. Mature granulocytes and monocyte are released into the bloodstream. Abnormalities in the normal blood cells differentiation and/or proliferation program result in hematological diseases, particularly leukemia.

Leukemia is a neoplastic proliferation of hematopoietic precursor cells, arises from a mutated myeloid progenitor or lymphoid progenitor cell. These cells infiltrate the blood-forming tissues and circulate in the bloodstream. Commonly, leukemia is divided into two main classes: acute and chronic leukemia, which are further classified into lymphoid and myeloid types, depending on the cell lineage represented by the leukemic clone.

Numerous transcription factors are involved in expression of genes during the progression of lymphoid cell precursors from the immature stage till they migrate into periphery. Though, mutations in transcription factors and/or overexpression of genes are tightly connected to lymphoid malignancies; for example mutations in the *PAX5, IKZF1 and EBF1* genes which are important for B-cell development and differentiation, and thus associated with B-cell acute lymphoblastic leukemia (B-ALL) (O'Brien et al. 2011, Mullighan 2013). In contrast, somatic mutations leading to overexpression or acquired deletions in transcription factors have been described for myeloid cell development. In most of these cases they lead to inhibition of proliferation, block of differentiation and/or lead to altered lineage commitments. For example, mutation in C/EBP alpha which regulates proliferation and controls terminal granulocytic differentiation is associated with AML (Ho et al. 2009).

1.3. Acute leukemia (AL)

AL is an aggressive and heterogeneous disease characterized by uncontrolled clonal proliferation and accumulation of poorly differentiated blast cells in the BM. AL shows a fast clinical pattern in comparison to chronic leukemia which is generally less aggressive. Without treatment AL can result in death within a few months. AL constitutes 95% of all childhood leukemias (Coebergh et al. 2006, Estey and Döhner 2006, Inaba et al. 2013). The severity of AL depends on leukemic cells infiltrating the BM and extramedullary organs and on the extent to the BM failure. Typical signs and symptoms of AL are fever, fatigue, pallor, bruises, bleeding, hepatosplenomegaly, lymphadenopathy, thrombocytopenia, coagulopathy, hyperleukocytosis and bone pain. In addition, central nervous system (CNS) involvement is possible (Reman et al. 2008, Nowak-Gottl et al. 2009). Overall, classification of AL plays an essential role in determining both treatment options and prognosis.

1.4. Acute lymphoblastic leukemia (ALL)

ALL is a malignant disease with clonal proliferation of lymphoid progenitor cells. It arises from recurrent genetic alterations that block precursor B and T cell differentiation and affect children (Teitell and Pandolfi 2009). ALL represents ~80% of childhood AL and ~25% of all childhood cancers (ages 0-15 years) but only ~20% of adult AL (Bassan et al. 2004, Inaba et al. 2013, ACS 2015). Worldwide, a sharp peak in incidence is observed among ALL children aged 2 to 5 years. In other words in Western Europe and in USA ALL appears in up to 40 cases / million and year, while in Eastern Europe and Japan the rate is only around 30 cases/ million and year; in sub-Saharan Africa, India, and in the Middle East the rate is only 20 cases/million and year. This suggests either that in the industrialized Western countries there are higher exposures to environmental leukemogenes or that the genetic backgrounds are different (Stiller 2004, Howard et al. 2008, Hrusak et al. 2002, Linabery and Ross 2008).

1.4.1. Classification of ALL

ALL was initially classified into three major subgroups: L1 (80%), L2 (15%), and L3 (5%) based on French-American-British (FAB) Cooperative Group criteria using morphological features of lymphoblasts. L1 was correlated with the best prognosis, higher relapse rates were found for L2 and for L3 cases an adverse prognosis was given (Bennett et al. 1981). As mentioned ALL also classified into B and T cell ALL according to the expression of specific antigens easily identifiable by flow cytometry (appendix Tab. 1.1). B-ALL constitutes 80-85% and T-ALL the remainder of ALL cases. B-ALL patients have a favorable prognosis with an overall complete remission (CR) rate of 95% for children between 1-15 years, and of 60% for adults. Adverse prognosis in T-ALL was correlated with male gender, older age, leukocytosis and mediastinal mass (Perez-Andreu et al. 2015, Faderl et al. 2010, Goldberg et al. 2003). Hence, immunophenotype (Benter et al. 2001) and genetic and cytogenetic classifications of ALL are important aspects of diagnosis, risk assessment, treatment and prognosis in ALL (Vardiman 2010). Nowadays, around 80% of ALL patients can be readily classified into therapeutically relevant subgroups based such data (appendix Tab. 1.2).

1.4.2. Clinical prognostic factors in ALL

Prognostic factors to be assessed during ALL diagnostics are:

Age: Children between 1 and <10 years of age with B-ALL tend to have favorable prognosis, while infants, adolescents, and adults are considered high-risk for treatment failure. For T-ALL patients no effect of age for clinical outcome is know, yet (Hilden et al. 2006, de Bont et al. 2004). **WBC count** is a crucial variable for describing the nature of leukemia. Children

who have WBC counts more than 50,000/µl are classified as a high risk of relapse and need more intensive treatment (Vaitkevičienė et al. 2011).

1.4.3. Cytogenetic aberrations in ALL

Cytogenetic chromosomal abnormalities are detected in 50-60% of ALLs and may be structural or numerical (Fig. 1.4). Such aberrations are prognostic factors, too. **Chromosome numbers:** High hyperdiploidy (51-65 chromosomes) has been connected with good survival and excellent outcome in B-ALL, while hypodiploidy (*<44 chromosomes*) has worse prognosis (Chilton et al. 2014, Holmfeldt et al. 2013). **Chromosomal translocations:** Patients with a translocation t(12;21)(p13;q22)/*ETV6/RUNX1* are more likely to be excellent cured, while those with a translocation t(9;22) or t(4;11) tend to have unfavorable outcomes (Bhojwani et al. 2012, Woo et al. 2014, Pui et al. 2003). In appendix Tab. 1.2 summarized the most common cytogenetic prognostic marker in ALL subtypes.

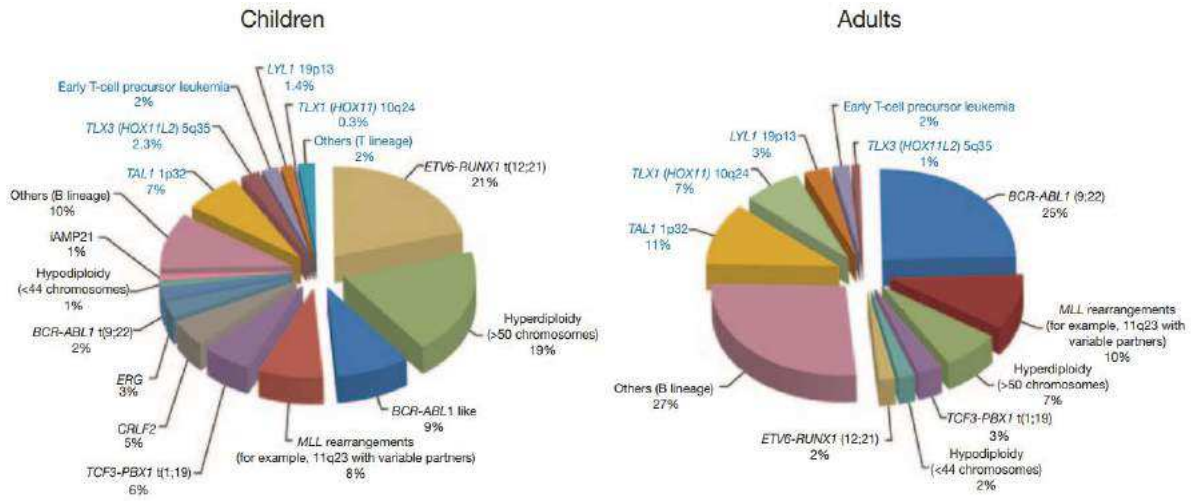

Figure 1.4. Summary of the frequency of cytogenetic and molecular genetic aberrations frequently detected in ALL. Left side refers to childhood ALL and right side to adulthood ALL; B-ALL aberrations are indicated in black letters while T-ALL in blue ones [adapted from Downing et al. 2012].

In ALL hyperdiploid karyotypes, the translocation t(9;22)(q34;q11), 11q23 (*MLL* gene*)* rearrangements, translocations t(12;21)(p13;q22), t(1;19)(q23;p13) and t(8;14)(q24;q32) are the most frequent structural cytogenetic abnormalities, while the genetic alterations associated with ALL hypodiploidy are: deletion in/of the genes *TP53*, *RB1*, and *IKZF1* (Paulsson et al. 2003, Chilton et al. 2014, Nachman et al. 2007, Holmfeldt et al. 2013). The most recurrent structural chromosomal aberrations in ALL are summarized in appendix Tab. 1.3.

1.4.3.1. Cytogenetically normal ALL (CN-ALL)

CN-ALL represent up to ~50% of ALL cases. T-ALL- showed a normal karyotype more frequently than B-ALL patients and accordingly here cytogenetic markers cannot be determined. Based on the knowledge that chromosomes in ALL show a low banding resolution and that a good part of ALL cases present with a normal karyotype, it is not far to seek, that small aberration can easily be missed when analyzing ALL derived chromosomes by banding cytogenetics (Karst et al. 2006, Mrózek et al. 2009).

1.4.3.2. Complex karyotypes in ALL

Complex karyotypes are also well known and typical for approximately 5% of ALL cases. Such complex karyotypes include more than three to five chromosomal abnormalities. This group has been reported to indicate a significantly increased risk of treatment failure. Still, this prognostic marker has been incorporated in the definition of high-risk ALL groups (Moorman et al. 2007).

1.4.4. Molecular genetics of ALL

CNAs are changes that alter the genome structure. They can be simple abnormal numbers of chromosomes (losses or gains) or, smaller, down to submicroscopic deletions or duplications. CNAs can be detected by technologies like MLPA, array-CGH, SNP-array-CGH and FISH using LSP. As submicroscopic CNAs have been revealed focal deletions, but also less frequently duplications or sequence/point mutations in genes that primarily serve as transcriptional regulators of the lymphoid development pathway (Mullighan 2012, Van Vlierberghe and Ferrando 2012, Inaba et al. 2013, Woo et al. 2014, Faderl et al. 2010). Common CNAs in ALL are listed in appendix Tab. 1.4. Numerous new genetic alterations have been discovered in ALL by using high throughput technologies such as NGS. Appreciation of these genomic abnormalities and mutations led to redefining subclassifications of ALL, recently (Pui et al. 2012, Mullighan 2013). For a number of target genes that play a key role in lymphoid development (e.g., *PAX5*, *IKZF1*, *EBF1*, *LMO2*) somatic mutations have been identified in B and T-ALL. For instance, deletion of *PAX5* has been detected in 30% of B-ALL (Mullighan et al. 2007). *JAK2* is a member of a family of tyrosine kinases involved in cytokine receptor signaling. Mutations in *JAK2* were identified in 10% of high-risk childhood B-ALL and frequently associated with other abnormalities, including deletions or mutations of *IKZF1* and overexpression the *CRLF2* gene (Mullighan et al. 2009a). In T-ALL, *NOTCH1*-activating gene mutation have been found in 60% and

FBXW7-inactivating gene mutation occurs in 20% of pediatric T-ALL (Gallo Llorente et al. 2014). Less commonly, mutations in *PTEN*, *WT1*, amplification of *MYB* and sequence mutations in ras signaling (*NRAS*, *KRAS*, and NF1) and tumor suppression (TP53) have been identified in ALL (Mullighan 2013).

1.5. Acute myeloid leukemia (AML)

AML is clinically and biologically a heterogeneous disease, characterized by clonal proliferation of myeloid precursors. These immature cells accumulated in BM or can escape into the peripheral blood, and infiltrate other organs (Ferrara and Schiffer 2013, Estey 2013). AML accounts for ~20% of childhood AL and is the most common AL type in adults over 60 years of age. AML represents ~80% of all adult AL. The frequency of AML remains stable throughout childhood with a slight increase during adolescence age can be observed. 4-10 cases per million children develop an AML annually (Stiller 2004, Belson et al. 2007). In advanced ages, the frequencies dramatically change: 3-10 cases per 100,000 per individuals over 65 years old per year are diagnosed with AML (Yamamoto and Goodman 2008, Dores et al. 2012).

1.5.1. Classification of AML

AML has been classified as to FAB into eight different subtypes (M0–M7) which depend on morphological and cytochemical evaluation. Some subtypes of AML tend to have a better outcome than others. For example, M3 subtype has a more favorable outcome, while undifferentiated AML-M0 and M7 are harder to treat effectively and have poorer outcome (Craig and Foon 2008, Vardiman et al. 2009). Cell surface and cytoplasmic expressed antigens help in diagnosis and classification of AML (appendix Tab. 1.5) (Vardiman et al. 2009). Recently, WHO classified seven subtypes of AML with recurrent (cyto)genetic abnormalities (appendix Tab. 1.6). Each of these translocations or inversions results in a fusion gene encoding a chimeric protein that participates in leukemogenesis (Vardiman et al. 2009, Dores et al. 2012).

1.5.2. Clinical prognostic factors in AML

Age: Children younger than 2 years suffering from AML have better prognosis than older children, while adult less than 60 years have favorable outcome with higher rates of achieving CR compared to those older than 60 years (Shah et al. 2013, Creutzig et al. 2008). **WBC count**: AML patients with WBC counts higher than 100,000/µl are classified as having a high risk of relapse and need more intensive treatment (Löwenberg et al. 1999).

1.5.3. Cytogenetic aberrations in AML

Abnormal karyotypes can be detected in 50-60% of AML patients. To date, many specific translocations and inversions have been described in AML (appendix Tab. 1.7). AML patients who have translocations t(15;17), t(8;21), t(16;16)/or inv(16) have better chances to become cured and receive a CR, whereas patients with monosomies of chromosomes 5 or 7, with 11q23 rearrangements, monosomic and/or complex karyotypes are associated with poor prognosis, and require hematopoietic stem cell transplantation (HSCT) during their first remission (appendix Tab. 1.8) (Grimwade et al. 2010, Kayser et al. 2012, Ferrara and Schiffer 2013). Gain of chromosome 8 (trisomy 8) and loss of chromosomes 5 and 7 (monosomy 5 or 7) are the most frequent numerical chromosomal abnormalities observed in different subtypes of AML. The recurrent loss of chromosome material proposes the existence of a putative tumor suppressor gene in these regions, as well the gain of chromosome result from the presence of potential oncogene that regulates myeloid precursor cells in proliferation and differentiation. Thus, loss of function or overexpression may leads to leukemic transformation (Braoudaki and Tzortzatou-Stathopoulou 2012, Schoch et al. 2006).

1.5.3.1. Cytogenetically normal AML (CN-AML)

CN-AML accounts 40-50% of de novo AML and up to 10% of sAML (secondary, therapy related AML). It is a very heterogeneous group of patients with variable age, morphological features, clinical course, and response to therapy. In this group patients are thought to have cryptic (cyto)genetic changes and categorized in the intermediate risk group (Gross et al. 2009, Grimwade et al. 2010, Walker and Marcucci 2012).

1.5.3.2. Complex karyotypes in AML

Complex karyotypes with three or more numerical and/or structurally altered chromosomes have been well recognized in AML, too with a high degree of genomic complexity with an average of 14 aberrations per case. Complex karyotypes occur in ~10% of AML patients. Noticeably, complex karyotypes may involve *TP53* deletions and/or mutations. Indeed, this subgroup does not appear to be associated with age, gender, or WBC count, and particularly abnormalities of 17p or *TP53* are predictive of a high risk of treatment failure in AML (Mrózek 2008, Rücker et al. 2012, Middeke et al. 2014).

1.5.4. Molecular genetics of AML

Mutations in certain genes include *FLT3*, *NPM1*, *IDH1/2*, *KIT*, *BAALC* and *CEBPA* have significant impact on the prognosis in adult AML, particularly in CN-AML (Walker and Marcucci 2012). Point mutations or amplification of oncogenes provided new insight into the pathogenesis of CN-AML and also are important for further clarifying prognosis (Ilyas et al. 2015). E.g. *NPM1* gene mutations were identified in ~35 and 50% of de novo AML and CN-AML, respectively. Sole mutation in *NPM1* has been found as well as accompanied with other gene mutations including *FLT3* and *IDH1/2* (Ferrara and Schiffer 2013, Schneider et al. 2012, Port et al. 2014).

1.6. Aim of study/Questions worked on

Normal karyotypes can be observed in AL in 40-50% of all cases studied by routine GTG-banding analysis. According to what was outlined in the introduction part, unknown cryptic changes must be suggested to be present in the leukemic cells of these patients. The aberrations to be expected are suggested to be on the submicroscopic level. Previous studies have found such so-called cryptic aberrations when using high resolution FISH approaches. The observed aberrations fall into two groups: a) such cases which were only detectable by FISH and b) such which would have been also possible to be picked up, if more or better metaphases would have been analyzed in routine cytogenetics (Karst et al. 2006, Gross et al. 2009). Besides, identification of additional aberrations (like point mutations or epigenetic changes) can be expected when using other, more molecular oriented approaches.

Thus, the aims of the present work were:

1 to identify overlooked and unknown cryptic chromosomal rearrangements in both CN-ALL (61 cases) and CN-AML (42 cases);
2 to characterize in detail here new identified tumor-associated acquired chromosomal breakpoints in CN-ALL and CN-AML cases;
3 to characterize in detail the tumor-associated acquired breakpoints also in complex aberrant karyotypes of one ALL and seven AML cases;
4 to detect submicroscopic structural CNAs in ALL and AML cases using MLPA and array-CGH;
5 to correlate the new tumor-associated acquired rearrangements with diagnostic, prognostic and therapeutic relevance.

Overall, the present work led to the numerous publications, 10 of which were selected for this thesis, which all deal with answering the questions raised before.

2. Results

2.1. Basic papers of thesis

1- Liehr T, **Othman MA,** Rittscher K, Alhourani E. **The current state of molecular cytogenetics in cancer diagnosis.** Expert Rev Mol Diagn, 2015;15(4):517-526.

2- **Othman MA,** Grygalewicz B, Pienkowska-Grela B, Rincic M, Rittscher K, Melo JB, Carreira IM, Meyer B, Marzena W, Liehr T. **Novel Cryptic Rearrangements in Adult B-Cell Precursor Acute Lymphoblastic Leukemia Involving the MLL Gene.** J Histochem Cytochem, 2015;63(5):384-390.

3- **Othman MA,** Melo JB, Carreira IM, Rincic M, Alhourani E, Wilhelm K, Gruhn B, Glaser A, Liehr T. **MLLT10 and IL3 rearrangement together with a complex four-way translocation and trisomy 4 in a patient with early T-cell precursor acute lymphoblastic leukemia:** A case report. Oncol Rep, 2015;33(2):625-630.

4- Al-Achkar W, Wafa A, **Othman MA,** Moassass F, Aljapawe A, Liehr T. **An adult B-cell precursor acute lymphoblastic leukemia with multiple secondary cytogenetic aberrations.** Mol Cytogenet, 2014;7:60.

5- **Othman MA,** Rincic M, Melo JB, Carreira IM, Alhourani E, Hunstig F, Glaser A, Liehr T. **A Novel Cryptic Three-Way Translocation t(2;9;18)(p23.2;p21.3;q21.33) with Deletion of Tumor Suppressor Genes in 9p21.3 and 13q14 in a T-Cell Acute Lymphoblastic Leukemia.** Leuk Res Treatment, 2014;2014:357123.

6- **Othman MA,** Grygalewicz B, Pienkowska-Grela B, Ejduk A, Rincic M, Melo JB, Carreira IM, Meyer B, Marzena W, Liehr T. **A novel IGH@ gene rearrangement associated with CDKN2A/B deletion in young adult B‑cell acute lymphoblastic leukemia.** Oncol Lett, 2016; 11(3): 2117-2122.

7- **Othman MA,** Melo JB, Carreira IM, Rincic M, Glaser A, Grygalewicz B, Gruhn B, Wilhelm K, Rittscher K, Meyer B, Silva ML, Marques-Salles Tde J, Liehr T. **High rates of submicroscopic aberrations in karyotypically normal acute lymphoblastic leukemia.** Mol Cytogenet, 2015;8:45.

8- **Othman MA,** Vujić D, Zecević Z, Đurišić M, Slavković B, Meyer B, Liehr T. **A cryptic three-way translocation t(10;19;11)(p12.31;q13.31;q23.3) with a derivative Y-chromosome in an infant with acute myeloblastic leukemia (M5b).** Gene, 2015;563(2):115-119.

9- Jancuskova T, Plachy R, Zemankova L, Hardekopf DW, Stika J, Zejskova L, Praulich I, Kreuzer KA, Rothe A, **Othman MA,** Kosyakova N, Pekova S. **Molecular characterization of the rare translocation t(3;10)(q26;q21) in an acute myeloid leukemia patient**. Mol Cytogenet, 2014;7:47.

10- Al-Achkar W, Aljapawe A, **Othman MA,** Wafa A. **A de novo acute myeloid leukemia (AML-M4) case with a complex karyotype and yet unreported breakpoints.** Mol Cytogenet, 2013;6:18.

2.2. Article .1

Liehr T, **Othman MA,** Rittscher K, Alhourani E. **The current state of molecular cytogenetics in cancer diagnosis.** Expert Rev Mol Diagn, 2015;15(4):517-526.

Review

The current state of molecular cytogenetics in cancer diagnosis

Expert Rev. Mol. Diagn. 15(4), 517–526 (2015)

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Cytogenetics and molecular cytogenetics are and will continue to be indispensable tools in cancer diagnostics. Leukemia and lymphoma diagnostics are still emphases of routine (molecular) cytogenetics and corresponding studies of solid tumors gain more and more prominence. Here, first a historical perspective of molecular tumor cytogenetics is provided, which is followed by the basic principles of the fluorescence *in situ* hybridization (FISH) approach. Finally the current state of molecular cytogenetics in cancer diagnostics is discussed. Nowadays routine diagnostics includes basic FISH approaches rather than multicolor-FISH. The latter together with modern high-throughput methods have their impact on research to identify new tumor-associated genomic regions.

Keywords: copy number variation • counseling • cytogenetics • fluorescence *in situ* hybridization • leukemia • lymphoma • molecular cytogenetics • oncogene • solid tumors • tumor suppressor gene

Even though they have been called outdated for decades [1], cytogenetics and molecular cytogenetics still are and will stay in future indispensable tools in diagnostics. This statement is true for clinical aspects of prenatal and postnatal patient care but also for patients suffering from neoplasia, in particular leukemia, lymphoma and solid tumors, as well. In this review, the development of cytogenetics and molecular cytogenetics is summarized, the basic technique of molecular cytogenetics is outlined together with an overview on the different kinds of probes available for fluorescence *in situ* hybridization (FISH) and the current state of molecular cytogenetics in cancer diagnostics is given. This includes especially the commercially available probe sets applied in routine neoplasia diagnostics and those multicolor FISH (mFISH) tools used in research to identify new tumor-associated critical genomic regions.

Cytogenetic & molecular cytogenetics

The history of human cytogenetics started not before the year 1879. At this time, microscopes of a certain quality were available, which were prerequisite to localize and identify chromosomes in a cell. All chromosomal studies between 1879 until approximately 1970 were retrospectively summarized as having been performed in the 'pre-banding era'. Only so-called 'classical cytogenetic studies' were possible in that time, that is, chromosomes could exclusively be distinguished by size and centromere index [2]; nowadays classical cytogenetics is still essential in animal [3] and plant cytogenetics [4]. However, the determination of the correct modal human chromosome number in 1956, the first characterization of inborn numerical chromosome aberrations (like Down syndrome) as well as the detection of first tumor-associated aberrations were all achieved during the early days of classical cytogenetics [2]. As summarized by E Gebhart (1989) [5], tumor-associated chromosomal anomalies were indeed already recognized by the first observer of human chromosomes, J Arnold in 1879. In 1890, it was D von Hansemann who highlighted that unusual, asymmetric mitosis can be observed only in cancer cells. Partially based on this, T. Boveri established in 1914 a 'chromosome theory of cancer development' [5], which turned out to be basically true many years later [6]. Between 1927 and 1956, there were multiple attempts to characterize chromosome content and numbers of tumor cells, which were basically hampered by the fact that the

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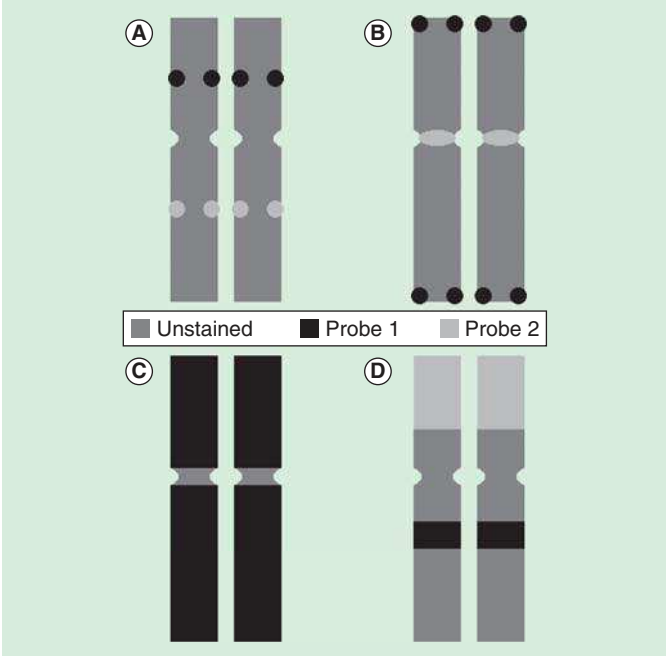

Figure 1. Schematic drawing depicting the four different kinds of fluorescence *in situ* hybridization-probes as differentiated in this review. (A) Locus-specific, single-copy probes, including subtelomeric probes. **(B)** Probes specific for repetitive sequences like telomeric (probe 1) and centromeric regions (probe 2). **(C)** A whole chromosome painting probe and **(D)** partial chromosome painting probes.

constitutional chromosome number in human was not determined (correctly) at that time. It is noteworthy that the chromosomal aberration being typical for chronic myelogenous leukemia, so-called Philadelphia chromosome, was already detected in the 'pre-banding era' (in 1960). The same holds true for characterization of monosomy 22 as being typically observed in meningioma (in 1967), and double minutes (in 1962) later being identified as one of the cytogenetic equivalents of oncogene amplification [5]. Interestingly, even G Mendel, the 'father of modern genetics' postulated the existence of linkage groups (in German 'Kopplungsgruppe') for the features he studied in peas [7]; and these linkage groups were nothing else than chromosomes.

Logically, after 'pre-banding era' came the 'pure banding era', starting with the invention of the Q-banding method by Lore Zech (Uppsala) in 1968 [8]. Based on this, the GTG-banding approach (G-bands by trypsin using Giemsa) was established in 1971, which remained the gold standard of all cytogenetic techniques until now [2,5]. Using banding cytogenetics, more chromosomal abnormalities, like translocations, inversions, deletions and insertions, could be detected and precisely characterized, which was impossible before. Many tumor-specific aberrations were clearly identified since then, like the aforementioned Philadelphia chromosome which was characterized to be the result of a reciprocal translocation t(9;22)(q34;q11) in 1973. Also the acquired translocation t(8;14)(q24;q32) detected in Burkitt's lymphoma in 1976 and the characterization of homogeneously staining regions in 1978 were important findings enabled due to banding cytogenetics [5].

As black and white banding pattern together with chromosome morphology are the only two parameters that can be evaluated in GTG-banding, origin of additional material in a derivative chromosome often remains unclear. In order to overcome this kind of limitations, molecular cytogenetic approaches were and are necessary. *In situ* hybridization allows for examination of nucleic acid sequences inside cells or on chromosomes and was first described in 1969 as a radioactive approach. As nonradioactive probe labeling was not invented before 1981, non-radioactive FISH was needed until 1986, until it was ready to be used in human cytogenetics. Apart from avoidance of health-threatening radioactivity, FISH speeds up analysis time and comprises the possibility to detect several targets simultaneously (see below in section "FISH-techniques") [2].

Thus, 'pure banding era' finished in 1986 with the first successful molecular cytogenetic experiment on human chromosomes by D Pinkel and colleagues. The period since then may be denominated 'banding and molecular cytogenetic era' as banding cytogenetics and molecular cytogenetics complemented each other and became important tools on an equal footing in many fields of human diagnostics, including the care of cancer patients. Initially, there were two basic approaches in molecular cytogenetics: FISH and primed *in situ* hybridization (PRINS). However, the latter never acquired the importance of FISH, as it is much less robust and was never developed in a multicolor variant [2,9].

Especially important for tumor cytogenetics was inventing a molecular cytogenetic approach called comparative genomic hybridization (CGH). In CGH, two genomes are analyzed for gains and losses of genomic material at a low resolution of 5–10 Mb. Even though a main feature of many solid tumors is their abnormal rapid *in vivo* growth, corresponding tumor cells often refrain from growing in cell culture. Thus, originally CGH gave first insights into chromosomal imbalances of many previously not cytogenetically analyzed solid tumor types. Indeed, CGH was applied more in research rather than as a diagnostic tool [10]. An advancement of this chromosome-based CGH approach is the so-called array-CGH, providing much higher resolution of approximately 50 kb or even less, and being used routinely in clinical rather than cancer diagnostics, however, applied in cancer research [2,11,12].

Before discussing molecular cytogenetic applications in cancer diagnostics, some aspects about how the FISH technique itself is performed need to be stressed.

FISH – technical aspects

DNA probes applied in FISH can be grouped in different ways; here we suggest doing it as follows:

- locus-specific, single-copy probes;
- probes specific for repetitive sequences;
- whole chromosome painting probes (wcp);
- partial chromosome painting probes (pcp) (Figure 1).

All four kinds of probes may be used in diagnostics and should be applied at least in two-color FISH experiments: one probe as specific for the region of interest, the second one as a control. Most commercially available probes are locus- and/or centromere-specific ones (see Tables 1–3) [2].

Besides, mFISH probe sets can be of importance in molecular tumor-cytogenetic diagnostics, and they are even more considerable in research. mFISH is defined as the simultaneous use of at least three different ligands or fluorochromes for the specific labeling of DNA, excluding the counterstain. The first commercially available and still diagnostically relevant mFISH probe sets were put together in 1996 by M Speicher and colleagues and E Schröck and coworkers, respectively, enabling the staining of each of the 24 human chromosomes in different colors using wcp probes. This kind of probe set was developed in parallel, with slight modifications and described under different names as mFISH (=multiplex FISH), SKY (=spectral karyotyping), multicolor FISH, COBRA-FISH (=COmbined Binary RAtio labeling FISH) or 24-color FISH [2]. A summary on possible applications besides cancer diagnostics can be found elsewhere [13].

As mFISH methods applying wcp probes are not suited for exact chromosomal breakpoint characterization, different approaches summarized as 'FISH banding methods' were developed. The latter 'are any kind of FISH technique, which provide the possibility to characterize simultaneously several chromosomal subregions smaller than a chromosome arm with resolution down to 5 Mb (excluding the short arms of the acrocentric chromosomes). FISH banding methods fitting that definition may have quite different characteristics, but share the ability to produce a DNA-specific chromosomal banding' [14]. The most often applied FISH-banding approach is the microdissection-based multicolor banding (MCB or m-band). Other mFISH probe sets such as for all subtelomeric regions (M-Tel-FISH) or variants of centromere-specific multicolor FISH (=cenM-FISH) are commonly not applied in cancer diagnostics [2]. Array-CGH and next-generation sequencing (NGS) methods are not considered as 'molecular cytogenetic' approaches, even though some authors surprisingly do this [15]. The latter may be warranted by the recent description of chromothripsis based on NGS [16]. However, it has to be emphasized that complex chromosomal rearrangements and even conditions like 'chromosome-pulverization', which may be one step of chromothripsis, are known for decades already from pre-banding era of cytogenetics [5].

Molecular cytogenetics in cancer diagnosis

It goes without saying that in neoplasia the identification of cytogenetic markers[1] is of high clinical significance for diagnostics, follow-up studies and prognosis [5,17,18]. In the first years after introduction of molecular cytogenetics into cancer

[1]A 'cytogenetic marker' is a set phrase in tumor cytogenetics. It can be, for example, a trisomy 8 as well as a translocation leading to oncogene activation or a deletion leading to tumor-suppressor gene loss.

Table 1. List of most important commercially available fluorescence *in situ* hybridization-probes for leukemia.

Leukemia subtype	Target region	Gene
Myelodysplastic syndrome	3q26	*EVI1*
	4q24	*TET2*
	5q31.2	*EGR1*
	6p22 and 9q34	*DEK/NUP214*
	7q22 and 7q31	*RELN/TES*
	11q21	*MAML2*
	16p13 and 16q22	*MYH11/CBFB*
	20q12 and 20q13.12	*PTPRT/MYBL2*
Chronic myeloid leukemia	4q12	*FIP1L1/CHIC2/PDGFRα*
	5q32~33	*PDGFRB*
	9p24	*JAK2*
	9q34 and 22q11	*BCR/ABL*
	11q22	*ATM*
	17p13	*P53*
Acute myeloid leukemia (AML)	3q26	*EVI1*
	4q12	*KIT*
	5q31.2	*EGR1*
	5q32	*CSF1R*
	5q35	*NPM1*
	6p22 and 9q34	*DEK/NUP214*
	6q23	*MYB*
	6q27	*MLLT4*
	7q22 and 7q31	*RELN/TES*
	9p24	*JAK2*
	9p21.3	*MLLT3*
	11p15	*NUP98*
	11q23	*MLL*
	15q24 and 17q21.2	*PML/RARα*
	16p13 and 16q22	*MYH11/CBFB*
	20q12 and 20q13.12	*PTPRT/MYBL2*
	21q22	*ERG*
	22q22 and 8q21	*RUNX1/RUNX1T1*
Chronic lymphocytic leukemia	3q26	*TERC*
	5q32	*CD74*
	6q21	*SEC63*
	6q23	*MYB*
	11q22	*ATM*
	11q13	*Cyclin D1*
	11q22 and 18q21	*BIRC3/MALT1*
	12q13	*GLI*
	13q14.3	*DLEU2* or *D13S25*
	14q32 and 11q13	*IGH/CCND1*
	17p13	*P53*
	19q13	*BCL3*
Acute lymphocytic leukemia	Xp22.3	*CRFL2*
	Xp22.3	*P2RY8*
	1p32	*SIL/TAL1*
	1q23 and 19p13.3	*PBX1/TCF3*
	4q21 and 11q23	*MLL/AFF1*
	5q35	*TLX3*
	6q23	*MYB*
	7q34	*TCRB*
	8q24	*C-MYC*
	9p21	*P16* or *CDKN2A*
	9p13	*PAX5*
	9q34 and 22q11	*BCR/ABL*
	10q23	*PTEN*
	10q24.3	*TLX1*
	11q23	*MLL*
	12p13 and 22q22	*TEL/AML1*
	14q11	*TCR A/D*
	14q32.13	*TCL1*
	14q32.3	*IGH*
	19p13	*E2A*
	22q22 and 8q21	*RUNX1/RUNX1T1*

Table 2. List of most important commercially available fluorescence *in situ* hybridization-probes for lymphoma.

Lymphoma subtype	Target region	Gene
Anaplastic large-cell l	2p23	*ALK*
	5q35	*NPM1*
Burkitt l	2p11	*IGK*
	8q24	*C-MYC*
	14q32.3	*IGH*
	17p13	*P53*
	21q11	*IGL*
Diffuse large B-cell l	2p16	*REL*
	2p11	*IGK*
	3q27	*BCL6*
	8q24	*C-MYC*
	9p21	*P16* or *CDKN2A*
	14q32 and 18q21.33	*IGH/BCL2*
	17p13	*P53*
	19q13	*BCL3*
	21q11	*IGL*
Follicular l	3q27	*BCL6*
	6q23	*MYB*
	9p21	*P16* or *CDKN2A*
	14q32 and 18q21.33	*IGH/BCL2*
	17p13	*P53*
Mantel cell l	5q32	*CD74*
	9p21	*P16* or *CDKN2A*
	11q22 and 18q21	*BIRC3/MALT1*
	13q14.3	*DLEU2*
	14q32 and 11q13	*IGH/CCND1*
	17p13	*P53*
	19q13	*BCL3*
Multiple myeloma	1q21 and 1p36	*c-MAF/SRD*
	1q21 and 8p21	*c-MAF*/n.a.
	4p16.3	*FGFR3*
	5q32	*CD74*
	6q23	*MYB*
	11q22	*ATM*
	13q14	*DLEU2*
	14q32 and 4p16	*IGH/FGFR3*
	14q32 and 11q13	*IGH/CCND1*
	14q32 and 16q23	*IGH/MAF*
	14q32 and 20q12	*IGH/MAFB*
	15q22 and 9q34	n.a. → detection of hyperdiploidy
	17p13	*P53*
Others	2p23	*ALK*
	3q12	*TFG*
	3q27	*BCL6*
	5q35	*NPM1*
	6q23	*MYB*
	10p11.2	*KIF5B*
	11q21 and 18q21	*API/MALT1*
	11q22	*ATM*
	13q14.3	*DLEU2*
	14q32 and 18q21.33	*IGH/BCL2*
	17p13	*P53*

l: Lymphoma; n.a.: Not available.

diagnostics, FISH was most often considered as a tool to continue and refine previous cytogenetic studies. This way to choose and apply corresponding FISH-probes represents still a major part of molecular cytogenetic diagnostics [19–21]. Besides, molecular cytogenetics is more and more performed independently from banding cytogenetic analyses in all kinds of tumors, too [22]. This development was, among others, supported by the fact that every cytogenetic analysis is in need of dividing cells to produce metaphase spreads. In other words, time-consuming cell culture is necessary. Thus, interphase-directed FISH (iFISH) analyses on tumor cell smear, touch preparations or tissue sections are more and more in use with the goal to achieve a quick result [23–25].

FISH approaches are especially suited to characterize chromosomal and subchromosomal copy number changes and gene fusions due to translocations or other rearrangements. All these features are characteristically found acquired aberrations in cancer [5,18,19].

In the following, different FISH-probe types and possible applications in cancer diagnostics are summarized to the best of our knowledge. Various FISH probes may be applied in a specific case due to a finding in banding cytogenetics, indication specific and/or in follow-up studies.

Application of centromeric probes

Exclusive probes directed against the centromeric regions of one specific human chromosome, each, are available for all human gonosomes and most autosomes except for #5, #13, #14, #19, #21 and #22 [26]. As centromeric probes provide dot-like signals after FISH, they can be evaluated in metaphase and interphase easily. They are commercially available and highly suited to determine and/or confirm mono-, tri- or tetrasomies of single chromosomes in tumor cells. Due to often low banding resolution of tumor chromosome, preparations such a metaphase-directed FISH test may even be necessary in routine diagnostics, for example, to determine or confirm the origin of a trisomic chromosome derived from C-group. Numerical aberrations may be observed for practically all human chromosomes in cancer. So just three examples where these probes may be of importance are given here as monosomy 7, trisomy 8 or tetrasomy 8, which may all be present in acute leukemia [27,28]. Another important field where especially gonosomal centromere-directed probes are regularly applied is follow-up of sex-mismatched bone marrow transplantation [29,30].

For application of all centromeric probes, one possible pitfall has to be highlighted here: centromeric regions may be subject to so-called chromosomal heteromorphisms. There are reports on false-positive and false-negative results after pure iFISH diagnostics using this kind of FISH-probes [26]. Thus, centromeric probes should only be applied if metaphase FISH was done at least once with the corresponding probes. Nowadays, locus-specific probes (see below) suited for iFISH are available for all human chromosomes, which should preferably be applied in all neoplastic samples of patients where no information is available on potential centromeric heteromorphisms.

Table 3. List of most important commercially available fluorescence *in situ* hybridization-probes for solid tumors.

Tissue type probe to cancer	Target region	Gene
Bladder	9p21	*P16* or *CDKN2A*
	17p13	*P53*
Bone and soft tissue	1p36.2 and 3q25	*CAMTA1/WWTR1*
	1p36	*PAX7*
	2q33	*CREB1*
	2q36	*PAX3*
	3q12	*TFG*
	6p21	*PHF1*
	7p21	*ETV1*
	9q22	*NR4A3*
	11p15.5	*CARS*
	11p13	*WT1*
	11q24 and 22q12	*FLI1/EWSR1*
	12q13	*DDIT3*
	12q13~q14	*CDK4*
	12q14	*HMGA2*
	12q15	*MDM2*
	13q14	*FOXO1*
	16p11	*FUS*
	17q21 and 22q13	*COL1A1/PDGFB*
	18q11.2	*SS18*
	21q22	*ERG*
	22q12	*EWSR1*
Breast	1q32	*MDM4*
	1q41	*CENPF*
	3q26	*SOX2*
	5q31.2	*EGR1*
	6q23	*MYB*
	6q25	*ESR1*
	7p12	*EGFR*
	8p11.2	*FGFR1*
	8q24	*C-MYC*
	10q23	*PTEN*
	10q26	*FGFR2*
	11q13	*CCND1*
	11q22.3	*ATM*
	12p12	*KRAS*
	12q14	*HMGA2*
	15q25	*NTRK3*
	17p13.1	*P53*
	17q11.2~12	*HER2/NEU1/ERBB2*
	17q21~22	*TOP2A*
	20q13	*ZNF217*
CNS	1p36.2 and 3q25	*CAMTA1/WWTR1*
	1p36	*MEGF6*
	1q25	*ABL2*
	1q41	*CENPF*
	2p24	*NMYC*
	3p25	*VHL*
	3q26	*SOX2*
	6q22	*ROS1*
	7p11.2	*EGFR*
	9p21	*CDNK2A*
	10q23	*PTEN*

Table 3. List of most important commercially available fluorescence *in situ* hybridization-probes for solid tumors (cont.).

Tissue type probe to cancer	Target region	Gene
	12q13~q14	*CDK4*
	15q25	*NTRK3*
	17p13	*P53*
	19p13	*ZNF44/ZNF*
	19q13	*CRX*
Colorectal	3q26	*SOX2*
	6q23	*MYB*
	6q24.3	*RREB1*
	7q34	*BRAF*
	10q23	*PTEN*
	12p12	*KRAS*
	17p13.1	*P53*
	18p11.32	*TYMS*
Esophagus	8q24	*C-MYC*
	9p21	*P16* or *CDKN2A*
	17p13.1	*P53*
	17q11.2~12	*HER2/NEU1/ERBB2*
	18p11.32	*TYMS*
	20q13	*ZNF217*
Eye	1q32	*MDM4*
	13q14	*RB1*
Head and neck	1q41	*CENPF*
	3p25	*VHL*
	5q32	*CD74*
	11q21	*MAML2*
	12p13.3	*FOXM1*
	19p13.2	*BRD4*
Kidney	Xp11.23	*TFE3*
	3p25	*VHL*
	3p14	*FHIT*
	6p21	*TFEB*
	7q31	*MET*
	10q23	*PTEN*
	17p13	*YWHAE*
Liver	4q12	*KIT*
	8q24	*CMYC*
	9p21	*P16*
	11q13.3	*FGF3,4,19*
	12p12	*KRAS*
	17p13.1	*P53*
	18q21	*BCL2*
Lung	1q32	*MDM4*
	2p23 and 2p21	*ALK/EML4*
	3p14	*FHIT*
	3q12	*TFG*
	3q26	*SOX2*
	4q12	*PDGFRA*
	5q32	*CD74*
	6q22	*ROS1*
	7p12	*EGFR*
	7q34	*BRAF*
	10p11.2	*KIF5B*
	10q26	*FGFR2*

Table 3. List of most important commercially available fluorescence *in situ* hybridization-probes for solid tumors (cont.).

Tissue type probe to cancer	Target region	Gene
Skin (melanoma)	6q23	*MYB*
	6p25	*RREB1*
	7p21	*ETV1*
	7q34	*BRAF*
	9p21	*P16*
	10q23	*PTEN*
	11q13	*CCND1*
	22q12	*EWSR1*
Stomach	3q26	*SOX2*
	4q12	*KIT*
	4q12	*PDGFRA*
	7q31	*MET*
	8q24	*CMYC*
	10q23	*PTEN*
	10q26	*FGFR2*
	11q22 and 18q21	*BIRC3/MALT1*
	17p13.1	*TP53*
	17q21	*ERBB2*
	18p11.32	*TYMS*
Ovary	3q26	*PIK3CA*
	8q24	*CMYC*
	9p21	*P16*
	10q26	*FGFR2*
	11q13	*CCND1*
	12p12	*KRAS*
	17p13.1	*P53*
	19q13	*CRX*
	20q13	*NCOA3(AIB1)*
Pancreas	5q32	*CD74*
	6q24.3	*RREB1*
	7q34	*BRAF*
	9p21	*P16*
	10q23	*PTEN*
	11q22.3	*ATM*
	12p12	*KRAS*
	17q13	*P53*
Prostate	Xq12	*AR*
	3p14	*FHIT*
	3q27	*ETV5*
	7p21	*ETV1*
	8q24	*C-MYC*
	9p21	*P16*
	10q23	*PTEN*
	12p13.3	*FOXM1*
	12q13q14	*CDK4*
	17p13.1	*P53*
	21q22	*ERG*
Thyroid gland	1q22~q23	*NTRK1*
	2q13	*PAX8*
	3q12	*TFG*
	7q34	*BRAF*
	10q11.2	*RET*
	10q23	*PTEN*
Uterus	3q26	*PIK3CA*
	5q32	*CSF1R*
	6p21.3	*PHF1*
	7p15	*JAZF1*
	8q24	*CMYC*
	9p21	*P16*
	10q23	*PTEN*
	10q26	*FGFR2*
	12p12	*KRAS*
	17p13	*YWHAE*
	17p13.1	*P53*
	17q12	*HER2/NEU1/ERBB2*
Others	1p36	*SRD*
	1p32 and 1q21	*CKS1B/CDKN2C*
	3p14	*FHIT*
	3q26	*TERC*
	5p15	*TERT*
	6q22	*MET*
	7q31	*ROS1*
	12p13.3	*FOXM1*

Application of locus-specific probes

In Tables 1–3 major parts of the presently commercially available locus-specific probes for metaphase FISH and iFISH applications in human cancer diagnostics are listed [31–37]. According to tumor type, application of one or more of these probes may be indicated.

The sheer amount of available locus-specific probes hampers a detailed discussion of each of them in this review. Use of locus-specific probes in neoplasia was reviewed before for leukemia [29,38–44], lymphoma [44–46] and solid tumors [44,47], like skin [44,47–49], lung [50] or breast cancer [51,52].

However, the commercially available probes can be categorized as follows (Figure 2):

- dual-color break-apart probes, detecting oncogene activation [5] by disruption of the corresponding tested gene;
- dual-color (dual) fusion probes, which normally are separated from each other in the human genome, but can come into close proximity due to different kinds of rearrangements, leading in the end also to oncogene activation [5];
- dual-color probes meant to detect deletion of tumor-suppressor genes [5];
- dual-color probes for detection of copy number alterations of parts of the genome – especially oncogene amplification [5];
- dual-color probes just for detection of copy number alterations of major parts of or the entire genome (hypo- or hyperdiploidy [5]) localized at different chromosomes.

The same probe may be suited to detect oncogene disruption, translocation and amplification or hyper-/hypodiploidy.

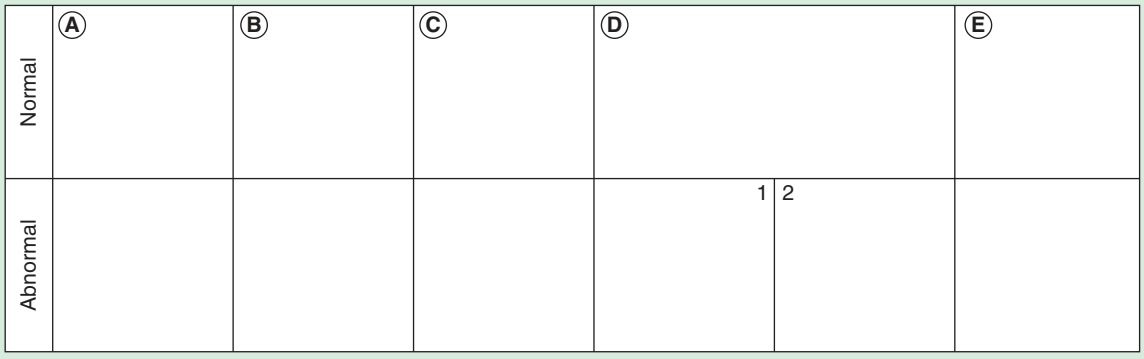

Figure 2. Schematic depiction of how locus-specific probes are normally combined in commercially available probe sets; the signal distribution as observed in an normal interphase cell is shown in the upper, the abnormal situation in the lower row. **(A)** Dual-color break-apart probe; **(B)** dual-color dual fusion probe; **(C)** dual-color probe-set for detection of a tumor-suppressor gene deletion; **(D)** dual-color probe-set for detection of an oncogene-amplification – in D1 a gene amplification due to double minutes and in D2 a corresponding amplicon due to a homogeneously staining region is shown; and **(E)** dual-color probe-set for detection hypo- or hyperdiploidy – here a triploidy is detected.

Here it must especially be stressed that molecular cytogenetic methods (except for CGH) are single-cell-directed tests. Thus, low-level mosaics can be detected that may be missed by molecular genetic approaches [53]. On the other hand, molecular approaches have the advantage of being inexpensive and able to cover more targets at once. An approach that could theoretically have the potential to partially replace (molecular) cytogenetics in tumor diagnostics is multiplex ligation-dependent probe amplification. This PCR-based technique can be used to screen for fusion genes, point mutations and copy number variations [54]. However, it has to be checked carefully when information on low-level mosaics can be renounced, and it is necessary for accurate patient care. This statement is true for all molecular approaches testing millions of cells at a time. Best may be to combine the available approaches in a tumor-specific scheme such as, for example, recently suggested for chronic lymphocytic leukemia [39].

Application of whole chromosome painting probes

Metaphase-directed two- or three-color FISH using wcp probes may be necessary in cancer diagnostics regularly, especially after derivative chromosomes were detected during banding cytogenetic analyses [55]. Still banding cytogenetics and/or the tumor-subtype need to provide clear hints that correct wcp probes are chosen for further characterization of an acquired derivative chromosome; otherwise, if available, mFISH using all wcp probes in different fluorochrome combinations may be indicated [56,57]. Of course, wcp probes may also be combined with other probes like pcp-, locus-specific or centromeric ones. Finally, it is a truism that wcp- and pcp-probes are not suited for routine iFISH studies [58].

Application of mFISH probe sets

In neoplasia, characterization of complex rearrangements (CCR) may also be necessary in routine diagnostics [57]. However, as CCR are considered to implicate an adverse diagnostics, often no further analyses are performed [5,17,18]. Besides, it is a matter of financial issues and of the technical possibilities available in the laboratory executing the diagnostics if expensive mFISH studies can be applied in a specific case. In a worldwide perspective, the majority of laboratories and oncologists will not be able to perform mFISH studies on a routine bases. Some countries in Western Europe, Northern America and some other more wealthy places around the world may be able to apply them on a routine base at present; these may be the same which can offer array-CGH and NGS as a routine setting [59–62].

In majority of cases, mFISH approaches (as well as array-CGH and NGS) will be applied only in individual cancer cases in research-associated settings [63–67]. Besides mFISH using wcp probes, also FISH-banding approaches and other probes will be used to resolve the individual case [68].

Clinical genetic aspects of molecular cytogenetics diagnostic performed in cancer diagnosis

Any kind of FISH study performed in a case with diagnosis cancer needs to be done according to the results of tumor cytogenetics and/or the input of the referring clinician. Genetic counseling will not be necessary in most of neoplastic cases. However, exceptions are the hereditary cancers, like breast cancer [69–71].

Moreover, one has to consider that during cytogenetic and molecular cytogenetic analysis incidental findings are possible. Mosaic Turner or Klinefelter syndrome or carriers of small supernumerary marker chromosomes may be detected [71,72]. Such findings, even though being rare, also should be expected by the clinician when a tumor-cytogenetic analysis has been requested.

Expert commentary

Molecular cytogenetics, together with cytogenetics provided, provides and will provide in future major input into the characterization of molecular defects in neoplasia. Morphological and clinical data, together with (molecular) cytogenetics and, as far as available, data from more sophisticated molecular approaches,

should all be considered to obtain correct diagnoses of studied malignancies. However, as in majority of the world, banding cytogenetics supplemented by the use of locus-specific probes is that what routine malignancy diagnostics consists of we clearly disagree with the statement of others [44] that FISH and mFISH approaches are 'early methods' for routine cancer diagnostics and 'recent high throughput genomic methods', that is, array-CGH and NGS are the new routine 'molecular cytogenetic' methods. Array-CGH and NGS are wonderful research tools. They will for sure lead in future to more insights into altered genome structure of malignancies. And maybe in some wealthy 'Western' countries these approaches, together with expensive mFISH techniques, may reach routine diagnostic status. The main importance of these sophisticated approaches in terms of implementation, and especially interpretation, will be the identification of new tumor-relevant genetic markers. The latter will be accessible by targeted and simpler tests, later.

Five-year view

In future, cytogenetics and molecular cytogenetics still will be a standard approach in cancer diagnostics. Specifically, the impact of metaphase as well as interphase-directed locus-specific FISH-probes will increase, especially as it can also be combined with immunohistochemistry [73]. This is among others highlighted by the fact that more and more companies enter the market offering increasing portfolios of tumor-related FISH-probes [31–37]. Thus, we expect molecular cytogenetics to remain a stable field in terms of necessity and application in cancer diagnostics. Thus, we suggest that not only for the next 5 years but for definitely longer, molecular cytogenetics would be a key diagnostic, prognostic and follow-up tool in routine.

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Key issues

- Molecular cytogenetics evolved in 1986 from cytogenetics.
- Cytogenetics started to gain major relevance in cancer diagnostics after identification of the first tumor-associated chromosomal aberration in 1960.
- Molecular cytogenetics uses different kinds of probes, such as locus-specific ones, whole and partial chromosome painting probes and probes specific for repetitive sequences.
- Two-color fluorescence *in situ* hybridization (FISH) is applied in routine cancer diagnostics, while multicolor FISH (mFISH) methods are applied more in research-associated settings.
- Locus-specific probes are routinely applied for the detection of tumor-suppressor gene deletion, oncogene amplification and/or gene fusions, as well as hypo- and hyperdiploidies.
- Molecular cytogenetics routine applications are used in leukemia, lymphoma and solid tumor diagnostics.
- Cytogenetics and molecular cytogenetics is single cell directed and thus able to detect even acquired low-level mosaics.
- One has to be prepared to meet also in cancer diagnostics from time to time hereditary cases, which need special attention.
- mFISH as well as array-comparative genomic hybridization and next-generation sequencing are highly suited for research settings, able to identify new tumor-relevant genetic markers.
- mFISH, array-comparative genomic hybridization and next-generation sequencing are and will in the near future be too expensive to become routine cancer diagnostic tools from a worldwide perspective.
- Cytogenetics and molecular cytogenetics are and will stay in the future indispensable tools in cancer diagnostics.

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2.3. Article .2

Othman MA, Grygalewicz B, Pienkowska-Grela B, Rincic M, Rittscher K, Melo JB, Carreira IM, Meyer B, Marzena W, Liehr T. **Novel Cryptic Rearrangements in Adult B-Cell Precursor Acute Lymphoblastic Leukemia Involving the MLL Gene.** J Histochem Cytochem, 2015;63(5):384-390.

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Novel Cryptic Rearrangements in Adult B-Cell Precursor Acute Lymphoblastic Leukemia Involving the MLL Gene

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Summary

MLL (mixed-lineage-leukemia) gene rearrangements are typical for acute leukemia and are associated with an aggressive course of disease, with a worse outcome than comparable case, and thus require intensified treatment. Here we describe a 69-year-old female with adult B cell precursor acute lymphoblastic leukemia (BCP-ALL) with hyperleukocytosis and immunophenotype CD10- and CD19+ with cryptic MLL rearrangements. G-banding at the time of diagnosis showed a normal karyotype: 46,XX. Molecular cytogenetics using multitude multicolor banding (mMCB) revealed a complex rearrangement of the two copies of chromosome 11. However, a locus-specific probe additionally identified that the *MLL* gene at 11q23.3 was disrupted, and that the 5′ region was inserted into the chromosomal sub-band 4q21; thus the aberration involved three chromosomes and five break events. Unfortunately, the patient died six months after the initial diagnosis from serious infections and severe complications. Overall, the present findings confirm that, by far not all *MLL* aberrations are seen by routine chromosome banding techniques and that fluorescence in situ hybridization (FISH) should be regarded as standard tool to access *MLL* rearrangements in patients with BCP-ALL.

Keywords

array-comparative genomic hybridization, B-cell precursor acute lymphoblastic leukemia, cryptic rearrangements, fluorescence in situ hybridization, *MLL*, mixed-lineage-leukemia gene

Introduction

B cell acute lymphoblastic leukemia (B-ALL) is a heterogeneous disease accounting for approximately 20% of adult leukemia. B-ALL is also the most common leukemia in pediatrics, representing up to 80% of childhood leukemia, with a peak of prevalence between the ages of 1 and 6 years (Zuckerman and Rowe 2014; Pui et al. 2008).

One of the most common recurrent chromosomal rearrangements in B-ALL (observed in approximately 50% of the rearrangements) is the balanced translocation t(4;11)(q21;q23), which leads to fusion of the *MLL* (mixed-lineage-leukemia) gene on 11q23 to the *AFF1* gene in 4q21 (Woo et al. 2014). *MLL* encodes for a protein with histone

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methyltransferase activity, which plays a critical role in the hematopoietic regulation of *HOXA* as well as embryonic development (Ansari and Mandal 2010). The translocation t(4;11) or *MLL/AFF1* gene fusion is almost exclusively seen in infant B-ALL (<1 year of age) and in highest frequency in childhood B-ALL. Up to 93% of affected infants under the age of 90 days harbor *MLL* rearrangements such as translocations t(4;11), t(11;19), or t(1;11), and most of these children cannot be rescued with the currently available therapies. These *MLL* rearrangements are also approximately four times more common in children than in adults (Braoudaki and Tzortzatou-Stathopoulou 2012; van der Linden et al. 2009), and the most frequently observed translocation t(4;11), has a dismal prognosis (Pui et al. 2002; Biondi et al. 2000).

Cryptic structural abnormalities often remain undetected by routine chromosomal banding techniques in acute leukemia. However, molecular (cyto)genetics has been proven to be a reliable tool for identification of such cryptic aberrations. Well known examples are the recurrence of cryptic translocation t(12;21)(p13;q22), which is solely associated with childhood B-ALL, and the cryptic translocation t(5;14)(q35;q32), which is known to be present in children and adolescents with T-ALL (Lazic et al. 2010; Su et al. 2006). Overall, chromosomal translocations found in childhood and/or adult B-ALL may result in the production of chimeric fusion proteins with leukemogenic potential.

Here, we report the case of a patient with adult BCP-ALL with a novel cryptic submicroscopic balanced translocation and an additional cryptic insertion of 5'*MLL* region into the *AFF1* locus at 4q21, with an unfavorable prognosis.

Materials & Methods

Clinical Description

A 69-year-old female presented in 2008 with hyperleukocytosis (white blood cell (WBC) count of 259.7×10^9/l; hemoglobin of 14.2 mmol/l and platelets of 103×10^9/l). The bone marrow (BM) aspiration showed hypercellularity, with 98% blasts. Immunophenotyping identified a variety of B-cell-specific antigens, with 96% of cells positive for CD15, CD19, CD22, CD34, CD45 and HLA-DR and all cells negative for CD10, CD13, CD20, and CD117. These findings were consistent with a diagnosis of BCP-ALL. It is noteworthy that the immunophenotypes CD10- and CD19+ as seen here are associated with *MLL* rearrangements in BCP-ALL. The patient was treated by induction therapy: Epi (4-epi-doxorubicin)/ VCR (vincristine)/ PEGAsp (polyethyleneglycole asparaginase)/ PDN (prednisone), two courses of consolidation and maintenance treatment (Mercaptopurin, Metotrexat). Unfortunately, she died six months after the initial diagnosis from serious infections and severe complications.

Diagnosis

Banding cytogenetic anaylsis was performed using an unstimulated bone marrow aspiration obtained at diagnosis and according to standard procedures (Claussen et al. 2002). A total of 20 metaphases were available for cytogenetic evaluation and analyzed on a level of 300 bands per haploid karyotype (Shaffer et al. 2013). Standard G-banding revealed a normal female karyotype as 46,XX and FISH test for a cryptic translocation t(9;22)(q34;q11.2) was negative.

Retrospective Analyses

Molecular Cytogenetics. FISH was performed according to standard procedures and/or to manufacturer's instructions. The probes and probe sets were made in-house. FISH-banding probe-sets were created using genome-wide multitude multicolor banding (mMCB) and chromosome specific array-proven multicolor-banding (aMCB) (Weise et al. 2003, 2008; Liehr et al. 2002). BAC (bacterial artificial chromosome) clones of interest were identified through the Human Genome Browser Database of the Genome Bioinformatics Group at the University of California at Santa Cruz (http://genome.ucsc.edu/) and Ensembl Genome Data Resources of the Sanger Institute Genome Database (http://www.ensembl.org/). DNA probes (Table 1) obtained from the Resources Center (Oakland, USA) were labeled by PCR with SpectrumGreen, SpectrumOrange or TexasRed-dUTP and applied in two- or three-color FISH-approaches.

Additionally, the following commercially available probes were used: LSI *MLL* (11q23 Break probe, Abbott Molecular/ Vysis, Mannheim, Germany), POSEIDON *NUP98* (*11p15* Break probe, Kreatech Diagnostics, Amsterdam, The Netherlands), SPEC *TFG* Break probe (*TFG* in 3q12.2, Zytovision, Bremerhaven, Germany), Centromere 4 (CEP4: 4p11-q11 Alpha Satellite DNA, Abbott Molecular/Vysis), and subtelomeric probes for 11p, and 11q (11p in D11S2071; 11q in D11S1037, Abbott Molecular/Vysis).

A total of 10–15 metaphase spreads were analyzed, using a fluorescence microscope (AxioImager.Z1 mot; Zeiss, Oberkochen, Germany) equipped with appropriate filter sets to discriminate between a maximum of five fluorochromes and the counterstain DAPI (Diaminophenylindol). Image capturing and processing were carried out using an ISIS imaging system (MetaSystems; Altlussheim, Germany).

DNA Isolation. Genomic DNA was extracted from cells fixed in acetic acid:methanol (1:3) by Puregene DNA Purification Kit (Gentra Systems; Minneapolis, MN). DNA concentration was determined using a Nanodrop spectrophotometer (NanoDrop Technologies, Inc., Thermo Scientific; Wilmington, DE). The quality of DNA was checked using agarose

Table 1. Results of Locus-Specific Probes Used for Breakpoint Characterization.

Cytoband	Location [hg18]	Probe	Result
3q12:2	Chr3:101,910,850-101,950,501	SPEC TFG	signal on der(3); no split signal
4p11q11	Chr4:48,200,001-52,700,000	CEP4	signal on der(4); no split signal
11p15.4	Chr11:2,907,721-3,231,290	SHGC-84145 to RH75370	signal on der(11p) and (11q); split signal
11p15.4	Chr11:3,193,128-3,312,588	RP11-11A9	signal on der(11p) and (11q); split signal
11p15.4	Chr11:3,652,816-3,775,468	NUP98	n.a.
11p15.4	Chr11:3,573,461-3,758,006	RP11-120E20	signal on both der(11)
11p15.4	Chr11:3,694,708-4,295,038	D11S4525 to SHGC-79113	signal on both der(11)
11p15.5~p15.4	Chr11:2,755,275-2,927,014	RP11-81K4	signal on both der(11q); no split signal
11p15.5	Chr11:872,364-1,051,564	RP11-401C19	signal on both der(11q); no split signal
11p15.5	Chr11:135,611-335,808	D11S2071	signal on der(11p) and (11q); no split signal
11q23.3	Chr11:117,812,415-117,901,146	LSI MLL	split signal on der(4) and der(11)
11q24.1	Chr11:120,790,892-120,960,991	RP11-142I2	signal on both der(11)
11q24.1	Chr11:121,326,327-121,516,640	RP11-166D19	signal on both der(11)
11q24.2	Chr11:123,265,105-123,469,312	RP11-485A5	signal on both der(11)
11q24.2	Chr11:124,585,478-124,761,531	RP11-100P11	signal on der(11q) and (11p); split signal
11q24.2	Chr11:125,827,475-126,006,340	RP11-432I22	signal on der(11q) and (11p); no split signal
11q24.3	Chr11:127,930,598-128,090,778	RP11-264E20	signal on der(11q) and (11p); no split signal
11q25	Chr11:133,964,875-134,130,595	RP11-267D5	signal on der(11q) and (11p); no split signal
11q25	Chr11:134,125,133-134,325,470	D11S1037	signal on der(11q) and (11p); no split signal

gel electrophoresis. DNA samples extracted from fixed cells of two healthy males and two healthy females by the same method were used as reference samples.

Multiplex Ligation-dependent Probe Amplification (MLPA). The P377-A1 Hematologic malignancies probemix and SALSA reagents were used for this study (MRC-Holland; Amsterdam, The Netherlands). Amplified probes and Genescan 500 ROX standard were separated by capillary electrophoresis using a 4-capillary ABI-PRISM 3130XL Genetic Analyzer (Applied Biosystems; Foster City, CA). Sizing of peaks and quantification of peak areas and heights were performed using GeneMarker v1.9 software (Applied Biosystems). A minimum of four healthy control samples were included in each run.

Array-Comparative Genomic Hybridization (aCGH). aCGH was performed using the Agilent SurePrint G3 Human Genome microarray 180 K (Agilent Technologies, Santa Clara, CA), an oligonucleotide microarray containing approximately 180,000 probes 60-mer with a 17 kb average probe spacing. Genomic DNA from the patient was cohybridized with a male control DNA (Agilent Technologies). Labeling was performed using the Agilent Genomic DNA enzymatic labeling kit according to the manufacturer's instructions. After hybridization, the aCGH slide was scanned on an Agilent scanner, processed with Feature Extraction software (v10.7) and results were analyzed using Cytogenomics (v2.9.1.3) using ADM2 as aberration algorithm.

Results

At diagnosis, banding cytogenetics at low resolution did not show any chromosomal aberrations. However, after subjecting the cytogenetic preparations in retrospective to FISH-banding probe-sets, mMCB identified a complex rearrangement for chromosome 11 involving reciprocal translocation and inversion (data not shown). The breakpoints were determined in more detail by further FISH experiments, such as aMCB, using a chromosome 11 specific probe set (Fig. 1) and by locus-specific FISH probes at 11p15.4 and 11q24.2 as shown in Table 1.

Additionally, dual-color FISH using a commercially available Break Apart Rearrangement probe specific for the *MLL* locus (LSI MLL) revealed an insertion of the 5′*MLL* gene into chromosome 4q21. According to the manufacturers of LSI MLL, a 350-kb portion (5' region) centromeric of the *MLL* gene breakpoint cluster region was labeled in SpectrumGreen and includes exons 1–6, whereas the ~190-kb portion of the *3' MLL* region is labeled by SpectrumOrange; the latter remained on one of the two derivative chromosomes 11, while the green-labeled part of LSI MLL went to the der(4) (Fig. 2). This cryptic insertion was observed as signal splitting of the probe LSI MLL in 6/6 metaphases and 158/200 interphase-nuclei. Thus, the 5′ *MLL* region was inserted most likely into the *AFF1* gene in chromosome 4q21.

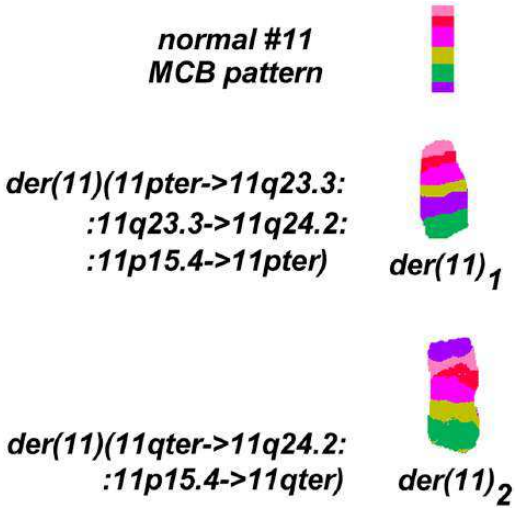

Figure 1. Result of the aMCB probesets for chromosome 11. Characterization of the complex rearrangements occurring in the derivative chromosomes. A normal chromosome 11 pattern (topmost) is provided as a comparison to the two derivative patterns of chromosome 11.

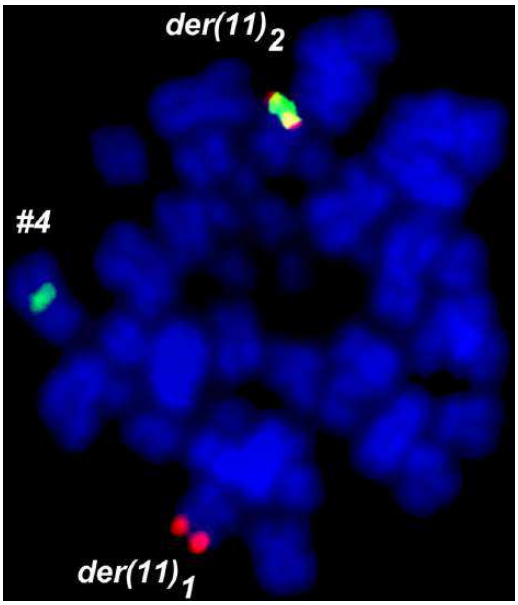

Figure 2. LSI MLL Break Apart probe showed one yellow fusion signal, and split of green signal and orange signal. Surprisingly the 5'MLL probe signal was inserted in a derivative chromosome 4.

The karyotype can be described as follows:

46,XX,der(4)(4pter->4q21.3::11q23.3->11q23.3::4q21.3->4qter),
der(11)(11pter->11q23.3::11q23.3->11q24.2::11p15.4->11pter), der(11)(11qter->11q24.2::11p15.4->11qter).

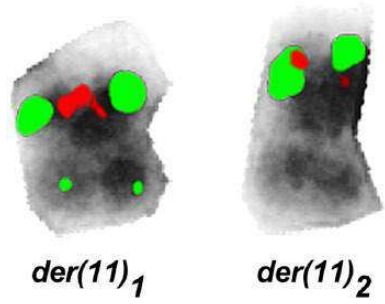

Figure 3. POSEIDON *NUP98* (*11p15* Break probe) revealed a split of green signal upstream of the NUP98 gene (see Table 1) and translocation to 11q24 due to an inversion (see Table 1).

In summary, the present case presents genetic changes involving three chromosomes and five break events.

The breakpoints in 11q24.2 and 11p15.4 were further delineated by locus-specific probes, as summarized in Table 1. The positions are given according to NCBI36/hg18, as a number of the used BAC-probes could not be found in later genomic browser versions.

For 11q24.2, the break was narrowed down to lying between the positions 124,585,487 and 124,761,531; one OMIM gene is located there: *PKNOX2* (PBX/KNOTTED 1 HOMEOBOX 2). The breakpoint in 11p15.4 was found to be spanned by a probe from locus SHGC-84145 to locus RH75370 that was part of a dual color/Break Apart probe from Kreatech (The Netherlands) flanking the NUP98 gene (Fig. 3). Additionally, BAC RP11-11A9 showed a split signal and the position of the break event can be given between 3,193,128 and 3,231,290; two OMIM genes are located there: *MRGPRE* and *MRGPRG*.

MLPA analysis showed no copy number variants; however, the array-CGH revealed an amplification of 83.4 Kb in the region of 3q12.2, which involves two genes, *GPR128* and *TFG*; the latter result was confirmed using locus-specific FISH probes, which showed intrachromosomal amplification in 12% of the interphase nuclei (data not shown).

Discussion

Structural chromosomal abnormalities can be readily detected by metaphase analysis or FISH in B-ALL. The most common balanced or unbalanced translocations have been correlated with variable prognostic significance. Here, included aberrations such as translocations t(4;11) (*MLL/AFF1*), t(12;21) (*ETV6/RUNX1*), t(1;19) (*E2A/PBX1*), and t(9;22) (*BCR/ABL*) (Zhou et al. 2012; Pui et al. 2008). These alterations can be found in different incidences in childhood and adult B-ALL (Lazic et al. 2010).

In the present B-ALL case, a normal karyotype was initially reported, since the here-described translocation and insertion events were submicroscopic and only identifiable by a combination of different molecular (cyto)genetic approaches.

The main problems hampering banding cytogenetics are the well-known difficulties in obtaining evaluable metaphases with well-spread chromosomes instead of clumsy ones or those that appear fuzzy with indistinct margins (Othman et al. 2014; De Braekeleer et al. 2011).

The patient whose case is presented here had high counts of WBC and blast cells, with a pre-B phenotype (CD19+, CD10-) -hallmarks of patients carrying a translocation t(4;11). Unfortunately, these hints were not further followed initially.

The *MLL* gene plays an important role in normal hematopoietic growth and differentiation. Abnormalities to this region can occur very early in hematopoietic stem cell development (Ansari and Mandal 2010; Ferrando et al. 2003). The translocation t(4;11)(q21;q23) is solely observed in B-ALL patients and presents in ~50% of *MLL* rearrangements as well as in the ins(4;11)(q21.3;q23.3) insertion as a typical variant of this translocation. In addition, an absence or low expression of CD10- in BCP-ALL and a very high WBC count are particularly common with the translocation t(4;11)(q21;q23) (Woo et al. 2014; De Braekeleer et al. 2011; Burmeister et al. 2009). *MLL* is well known to be rearranged in myeloid and lymphoid leukemia and can be classified into two groups. The first group includes *MLL* rearrangements, such as translocations or insertions, some of which are cryptic. These rearrangements result in the generation of in-frame fusion transcripts with various partner genes, with more than 120 loci already identified. The second group comprises amplification of 11q23, leading to the presence of multiple copies of the *MLL* gene located either intrachromosomally as a homogeneously staining region (hsr), or extrachromosomally in double minutes (dmin) (Meyer et al. 2013; De Braekeleer et al. 2011). The prognosis of *MLL* rearrangements in infants (<1 year of age) is extremely poor due to a high risk of treatment failure. Young children (1 to <10 years) have a better response to therapy than infants. Finally, for adults, event-free survival (EFS) is seen in 80% of cases. In general, the outcomes for adolescents and adults have improved significantly over time (van der Linden et al. 2009; Bassan 2005; de Bont et al. 2004; Pui et al. 2002; Morel et al. 2003). The present case, which involves 3 chromosomes and 5 break events in connection with an *MLL* gene rearrangement, is more complex than other comparable cases, but still belongs to the aforementioned first group.

Interestingly, it is considered that the fusion product of *MLL-AFF1* is transcribed from the der(4) and not from the der(11), which supports the idea that the *MLL-AFF1* is a protein with oncogenic potential. A review of the literature revealed that 10 cases with an insertion of chromosome 11 material in chromosome 4 have been identified in six children (all females) and four adult (3 elderly females and one male) B-ALL patients (Mitelman et al. 2014). Still, no other comparable cases have shown an additional reciprocal translocation between the two homologous chromosomes 11 and amplification in 3q12.2.

The chromosomal breakpoint 11p15 is recurrently involved in translocations in acute leukemia. The gene *NUP98* can fuse with *DOX10* in 11q22 or with *MLL* in 11q23 in acute myeloid leukemia (AML) (Kaltenbach et al. 2010; Romana et al. 2006). In the present case, the breakpoint at 11p15.4 involved two other genes *MRGPRE* and *MRGPRG*, which are related to the *MAS1* oncogene and mainly expressed in sensory neurons. The proteins derived from the *MRG* gene contain transmembrane, extracellular, and cytoplasmic domains that regulate nociceptor function (Dong et al. 2001). In the second breakpoint observed here, 11q24.2, there is only one OMIM gene located: *PKNOX2*. PKNOX2 belongs to a homeodomain protein superfamily comprising a large number of sequence-specific transcription factors that share a highly conserved DNA-binding domain; they play fundamental roles in cell proliferation, differentiation, and death (Imoto et al. 2001). Thus, it can be speculated that *MRGPRE* and/or *MRGPRG* fused with *PKNOX2* may lead to gene expression with oncogenic potential.

In the present case, it remains rather unclear which of the rearrangements—*MLL* with *MRGPRE* and/or *MRGPRG*, fusion of *MLL* with *AFF1* or 3q12.2 amplification—were causative in the adverse outcome. In terms of the latter alteration, the *TFG* gene located at 3q12.2 is known to play a role in the NF-κB pathway and, thus, multiple copies of the gene may have contributed to oncogenic potential of the tumor cells. Indeed, translocations involving this gene have been observed in hematological malignancies (Chase et al. 2010).

Overall, this case shows that it is necessary to screen for further unbalanced submicroscopic abnormalities by molecular approaches such as MLPA and aCGH in acute leukemia. The present report highlights that *MLL* gene rearrangements should be considered and tested by molecular approaches in case of a normal cytogenetic result. This holds especially true for such patients with a BCP-ALL who are diagnosed as a result of high WBC counts and CD10-negative staining. However, if, in such cases, MLL rearrangements are detected, further cryptic aberrations with potential influence on the disease may be present. Overall, a normal routine chromosome banding karyotype in acute leukemia needs to be considered as a stimulus and reason for more detailed molecular (cyto) genetic analyses.

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2.4. Article .3

Othman MA, Melo JB, Carreira IM, Rincic M, Alhourani E, Wilhelm K, Gruhn B, Glaser A, Liehr T. **MLLT10 and IL3 rearrangement together with a complex four-way translocation and trisomy 4 in a patient with early T-cell precursor acute lymphoblastic leukemia:** A case report. Oncol Rep, 2015;33(2):625-630.

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MLLT10 and IL3 rearrangement together with a complex four-way translocation and trisomy 4 in a patient with early T-cell precursor acute lymphoblastic leukemia: A case report

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Abstract. Cytogenetic classification of acute lymphoblastic leukemia (ALL) is primarily based on numerical and structural chromosomal abnormalities. In T-cell ALL (T-ALL), chromosomal rearrangements are identified in up to 70% of the patients while the remaining patients show a normal karyotype. In the present study, a 16-year-old male was diagnosed with T-precursor cell ALL and a normal karyotype after standard GTG-banding, was studied retrospectively (>10 years after diagnosis) in frame of a research project by molecular approaches. In addition to molecular cytogenetics, multiplex ligation-dependent probe amplification (MLPA) and high resolution array-comparative genomic hybridization (aCGH) were also applied. Thus, the following yet unrecognized balanced chromosomal aberrations were detected: der(3)t(3;5)(p23;q31.1), der(5)t(3;5)(p23;q35.3), der(5)t(5;10)(q31.1;p12.3) and der(10)t(5;10)(q35.3;p12.3). The oncogene *MLLT10* was involved in this rearrangement as was the *IL3* gene; in addition, trisomy 4 was present. All of these clonal aberrations were found in 40% of the cells. Even if this complex karyotype would have been identified at the time of diagnosis, most likely no other protocol of anticancer therapy (ALL-BFM 95) would have been applied. Three months after the end of a successful 2-year treatment, the patient suffered from isolated bone marrow relapse and died of sepsis during ALL-REZ-BFM protocol treatment.

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Key words: early T-cell precursor acute lymphoblastic leukemia, molecular cytogenetics, MLLT10, IL3, array-CGH

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive leukemia derived from malignant transformation of T cell progenitors and is more common in males than in females. T-ALL affects mainly older children and adolescents and represents 10-15% of pediatric and 25% of young adult ALL cases (1). Hyperdiploidy (>46 chromosomes) is found in 30% of childhood and 10% of adulthood ALL cases. Notably, high hyperdiploidy (51-65 chromosomes) has been connected with high survival rates and excellent outcome (2,3), while low hyperdiploidy (47-50 chromosomes) has been associated with worse prognosis (4). The most commonly gained chromosomes in ALL are #4, #6, #10, #14, #17, #18, #21 and X (5). Trisomy 4 is rarely observed as a sole cytogenetic abnormality in T-ALL (6). However, the mechanism for chromosomal gains in ALL and their role in leukemogenesis are still ambiguous (7,8). In hyperdiploid karyotypes, the t(9;22)(q34;q11), 11q23 (*MLL* gene) rearrangements, t(12;21)(p13;q22), t(1;19)(q23;p13) and t(8;14)(q24;q32) are the most common structural cytogenetic abnormalities in ALL. However, in T-ALL, involvement of the T cell receptor (*TCR*) gene in 14q11 in rearrangements such as t(1;14)(p31;q11), t(10;14)(q24;q11) or t(8;14)(q24;q11) are frequently observed; also del(6)(q15) and del(1)(p32) have been described (3,9-11).

Still, cryptic structural chromosomal abnormalities were and are a challenge in the cytogenetics of T-ALL. For example, as the cryptic t(5;14)(q35;q32) is known to be present in ~20% of childhood and in 13% of adult T-ALL cases, this aberration is currently routinely tested by molecular (cyto)genetics, addressing the breakpoint on the *TLX3* (*HOX11L2*) gene in 5q35 and to the promoter of the *BCL11B* gene in 14q32 (12). In addition, recent reports on newly detected cryptic chromosomal rearrangements such as the *MLLT10* gene (previously *AF10*, in 10p13), and *MLL* (in11q23) or *PICALM* (in 11q14) highlight the necessity to further study clinical cases as detailed as possible (13,14). The goal of these studies must be, on the one hand, to provide the most accurate diagnosis to each

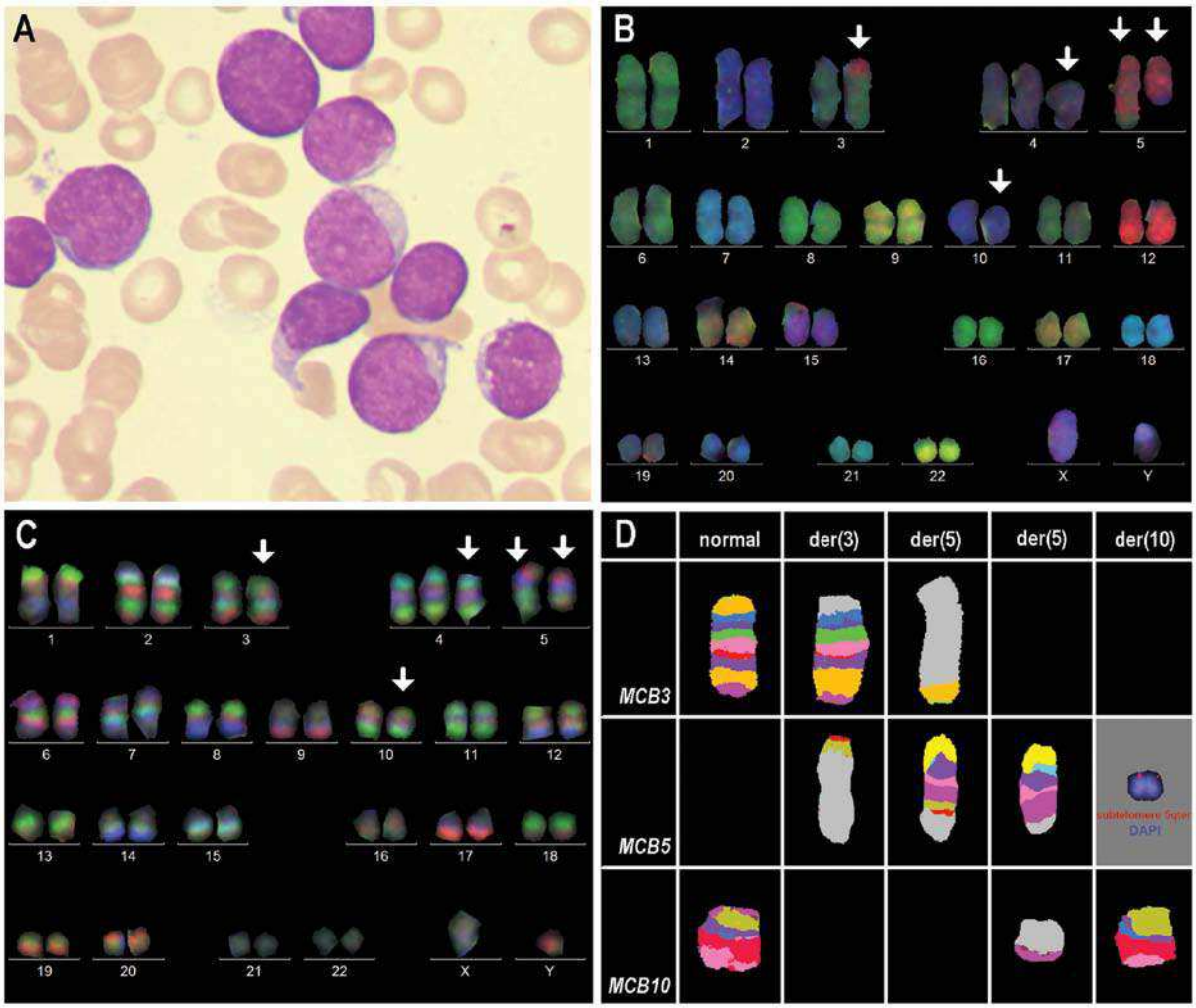

Figure 1. (A) Early T-cell precursor ALL cells of the presented patient depicted after Pappenheim staining. (B) Application of M-FISH revealed derivative chromosomes 3, 5, 5 and 10 (arrows). (C) mMCB results are shown as an overlay of three of the six used color channels. Evaluation was carried out as previously reported (21) using all 6 color channels and pseudocoloring. Breakpoints were determined as 3p23, 5q31.1, 5q35.3X and 10p12.3. (D) aMCB probesets for chromosomes 3, 5 and 10 confirmed the observed breakpoints after mMCB application. The breakpoint in 5q35.5 was confirmed by a subtelomeric probe 5qter.

individual patient and, on the other hand, to achieve insights into the biology and pathogenesis of T-ALL.

In the present study, an adolescent T-precursor cell ALL case with an *MLLT10* and *IL3* gene rearrangement together with trisomy 4 in complex four-way translocation is characterized in detail retrospectively using molecular cytogenetics and molecular genetics. This leukemia subtype would currently be classified as early T-cell precursor ALL (15-17).

Case report

Clinical description. A 16-year-old male presented in 1998 for diagnostics due to fever and unclear symptoms of malaise. Immunophenotypic analysis of bone marrow cells revealed the following results: HLA-DR$^+$, TdT$^+$, cyCD3$^+$, CD5 weak, CD7$^+$, CD8$^+$, CD10$^+$, CD13$^+$, CD33$^+$ and CD34$^+$. This supported a diagnosis of early T-ALL; at present, it would be classified as early T-cell precursor ALL (Fig. 1A).

The patient was treated according to the ALL-BFM 95 protocol; the continuation therapy was completed 24 months after the initial diagnosis. Three months later an isolated bone marrow relapse with acute thrombocytopenia was diagnosed, and treatment according to the ALL-REZ-BFM protocol was initiated. One month later the patient died due to an *Aspergillus* sepsis and still with 100% blasts in the bone marrow.

Tests conducted at diagnosis. Banding cytogenetic analysis was performed on an unstimulated bone marrow aspirate according to standard procedures. A total of 20 metaphases were available for cytogenetic evaluation and analyzed on a banding level of 300 bands per haploid karyotype (22). GTG-banding revealed a normal male karyotype in our laboratory, and also a second cytogenetic analysis on 25 metaphases performed 4 months after the initial diagnosis in another laboratory confirmed this test result. Molecular diagnostic PCR tests for gene fusions *BCR/ABL*, *MLL/AF4* and *TEL/AML1* were negative (data not shown).

Test conducted in retrospect

Molecular cytogenetics. Fluorescence *in situ* hybridization (FISH) was performed according to standard procedures and/or according to the manufacturer's instructions.

Table I. Results of the locus-specific probes used for breakpoint analyses in metaphase FISH are listed.

Cytoband	Position [hg18]	Genes/locus	Probe	Results (signals on…)
3pter	chr3:131,486-331,767	D3S4559	3pTEL (Vysis)	der(5)t(3;5)
3p24.1	chr3:30,275,517-30,447,565	n.d.	RP11-69K20	der(5)t(3;5)
3p24.1	chr3:30,541,893-**30,705,070**	*STT3B*	RP11-7I16	der(5)t(3;5)
3p22.3	chr3:**32,453,732**-32,650,841	*GPD1L* *GADL1* *OSBPL10* *CMTM7* *CMTM8*	RP11-524O15	der(5)t(3;5)
3p22.2	chr3:38,928,115-39,088,251	n.d.	RP11-159A17	der(3)t(3;5)
5q22.2	chr5:112,073,070-112,236,540	n.d.	RP11-107C15	der(5)t(5;10)
5q23.1	chr5:117,308,035-117,479,091	n.d.	RP11-567A12	der(5)t(5;10)
5q23.3	chr5:126,045,879-126,232,850	n.d.	RP11-434D11	der(5)t(5;10)
5q23.3~q31.1	chr5:130,306,745-**130,460,728**	5' of IL3	RP11-114H7	der(5)t(5;10)
5q31.1	chr5:131,424,246-131,426,795	IL3	n.a.	n.a.
5q31.1	chr5:**131,817,004**-131,977,063	3' of IL3	RP11-729C24	der(3)t(3;5)
5q31.1	chr5:135,739,999-135,916,051	n.d.	RP11-114H21	der(3)t(3;5)
5q31.2	chr5:137,829,080-137,832,903	EGR1	LSI EGR1	der(3)t(3;5)
5q32.1	chr5:149,473,595-149,515,615	PDGFRB	POSEIDON PDGFRB (Kreatech)	der(3)t(3;5)
5q35.1	chr5:170,996,421-171,159,856	n.d.	RP11-20O22	der(3)t(3;5) and der(5)t(3;5)
5q35.2	chr5:173,985,900-174,153,222	n.d.	RP11-47J7	der(3)t(3;5) and der(5)t(3;5)
5q35.2	chr5:175,502,694-175,558,904	n.d.	RP11-844P9	der(3)t(3;5) and der(5)t(3;5)
5q35.3	chr5:176,550,923-176,735,050	n.d.	RP11-265K23	der(3)t(3;5) and der(5)t(3;5)
5q35.3	chr5:178,243,600-**178,455,573**	5' HNRNPH1	RP11-281O15	der(3)t(3;5) and der(5)t(3;5)
5q35.3	chr5:178,973,785-178,983,328	HNRNPH1	n.a.	n.a.
5q35.3	chr5:**179,360,362**-179,524,360	3' HNRNPH1	RP11-39H3	der(5)t(5;10) and der(5)t(3;5)
5q35.3	chr5:180,142,710-180,335,838	n.d.	RP11-516K1	der(5)t(5;10) and der(5)t(3;5)
5qter	chr5:180,510,748-180,711,420	D5S2907	5pTEL (Vysis)	der(5)t(5;10) and der(5)t(3;5)
10pter	chr10:292,280-292,670	Z96139	10pTEL (Vysis)	der(5)t(3;5)
10p12.31	chr10:20,782,567-20,938,614	n.d.	RP11-51E20	der(5)t(3;5)
10p12.31	chr10:21,321,413-**21,495,264**	5' MLLT10	RP11-165O3	der(5)t(3;5)
10p12.31	chr10:21,863,580-22,072,560	MLLT10	n.a.	n.a.
10p12.31	chr10:**22,399,352**-22,575,929	3' MLLT10	RP11-108B14	der(5)t(5;10)

n.d., not determined; n.a., not available.

The following homemade probes and probe sets were used: i) 24-color-FISH using all human whole chromosome painting (WCP) probes (19); ii) FISH-banding probe sets as follows: genome-wide multitude multicolor banding (mMCB) and chromosome-specific high resolution array-proven multicolor banding (aMCB) (20-22); iii) DNA from bacterial artificial chromosome (BAC) probes (Table I) obtained from Resources Center (Oakland, CA, USA) were labeled by PCR with SpectrumGreen, SpectrumOrange or TexasRed-dUTP and applied in two- or three-color FISH approaches.

Additionally, the following commercially available probes were used: LSI EGR1/D5S23, D5S721 (*EGR1* in 5q31; D5S23, D5S721 in 5p15.2; Abbott Molecular/Vysis, Mannheim, Germany), POSEIDON PDGFRB (*5q33* Break probe; Kreatech Diagnostics, Amsterdam, The Netherlands), and subtelomeric probes for 3p, 5p, 5q and 10p (3p in D3S4559; 5p in C84c11/T3, 5q in D5S2907; 10p in Z96139; Abbott Molecular/Vysis).

A total of 10-15 metaphase spreads were analyzed, using a fluorescence microscope (Axio Imager Z1 mot; Carl Zeiss AG) equipped with appropriate filter sets to discriminate between

a maximum of five fluorochromes and the counterstain DAPI (diaminophenylindol). Image capturing and processing were carried out using an ISIS imaging system (MetaSystems, Altlussheim, Germany).

DNA isolation. Genomic DNA was extracted from cells fixed in acetic acid-methonal (1:3) using the Puregene DNA purification kit (Gentra Systems, Inc., Minneapolis, MN, USA). DNA concentration was determined by a NanoDrop spectrophotometer. The quality of DNA was checked using agarose gel electrophoresis. DNA samples extracted from fixed cells of 2 healthy males and 2 healthy females by the same method were used as reference samples.

Multiplex ligation-dependent probe amplification (MLPA). The P377-A1 hematologic malignancies probemix and SALSA reagents were used for the present study (MRC-Holland, Amsterdam, The Netherlands). Amplified probes and GeneScan 500 ROX standard were separated by capillary electrophoresis using a 4-capillary ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sizing of peaks and quantification of peak areas and heights were performed using the GeneMarker v1.9 software (Applied Biosystems). A minimum of 4 healthy control samples were included in each run.

High resolution array-comparative genomic hybridization (aCGH). aCGH was performed using the Agilent SurePrint G3 Human Genome Microarray 180K (Agilent Technologies, Santa Clara, CA, USA), an oligonucleotide microarray containing ~180,000 probes 60-mer with a 17-kb average probe spacing. Genomic DNA of the patient was co-hybridized with a male control DNA (Agilent Technologies). Labeling was performed using the Agilent Genomic DNA Enzymatic Labeling kit (Agilent Technologies) according to the manufacturer's instructions. After hybridization, the aCGH slide was scanned on an Agilent scanner, processed with the Feature Extraction software (v.10.7) and results were analyzed using Cytogenomics (v2.9.1.3) using ADM2 as aberration algorithm.

Results of the retrospective analyses. Genome-wide 24-color FISH using all human WCP probes and FISH-banding analysis using the mMCB probe set were applied as initial tests in this retrospective case. Thereby, a previously unrecognized numerical aberration, trisomy 4, and balanced translocations were identified between one chromosome 3 and 10, each, and both chromosomes 5. Overall, an abnormal karyotype was characterized as 47,XY,+4,der(3)t(3;5)(p23;q31.1),der(5)t(3;5)(p23;q35.3), der(5)t(5;10)(q31.1;p12.3),der(10)t(5;10)(q35.3;p12.3)[8]/46,XY[13] (Fig. 1B and C).

Chromosome-specific aMCB confirmed these results (Fig. 1D) and locus-specific probes narrowed down the breakpoints according to NCBI36/hg18 as follows (Table I). i) The breakpoint in 3p23 was determined between the positions 30,705,070 and 32,453,732; 6 OMIM genes are located there: *STT3B*, *GPD1L*, *GADL1*, *OSBPL10*, *CMTM7* and *CMTM8*. ii) The breakpoint 5q31.1 locates between positions 130,460,728 and 131,817,004 and those flank the gene *IL3* (interleukin 3 precursor) in 131,424,246-131,426,795. iii) The second breakpoint on chromosome 5 in subband q35.3 was mapped to positions 178,455,573 to 179,360,362; here the *HNRNPH1* (heterogeneous nuclear ribonucleoprotein H1) gene is included in 178,973,785-178,983,328. iv) Finally, the breakpoint in 10p12.3 was narrowed down to localize between positions 21,495,264 and 22,399,352, where the *MLLT10* (myeloid/lymphoid or mixed-lineage leukemia) gene has been mapped to 21,863,580-22,072,560.

No submicroscopic changes were detected by MLPA and aCGH; only the trisomy 4 was observed in aCGH (data not shown).

Discussion

Chromosomal translocations in ALL may be missed in banding karyotyping due to several reasons. They may be cryptic, as they are not resolvable due to a similar or identical GTG-banding pattern; an example is the t(12;21)(p13;q22) in childhood ALL (23). In addition, known aberrations may be masked in a complex karyotype (24). Finally, it may simply be difficult to obtain evaluable metaphases where chromosomes are well-spread and not clumsy or appearing as fuzzy with indistinct margins (25). In the present case the latter was the major problem. In the reanalyses, all well-spread metaphases were normal and all aberrant metaphases were clumsy and not evaluable in standard GTG-banding. Thus, cytogenetic analyses in two different laboratories missed the aberrations present in this case. Otherwise gross structural and a numerical aberration would not have been overlooked like in this case which were detected in retrospect by molecular cytogenetics.

Trisomy 4 as a sole abnormality is rare in acute myeloid leukemia (AML) (26) but is scarce in ALL and is not associated with a clear prognosis (6,27,28). In pediatric ALL, trisomy 4 has been reported to be associated with a favorable outcome suggesting that children who have trisomies of both chromosomes 4 and 10 may have a particularly low risk of treatment failure (3,5). Here, trisomy 4 was observed together with additional structural chromosomal aberrations. Most likely the oncogene *MLLT10* in 10p12.31 was activated by the strong promoter of *HNRNPH1* in 5q35.3. In addition, the translocation of 5q31.1 to 3p23 brought in close proximity the gene *IL3*, which has been shown to have an oncogenic effect on hematopoietic cells (29), to 6 OMIM genes listed in Table I, which could also potentially lead to overexpression of IL3.

MLLT10 gene. Rearrangements have previously been identified in both child and adulthood acute leukemia (30). The t(10;11) is a recurrent reciprocal translocation present in two common variants: t(10;11)(p12;q23) and t(10;11)(p12;q21); the latter tending to be more frequent in T-ALL patients (31). In addition, the t(10;11)(p12;q23) mainly found in childhood AML is rarely observed in B-ALL and T-ALL (32). The *MLLT10* gene encodes a leucine zipper protein that functions as a transcription factor. *MLLT10* gene rearrangements are associated with a poor outcome due to the poor response to therapy (33,34).

HNRNPH1 gene. While unbalanced structural aberration of chromosome 5 are common in myelodysplastic syndrome or AML (35,36), they are less common in ALL. Still Brandimarte *et al* (14) previously identified the *HNRNPH1*

gene as a new *MLLT10* fusion partner in pediatric T-ALL, as we observed in our case of T-precursor cell ALL.

IL3 gene. Located in 5q31.1, the *IL3* gene is a multipotent hematopoietic growth factor produced by activated T cells (37). Its involvement in malignancies was previously reported in B-ALL cases due to a t(5;14)(q31;q32). Overexpression of *IL3* was associated with unfavorable outcome in such cases (38).

3p23 region. Six OMIM genes are located in the breakpoint region of chromosome 3 in subband p23. These include: STT3B (source of immunodominant MHC-associated), *GPD1L* (glycerol-3-phosphate dehydrogenase 1-like), *GADL1*, (glutamate decarboxylase-like 1), *OSBPL10* (oxysterol-binding protein-like protein 10), *CMTM7* (CKLF-like MARVEL transmembrane domain containing 7) and *CMTM8* (CKLF-like MARVEL transmembrane domain containing 8). It is difficult to determine which one might have provided a strong promoter for *IL3* gene expression.

In conclusion, the study in particular of ALL cases with unexpectedly adverse outcome in retrospect and in detail by high resolution molecular approaches is warranted. In the present case the combination of FISH-banding, FISH with locus-specific probes and aCGH revealed trisomy 4 but apart from that a balanced aberrant karyotype, explaining the severe course of the disease in this case with adverse outcome. Even if this complex karyotype would have been identified at the time of diagnosis most likely no additional therapy other than the applied protocol (ALL-BFM 95) would have been used. Yet, the recurrence may have been detected much earlier in the case of available cytogenetic markers. Thus, the most comprehensive molecular (cyto)genetic analyses should be offered to each individual ALL case. Even though aCGH would not have detected the balanced translocations, the detectable trisomy 4 would have hinted at the malignant clone missed by banding cytogenetics. In conclusion, the present case is the first one presenting with combined trisomy 4 with a four-way translocation activating *IL3* together with *MLLT10*.

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2.5. Article .4

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MOLECULAR CYTOGENETICS

CASE REPORT Open Access

An adult B-cell precursor acute lymphoblastic leukemia with multiple secondary cytogenetic aberrations

Walid AL-Achkar[1*], Abdulsamad Wafa[1], Moneeb Abdullah Kassem Othman[3], Faten Moassass[1], Abdulmunim Aljapawe[2] and Thomas Liehr[3]

Abstract

Background: We report a clinically diagnosed acute lymphoblastic leukemia (ALL) with yet unreported secondary chromosomal aberrations.

Results: A complete cytogenetic and molecular cytogenetic analysis, using GTG banding, fluorescence in situ hybridization (FISH) and array-proven multicolor banding (aMCB), for a female patient with clinically diagnosed ALL and immunophenotypically confirmed pre-B ALL (FAB classifications), revealed the presence of a complex structural rearrangement, der (2) (20qter- > 20q13.33::2q21- > 2p14::2q21 > 2qter) along with t (9;22) (q34;q11), t (12;14) (q12;p12) and a monosomy of chromosome 7.

Conclusions: Molecular cytogenetic studies are suited best for identification and characterization of chromosomal rearrangements in acute leukemia. Single case reports as well as large scale studies are necessary to provide further insights in karyotypic changes taking place in human malignancies.

Keywords: Acute lymphoblastic leukemia, Secondary chromosomal abnormalities, Philadelphia chromosome, Fluorescence in situ hybridization, Array-proven multicolor banding, Prognostic factors

Background

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease characterized by multiple subtypes [1]. To date, several structural and numerical chromosomal abnormalities have been characterized in ALL and according to the WHO classification the following, seven genetic subtypes are defined for B lymphoblastic leukemia, t (9:22) (q34;q11.2), 11q23 traslocations, t (12;21) (p13;q22), t (1;19) (q23;p13.3), t (5;14) (q31;q32), hyperdiploidy and hypodiploidy [2]. Among the genetic subtypes, Philadelphia (Ph) chromosome, which results from a reciprocal translocation between Abelson (*ABL1*) from chromosome 9 and breakpoint cluster region (*BCR*) from chromosome 22, is the most frequent cytogenetic aberration which is found in ~ 25% of adult ALL cases, and in more than 50% of patients, aged 50 years or more [3,4]. The presence of the BCR-ABL1 rearrangement worsens the prognosis of ALL and represents the most significant adverse prognostic marker that influences the disease outcome [5]. Ph positive (Ph+) ALL is a more aggressive disease than chronic myeloid leukemia (CML), indicating that other factors than BCR-ABL1 are involved in its development and progression [5,6]. Ph + precursor-B-ALL is highly aggressive, frequently resistant to chemotherapy and with a short survival time [6,7]. Here, we are presenting a Ph + pre-B-ALL case with yet unreported translocation events involving six different chromosomes and a monosomy 7. These chromosomal rearrangements appeared after unsuccessful chemotherapy treatment.

Case presentation

A 31-year-old woman was diagnosed as suffering from ALL in September 2011. Anemia, thrombocytopenia, diarrhea, fatigue and weight loss were the indicative symptoms. She was treated as follows: after the first GM-ALL protocol (phase I and II) failed, Flag-IDA protocol was

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used, which also did not succeed. Then again GM-ALL protocol (phase I and II) was applied and after being unsuccessful hyper-CVAD was applied. At this point the first cytogenetics and hematology were determined. The patient's hematologic parameters were white blood cells (WBC) at 123×10^9/l, consisting of 12% neutrophils, 75% lymphocytes, 11% monocytes and 1% basophiles. Red blood cell (RBC) count was $3.26\times10^6/mm^3$, hemoglobin level 9.7 g/dl and the platelet count 34×10^9/l. Serum lactate dehydrogenase (LDH) value was 2,712 U/l (normal value up to 480 U/l), serum alkaline phosphates value 208 U/l (normal value up to 128 U/l), serum alanine aminotransferase 198 U/l (normal value up to 40 U/l) and serum aspartate aminotransferase value 139 U/l (normal value up to 40 U/l). The patient was treated further according to standard ALL chemotherapy protocols for fourteen months, however, without clinical success of chemotherapy. Unfortunately she died under the treatment.

Results

A sample of a female patient diagnosed as pre B-ALL, according to FAB classifications, was received after the completion of three different protocols of chemotherapy. The conventional cytogenetics analysis by GTG banding revealed the karyotype as 45, XX, -7, der (2) t (2;20) (?;?), t (9;22) (q34;q11), t (12;14) (q?;p?) [12] / 46, XX, t (12;14) (q?;p?) [10] (Figure 1). The dual color FISH using the probe specific for BCR and ABL and WCP probes specific for chromosomes 2, 7, 12, 14 and 20 confirmed the presence of BCR/ABL fusion on der (22) (data not shown), and the presence of the other rearrangements. To further characterize the breakpoints, aMCB was performed, as previously reported [8] (Figure 2) and the final karyotype was redefined as: 45, XX,-7, der (2) (20qter- > 20q13.33::2q21- > 2p14::2q21 > 2qter), t (9;22) (q34;q11), t (12;14) (q12;p12) [12] / 46, XX, t (12;14) (q12;p12) [10].

The abnormal cell population showed the following immunophenotype, which was consistent with pre-B-ALL (FAB classifications): CD45+, HLADr+, CD117+, CD34+, CD19+, CD10+, CD38+ and expressed CD123 and CD11c (52%) heterogeneously. The abnormal cells negatively reacted with antibodies to CD5, CD64 and CD3.

Conclusions

We characterized a Ph + adult pre-B-ALL case with a complex secondary chromosomal abnormality, a translocation and a monosomy 7. According to the literature, not a single case of ALL showed a der (2) (20qter- > 20q13.33::2q21- > 2p14::2q21- > 2qter) plus a t (12;14) (q12;p12) [9]. Moreover, a t (12;14) (q12;p12) was observed only in two cases of mantle cell lymphoma [9] and in a case of acute myeloid leukemia [10]. On the other hand, the chromosomal bands, 2p14, 2q21, 12q12, and 14p12 are listed in 5, 32, 20, and 4 cases, respectively, in other rearrangements involving different chromosomes than the ones which are involved in the present case, in previously reported ALL cases [9]. In addition, inv (2) with 2q21 as one of the breakpoints has also been reported in 3 cases of ALL [9].

Till date, several chromosomal aberrations such as t (9;22), t (4;11), t (1;9), and hyperdiploid or hypodiploid karyotype have been associated with the prognostic

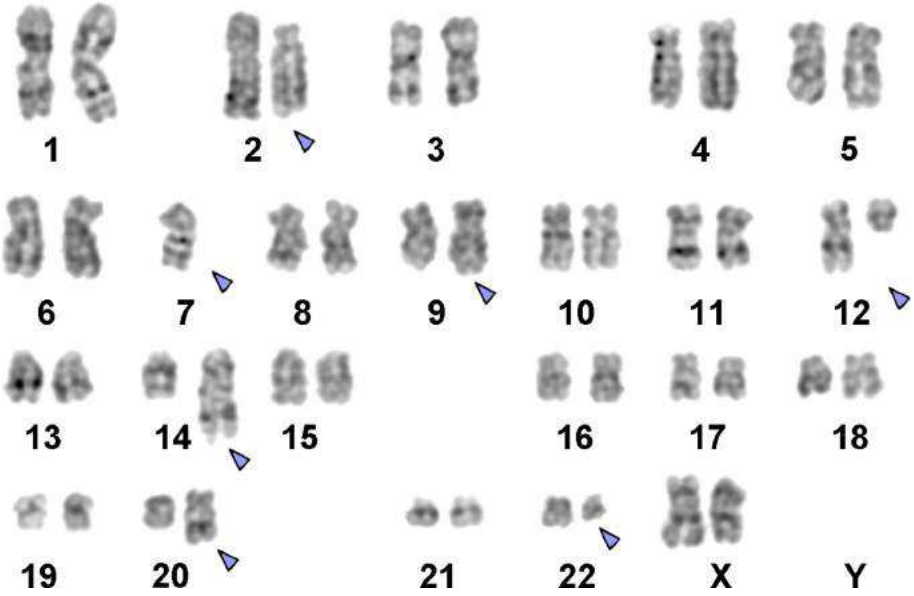

Figure 1 GTG-banding revealed a 45, XX-7, der (2) t (2;20), t (9;22), t (12;14). All derivative chromosomes are shown with arrows.

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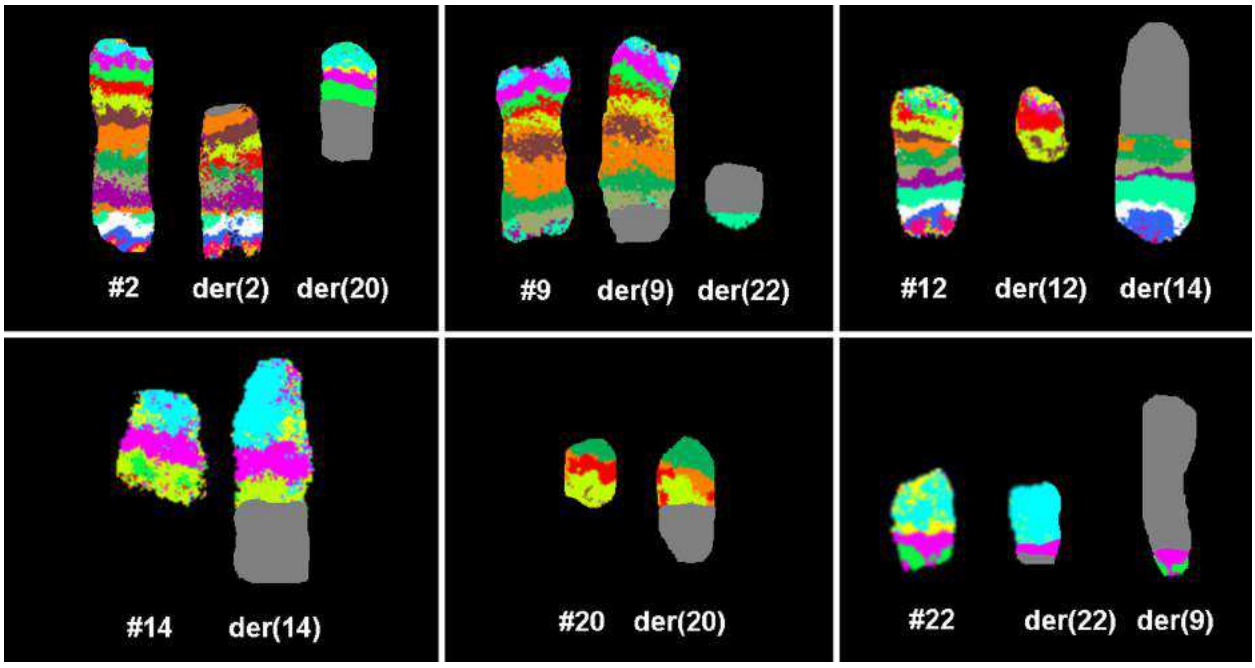

Figure 2 Array-proven multicolor banding (aMCB) was applied to characterize the breakpoint locations. Each image shows the results of MCB analysis using probe sets for chromosomes 2, 9, 12, 14, 20 and 22. The normal chromosomes are shown in the left side of each image and the derivative chromosomes on the right. The MCB-probes unstained regions on the derivative chromosomes are shown in gray. Abbreviations: # = chromosome; der = derivative chromosome; Ph = Philadelphia chromosome.

outcome in ALL cases. Apart from t (9;22) (q34;q11)/BCR-ABL and t (4;11) (q21;q23)/MLL-AF4, an elevated white blood cell count, age over 40 and non-responders/slow responders to chemotherapy are commonly regarded as high risk criteria in ALL [11]. Monosomy 7, as a sole secondary abnormality, is also related with a poor prognosis and shorter survival in adult ALL cases [12,13]. In addition, deletions of 7p confer with an inferior outcome in children with ALL, regardless of the presence of other poor prognostic features, whereas deletions of 7q are not associated with an adverse outcome [14]. The tendency for an adverse prognosis in patients with secondary loss of chromosome 7 or 7p in Ph + ALL may be the cumulative result of these events. Mullighan et al. [15] recently described a deletion of IKZF1 gene which encodes the transcription factor Ikaros, located on 7p12 in 83.7% of Ph + ALL cases but not in chronic-phase CML, suggesting that loss of Ikaros, a prototypical member of the Krüppel-like zinc finger (ZnF) transcription factor subfamily, which is required for normal hematopoietic differentiation and proliferation, particularly in lymphoid lineages, [16-18] is an important step in the progression of Ph + ALL. Recently, two of seven myeloproliferative neoplasms patients with loss of IKZF1 due to monosomy 7 have also been reported which suggests that IKZF1 may represent an important tumor-suppressor gene affected by monosomy 7 [19].

The presence of the underlying BCR/ABL gene rearrangement in CD10 B-cell precursor ALL has been reported previously [20] and it has already been demonstrated that the occurrence of BCR-ABL positive ALL in comparison to BCR-ABL negative disease represents a subgroup with a worse prognosis within the CD10+ B-lineage ALL [21].

In conclusion, the present case is a de novo case of adult pre-B-ALL with yet unreported translocation events involving six different chromosomes in addition to monosomy 7.

Materials and methods

Chromosome analysis

Chromosome analysis using GTG-banding was performed according to standard procedures [22] 12 months after ignition of the chemotherapeutic treatment. A minimum of 20 metaphase cells derived from unstimulated bone marrow culture were analyzed. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature [23].

Molecular cytogenetics

Fluorescence in situ hybridization (FISH) using LSI BCR/ABL three-color dual-fusion translocation probe (Abbott Molecular/Vysis, Des Plaines, IL, USA) was applied according to manufacturer's instructions together

with a whole chromosome painting (WCP) probe for chromosomes 2, 7, 12, 14 and 20 (MetaSystems, Altlussheim, Germany) [22]. FISH using the corresponding chromosome specific array-proven multicolor banding (aMCB) probe sets based on microdissection derived region-specific libraries was performed as previously reported [8]. A minimum of 20 metaphase spreads were analyzed, using a fluorescence microscope (AxioImager.Z1 mot, Carl Zeiss Ltd., Hertfordshir, UK) equipped with appropriate filter sets to discriminate between a maximum of five fluorochromes plus the counterstain DAPI (4',6- diamino-2-phenylindole). Image capture and processing were performed using an ISIS imaging system (MetaSystems).

Flow cytometric immunophenotype

Flow cytometric analysis was performed using a general panel of fluorescent antibodies against the following antigens typical for different cell lineages and cell types: CD1a, CD2, CD3, CD4, CD5, CD8, CD10, CD11b, CD11c, CD13, CD14, CD15, CD16, CD19, CD20, CD22, CD23, CD32, CD33, CD34, CD38, CD41a, CD45, CD56, CD57, CD64, CD103, CD117, CD123, CD138, CD209, CD235a and CD243; In addition to antibodies to Kappa and Lambda light Chains, IgD, sIgM, and HLADr. All antibodies were purchased from BD Biosciences. Samples were analyzed on a BD FACSCalibur™ flow cytometer. Autofluorescence, viability, and isotype controls were included. Flow cytometric data acquisition and analysis were conducted by BD Cellquest™ Pro software.

Consent

Written informed consent was obtained from the patient for publication of this Case Report. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AW and FM provided the case and/or did primary cytogenetic and main part of the FISH-tests; AA did the flow cytometry analysis; TL and MAKO did detailed FISH studies. WA supervised the cytogenetic analysis as Director of the MBBD HGD. WA and TL drafted the paper and all authors read and approved the final manuscript.

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2.6. Article .5

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Research Article

A Novel Cryptic Three-Way Translocation t(2;9;18)(p23.2;p21.3;q21.33) with Deletion of Tumor Suppressor Genes in 9p21.3 and 13q14 in a T-Cell Acute Lymphoblastic Leukemia

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Acute leukemia often presents with pure chromosomal resolution; thus, aberrations may not be detected by banding cytogenetics. Here, a case of 26-year-old male diagnosed with T-cell acute lymphoblastic leukemia (T-ALL) and a normal karyotype after standard GTG-banding was studied retrospectively in detail by molecular cytogenetic and molecular approaches. Besides fluorescence in situ hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA) and high resolution array-comparative genomic hybridization (aCGH) were applied. Thus, cryptic chromosomal aberrations not observed before were detected: three chromosomes were involved in a cytogenetically balanced occurring translocation t(2;9;18)(p23.2;p21.3;q21.33). Besides a translocation t(10;14)(q24;q11) was identified, an aberration known to be common in T-ALL. Due to the three-way translocation deletion of tumor suppressor genes *CDKN2A/INK4A/p16, CDKN2B/INK4B/p15*, and *MTAP/ARF/p14* in 9p21.3 took place. Additionally *RB1* in 13q14 was deleted. This patient, considered to have a normal karyotype after low resolution banding cytogenetics, was treated according to general protocol of anticancer therapy (ALL-BFM 95).

1. Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is a quite rare and heterogeneous disease, more common in males than in females. It accounts for 15% of childhood and 25% of adult ALL cases [1]. Underlying genetic causes of T-ALL are poorly understood and this is highlighted by the fact that T-ALL is associated with a normal karyotype in 30–50% of the cases [2, 3]. In abnormal karyotypes recurrent chromosomal aberrations are reported [4]. Regularly, promoter and enhancer elements of genes involved in T-cell development are juxtaposed with translocations in close proximity of oncogenes [5, 6]. The most common structural chromosomal abnormalities in T-ALL are TCR (T-cell receptor) loci rearrangements. Breakpoints in 14q11 (TCRA/D) and 7q34 (TCRβ) are observed frequently. Besides, deletions in the long arm of chromosome 6 may be found; the common deleted region involves mainly subband 6q16; however, candidate gene(s) have not been formally identified yet [7, 8]. Also tumor suppressor genes have been seen to be involved in T-ALL [9].

Cryptic structural chromosomal abnormalities are still a challenge in cytogenetic standard diagnostics of acute

leukemia. However, many cryptic aberrations have been identified by molecular cytogenetics already. Examples in T-ALL are cryptic deletions in 9p21 involving the genes *CDKN2A/INK4A/p16*, *CDKN2B/INK4B/p15*, and *MTAP/ARF/p14* leading to loss of G1 checkpoint control of the cell cycle or the *RB1* locus in 13q14, which also plays a role as tumor suppressor gene in cell cycle regulation [9].

Here, a case of a young adult T-ALL patient with a novel cryptic three-way translocation, a reciprocal translocation, and submicroscopic deletions is reported.

2. Material and Methods

2.1. Clinical Description. A 26-year-old male presented in 1998 initially with a total white blood cell count of 20.2×10^9/L, hemoglobin of 9.2 mmol/L, and platelets of 126×10^9/L. Bone marrow examination was consistent with T-ALL having 91% blast cells. According to flow cytometry the immunophenotype of bone marrow lymphocytes was as follows: the cells were positive for CD2 (96%), CD8 (96%), CD4 (92%), CD7 (92%), CD1A (89%), CD10 (87%), CyCD3 (86%), and TdT (85%) and negative for αF1, βF1, CD3, CD13, CD19, CD20, CD24, CD33, CD34, HLA-DR, MPO-7, slg, TZR-α/β, and TZRγ/δ. The patient was treated according to ALL-BFM 95 protocol and died eight months after initial diagnosis from serious infections and severe complications while being in complete hematological remission.

2.2. Test Done at Diagnosis. GTG-banding was done according to standard procedures. A total of 7 metaphases were available for cytogenetic evolution derived from unstimulated bone marrow of the patient and were analyzed on a banding level of 180–250 bands per haploid karyotype [11] and determined as 46,XY [7, 12]. RT-PCR performed for *TEL/AML1* and *BCR/ABL* fusion genes was reported to be negative and fluorescence in situ hybridization (FISH) analysis carried out according to manufacturer's instructions for the same loci was negative (probes used: LSI BCR/ABL and LSI TEL/AML1, Abbott Molecular/Vysis, Mannheim, Germany).

2.3. Test Done in Retrospective

2.3.1. Molecular Cytogenetics. FISH was done according to standard procedures and manufacturer's instructions for the following commercially available probes: LSI 13 in 13q14.2 (*RB1*, Abbott Molecular/Vysis, Mannheim, Germany), LSI IGH/BCL2 (*IGH* in 14q32; *BCL2* in 18q21, Abbott Molecular/Vysis, Mannheim, Germany), SPEC ALK/2q11 (*ALK* in 2p23, Zytovision GmbH, Bremerhaven, Germany), SPEC p16/CEN9 (p16 in 9p21.3, Zytovision GmbH, Bremerhaven, Germany), SPEC BIRC3/MALT1 (*BIRC3* in 11q22.2, *MALT1* in 18q21.32, Zytovision, Bremerhaven, Germany), and POSEIDON MLL/MLLT3 (*MLL* in 11q23.3, *MLLT3* in 9p21.3; Kreatech Diagnostics, Amsterdam, Netherland).

Whole chromosome painting (WCP) probe for chromosomes 2, 9, 10, 14, and 18 and bacterial artificial chromosome probes (BACs) for chromosomes 2 and 9 (Table 1) were homemade [13]. The homemade multitude multicolor-banding (mMCB) and chromosome specific high resolution array-proven multicolor-banding (aMCB) probe sets were also applied as previously reported [10, 14, 15].

A total of 10–15 metaphase spreads were analyzed, using a fluorescence microscope (AxioImager.Z1 mot, Zeiss) equipped with appropriate filter sets to discriminate between a maximum of five fluorochromes and the counterstain DAPI (Diaminophenylindol). Image capturing and processing were carried out using an ISIS imaging system (MetaSystems, Altlußheim, Germany).

2.3.2. DNA Isolation. Genomic DNA was extracted from cells fixed in acetic acid : methanol (1 : 3) by Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA). DNA concentration was determined by a Nanodrop spectrophotometer. The quality of DNA was checked using agarose gel electrophoresis. DNA samples extracted from fixed cells of 2 healthy males and 2 healthy females by the same method were used as reference samples.

2.3.3. Multiplex Ligation-Dependent Probe Amplification (MLPA). The P377-A1 hematologic malignancies probemix and SALSA reagents were used for this study (MRC-Holland, Amsterdam, The Netherlands). Amplified probes and Genescan 500 ROX standard were separated by capillary electrophoresis using a 4-capillary ABI-PRISM 3130XL Genetic Analyzer (Applied Biosystems, Foster City, USA). Sizing of peaks and quantification of peak areas and heights were performed using GeneMarker v1.9 software (Applied Biosystems). A minimum of 4 healthy control samples were included in each run.

2.3.4. High Resolution Array-Comparative Genomic Hybridization (aCGH). aCGH was performed using Agilent SurePrint G3 Human Genome microarray 180 K (Agilent Technologies, Santa Clara, CA, USA), an oligonucleotide microarray containing approximately 180,000 probes 60-mer with a 17 kb average probe spacing. Genomic DNA of patient was cohybridized with a male control DNA (Agilent Technologies, Santa Clara, CA, USA). Labeling was performed using Agilent Genomic DNA enzymatic labeling kit (Agilent) according to the manufacturers' instructions. After hybridization, the aCGH slide was scanned on an Agilent scanner and processed with Feature Extraction software (v10.7) and results were analyzed using Cytogenomics (v2.9.1.3) using ADM2 as aberration algorithm.

3. Results of Retrospective Analysis

As an initial test of retrospective analysis a genome wide FISH-banding applying mMCB was performed. Thereby, a previously unrecognized reciprocal and apparently balanced translocation between the three chromosomes 2, 9, and 18 was identified. Besides a known recurrent translocation of chromosomes 10 and 14 was recognized and the karyotype was suggested as 46,XY,t(2;9;18)(p23.2;p21.3;q21.33), t(10;14)(q24;q11) (Figure 1). aMCB and WCP probes as

Table 1: (a) Probes used for characterization of the three-way translocation, their location, and obtained results. (b) Probes used for characterization of the in aCGH detected deletions, their location, and obtained results.

(a)

Cytoband	Location [hg19]	Probe	Result for derivative chromosomes
2p24.3	chr2: 16,014,784–16,140,647	RP11-119F22	Signal on der(9); no split signal
2p23.3	chr2: 26,967,697–27,136,688	RP11-106G13	Signal on der(9); no split signal
2p23.2	chr2: 29,415,640–29,447,593	SPEC ALK	Signal on der(9); no split signal
9p22.1	chr9: 18,717,972–18,718,524	RP11-503K16	Signal on der(18); no split signal
9p22.1	chr9: 19,371,384–19,371,943	RP11-513M16	Signal on der(18); no split signal
9p21.3	chr9: 20,182,493–20,361,132	RP11-15P13	Signal on der(18); no split signal
9p21.3	chr9: 20,344,968–20,621,872	MLLT3	MLLT3-gene signal on der(18); no split signal
9p21.3	chr9: 21,967,751–21,975,132	SPEC p16	Deletion on der(9) and/or der(18)
9p21.3	chr9: 23,608,612–23,790,449	RP11-946B6	Deletion on der(9) and/or der(18) ish 9p21.3(RP11-946B6x0)[8]
9p21.2	chr9: 27,937,615–27,944,495	RP11-438B23	Signal on der(9); no split signal
18q21.32	chr18: 56,338,618–56,417,370	MALT1	MALT1-gene signal on der(18); no split signal
18q21	chr18: 60,985,282–60,985,899	BCL2	BCL2-gene signal on der(2); no split signal

(b)

Cytoband	Location [hg19]	Probe	Result for derivative chromosomes
9p21.3	chr9: 21,967,751–21,975,132	SPEC p16	ish 9p21.3(p16x1)[4] nuc ish 9p21(p16x0)[64]/9p21(p16x1)[83]/9p21(p16x2)[53]
13q14.2	chr13: 48,920,000–49,140,000	LSI 13 = *RB1*	nuc ish 13q14.2(*RB1*x0)[36]/13q14.2(*RB1*x1)[43]/13q14.2(*RB1*x2)[121]

shown in Figure 2 confirmed these suggestions. Locus specific probes narrowed down the breakpoints as shown in Table 1(a). Unfortunately there was no sufficient cell pellet available to characterize the breakpoints in more detail than listed in Table 1(a). Even though closely located to the observed chromosomal breakpoints, direct involvement of the following oncogenes was excluded using locus specific FISH-probes for *ALK* in 2p23.2, *MLLT3* in 9p21.3, and *MALT1* and *BCL2* in 18q21.33. However, MLPA (result not shown) and aCGH (Figure 3) revealed that the t(2;9;18) is not really balanced: a deletion in 9p21.3 including *CDKN2A/INK4A/p16*, *CDKN2B/INK4B/p15*, and *MTAP/ARF/p14* could be found as chr9: 21,252,517–21,798,676x1 and 21,817,082–23,515,821x0 (hg19) (Figure 3; Table 1(b)). Moreover, a deletion in 13q14.2 was detected as chr13: 48,982,000–49,062,000x1 (hg19, Figure 3). FISH showed a mosaic condition of mixed heterozygous and homozygous deletion of 9p21.3 and 13q14.2 (Table 1(b)).

4. Discussion

Chromosomal translocations are considered to be the primary cause of leukemia for both acute and chronic phase. In this study, we retrospectively identified previously undetected balanced and unbalanced chromosomal and subchromosomal changes by application of molecular cytogenetics including FISH-banding, locus-specific FISH-probes, and aCGH plus MLPA. FISH-banding, especially mMCB, allows the identification of balanced and unbalanced inter- and intrachromosomal rearrangements of the whole human karyotype

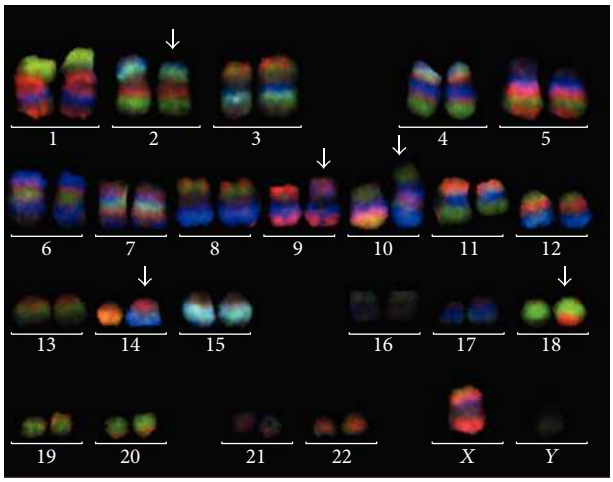

Figure 1: Application of mMCB showed no normal karyotype but derivative chromosomes 2, 9, 10, 14, and 18 (arrows). mMCB results are shown as overlay of three of the six used color channels. Evaluation was done as previously reported [10] using all 6 color channels and pseudocoloring. Breakpoints were determined as 2p23.2, 9p21.3, 10q24, 14q11, and 18q21.33.

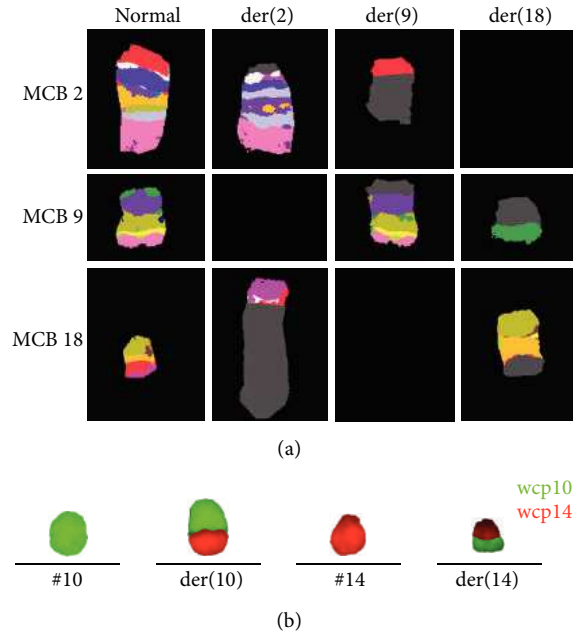

Figure 2: (a) Results of aMCB probe sets for chromosomes 2, 9, and 18 are shown in pseudocolor depiction, which confirmed the characterization of these three chromosomes involving rearrangement as t(2;9;18)(p23.2;p21.3;q21.33). (b) Whole chromosome paints (wcp) for chromosomes 10 and 14 confirmed that the t(10;14)(q24;q11) was independent of the t(2;9;18).

in one single experiment [10]. It might be indicated to apply mMCB or comparable FISH-banding approaches routinely in T-ALL cases exhibiting poor quality of the metaphase, that is, not well spreading ones with chromosomes appearing as fuzzy with indistinct margins [16, 17].

In this study one well-known and one yet unreported balanced translocation event were identified for a T-ALL as t(10;14)(q24;q11) and t(2;9;18)(p23.2;p21.3;q21.33), respectively. While a direct involvement of the cancer-related oncogenes *ALK* in 2p23.2, *MLLT3* in 9p21.3, and *BCL2* in 18q21.33 could be excluded, loss of two tumor suppresser gene loci in 9p21 and in 13q14 was found.

Data from the literature confirmed that the oncogenes tested and located nearby the chromosomal breakpoints of the three-way translocation were not yet found to be involved in T-ALL: *ALK* located in 2p23.2 was previously detected in a variety of B- and T-cell lymphomas and nonhematopoietic solid tumors [18–23], the *BCL2* gene is overexpressed in lymphomas [24, 25], and the *MLLT3* gene was one of the most highly upregulated transcripts and the most common fusion partner of *MLL* in *de novo* acute myeloid leukemia (AML) subtype M5 and therapy-related AML [26–28]; however, Meyer et al. [29] found that *MLLT3* also plays a role in pediatric rather than adult ALL.

In the present case, an additional chromosomal translocation t(10;14)(q24;q11), known as sole abnormality in 10% of T-ALL patients, was identified. Also it is present in 5% of pediatric and 30% of adult T-ALL [20, 30, 31]. The *TLX1* gene at 10q24 is a transcription factor becoming overexpressed as oncogene due to its juxtaposition to a strong promoter and enhancer elements of the TCR loci at 14q11 [5, 32–34]. A favorable outcome was reported in pediatric and adult T-ALL to be associated with the t(10;14) or TLX1 gene overexpression [5, 20, 35].

Even though balanced rearrangements are known to be typical for hematopoietic malignancies to date, only a limited number of studies have used whole genome directed FISH approaches to identify cryptic chromosomal abnormalities in ALL patients [36–38]. Still, in ALL it is uncommon to see three-way translocations. However, due to low metaphase resolution in ALL the real incidence of three-way translocations is currently unknown.

The present report highlights that after identification of apparently balanced chromosomal aberrations, it is still necessary to screen for further unbalanced submicroscopic abnormalities by molecular approaches such as MLPA and aCGH. However, also a confirmation of the results by molecular cytogenetics is necessary, as aCGH was partially misclassified a mix of homo- and heterozygote deletions as pure homozygote ones.

9p21.3 deletions, which lead to the loss of *CDKN2A/INK4A/p16*, *CDKN2B/INK4B/p15*, and *MTAP/ARF/p14* tumor suppressor genes expression, are the most predominant aberrations seen in precursor B-cell ALL (~20% of the cases) and T-ALL (>60% of the case) [39–42]. Besides also a deletion of *RB1* gene resulting in inactivation of another tumor suppressor gene expression was identified. *RB1* is rarely reported to be deleted in T-ALL. In contrast, deletion of *RB1* has been detected in 30% of B-ALL and nearly to 60% in B-CLL cases [43, 44]. Thus, *RB1* pathway was identified as potential targets for therapy of ALL [45, 46].

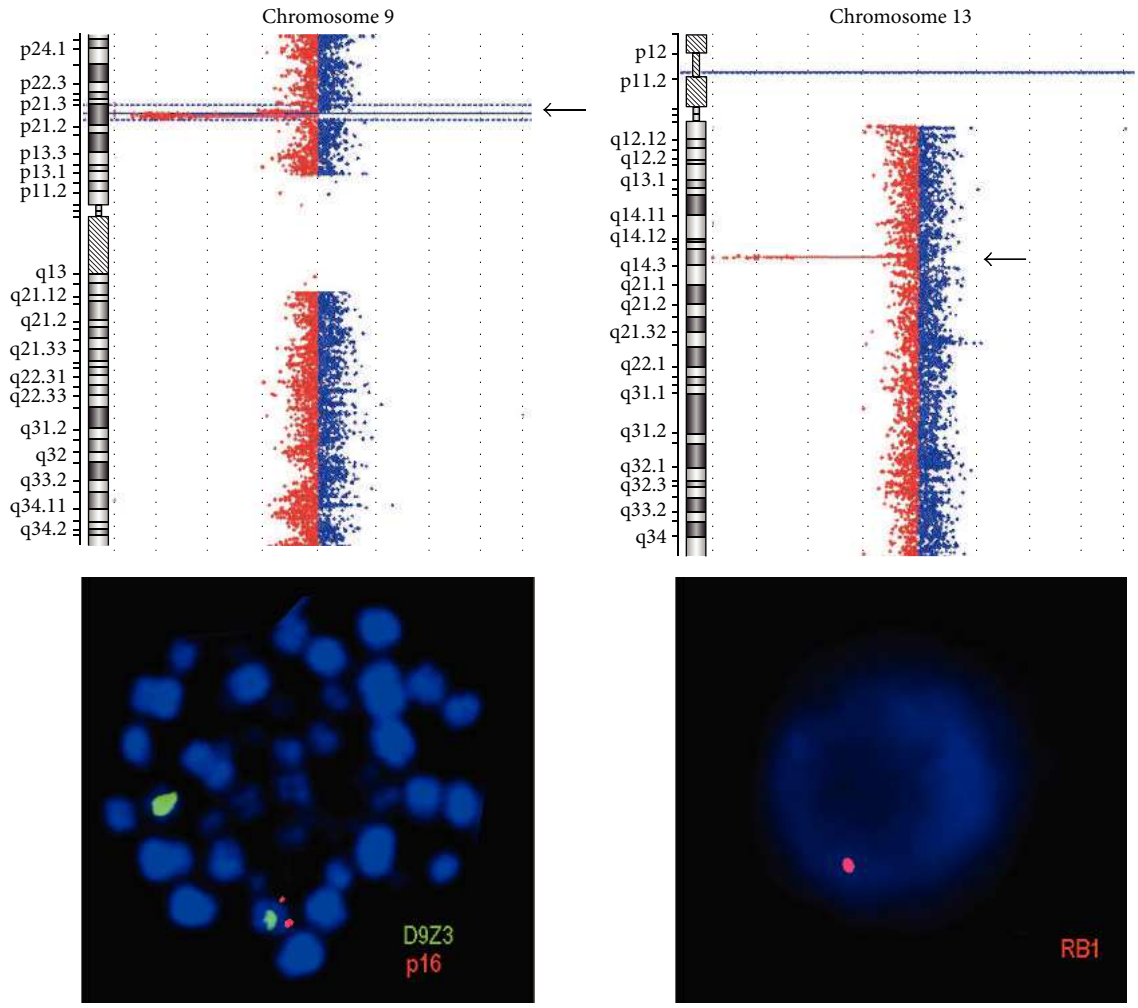

Figure 3: aCGH confirmed deletions in 9p21.3 and 13q14.2 (arrows) detected initially by MLPA (result not shown). FISH confirmed presence of these deletions in metaphase and/or interphase. Examples for heterozygote deletions of 9p21.3 and 13q14.2 are depicted; probes specific for the corresponding tumor suppressor genes were labeled in red; centromeric probe for chromosome 9 (D9Z3) was labeled in green.

5. Conclusion

In conclusion, we report a case of T-ALL with complex chromosomal aberrations. Even if at time of diagnosis the deletion on 9p21.3 would have been detected and accordingly treated, it remains unclear what influence the other tumor suppressors and oncogenes (possibly) activated by the complex rearrangements would have had for the clinical outcome. Overall, the present case stresses the necessity to study hematological malignancies by different means to get a comprehensive picture of the genetic changes in connection with the acquired disease, as aCGH or MLPA alone would only have identified the imbalanced rearrangements, while molecular cytogenetics predominantly gave hints on the presence of balanced rearrangements.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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2.7. Article .6

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A new *IGH@* gene rearrangement associated with CDKN2A/B deletion in a young adult B-cell acute lymphoblastic leukemia (B-ALL)

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Abstract

Acquired copy number changes are common in acute leukemia. They are reported as recurrent amplification or deletion and may be indicative for involvement of oncogenes or tumor suppressor genes in the acquired disease and can serve as potential biomarkers for prognosis or even as a target for molecular therapy. Here, we report a gain of copy numbers of 14q13 to 14q32 leading to an *IGH@* locus splitting in a young adult female, present as a yet unreported rearrangement in B-cell acute lymphoblastic leukemia (B-ALL). Low resolution banding cytogenetics at the time of diagnosis revealed a normal karyotype. However, retrospective application of fluorescence in situ hybridization-(FISH-) banding, locus specific FISH-probes, as well as multiplex ligation-dependent probe amplification and high resolution array-comparative genomic hybridization revealed previously cryptic aberrations. Overall a karyotype 46,XX,del(9)(p21.3p21.3),der(14)(pter->q32.33::q32.33->q13::q32.33->qter) was determined. The patient was treated according to PALG 5-ALL7-3 protocol and achieved complete remission. These findings indicate that a favorable prognosis is linked to these aberrations under the mentioned treatment.

Introduction

B-cell acute lymphoblastic leukemia (B-ALL) is a malignant neoplasm derived from B-cell progenitors. It is the most common malignancy in pediatric patients, accounting for up to 80% of childhood leukemia. Thus, it is the leading cause of cancer-related death in children and young adults (1-2).

Rearrangements involving the immunoglobulin heavy chain (*IGH@*) locus on chromosomal band 14q32.33 are rare in B-ALL, occurring in <5% of the childhood cases and detected in approximately 10% of adult patients (3-4). *IGH@* rearrangements occur more frequently in adolescent and appear to have a favorable clinical outcome. The same holds true for such cases of B-ALL associated with genetic aberrations like deletion in 9p21.3 (*CDKN2A/B*) and 9p13.3 (*PAX5*) (5). In B-ALL, the most common *IGH@* rearrangement is translocation to partner genes like *C-MYC* in 8q24 as the well characterized translocation t(8;14)(q24.1;q32). Another possible partner is the inhibitory transcription factor *ID4* in 6p22 being cytogenetically visible as translocation t(6;14)(q32;p22). The translocation t(14;19)(q32;q13) leads to overexpression of the *CEBP* (CCAAT/enhancer binding protein) gene family, the translocation t(5;14)(q31;q32) involves *IL3* in 5q31, and the translocation t(X;14)(p22;q32) or translocation t(Y;14)(p11.2;q32) result in deregulated expression of *CRLF2* (cytokine receptor-like factor 2). Translocations between *IGH@* and *EPOR* (erythropoietin receptor) in 19p13 have also been reported together with other translocations appearing less frequently (6-8).

In all of these translocations an oncogene located near the breakpoint of the translocation partner is activated by juxtaposing to *IGH@* regulatory sequences (4). Interestingly, all rearrangements involving *IGH@* at 14q32.33 have unique biological characteristics and correlate with clinical, morphological, and immunophenotypic features.

Cryptic deletions in chromosomal band 9p21.3 involve the *CDKN2A* gene which encodes for two transcripts: *p16/INK4A* and *p14/ARF*, and the *CDKN2B* gene (*p15/INK4B*). Their functions in cell cycle are to control the transition of G1 phase to S phase. The size of 9p21.3 deletions in ALL patients seem to vary substantially, but in most cases *CDKN2A* is co-deleted with *CDKN2B* and *MTAP* (9-11).

We report here a new *IGH@* rearrangement in a young adult of B-ALL associated with deletion in *CDKN2A/B*. The way how the chromosome 14 rearrangement may have been evolved is also discussed.

Material and Methods

Clinical description

A 20-year-old female presented in 2008 with white blood cell (WBC) count of $3.7x10^9$/l, hemoglobin of 11.0 mmol/l and platelets of 334 $x10^9$/l. In bone marrow about 93% of blast cells were observed. Immunophenotype was characterized by the expression of a variety of B-cell-specific antigens being positive for CD10, CD19, CD22, CD34, CD38, CD45, CD52, CD79a, TdT, HLA-DR, and being negative for CD2, CD15, CD20, CD33, CD56, CD66c, and cIgM. These findings were consistent with common acute B-cell lymphoblastic leukemia (B-ALL).

The patient was treated by induction therapy according to PALG 5-ALL7-3 (Epirubicin, Vincristine, PEG Asparaginaza, steroids), two courses of consolidation and maintenance treatment. From December 2011 till to date patient is under the observation in out-patient clinic with complete remission 1 (CR) and without signs for mininal residual disease (MRD).

Cytogenetic results at diagnoses

Banding cytogenetic analyses was performed on unstimulated bone marrow aspirate according to standard procedures (12). A total of 25 metaphases were available for cytogenetic evaluation and analyzed on a banding level of 300 bands per haploid karyotype (13). GTG-banding revealed a normal female karyotype as 46,XX.

Retrospective analyses

Molecular cytogenetics

Fluorescence in situ hybridization (FISH) was done according to standard procedures and/or according to manufacturer' s instructions.

Homemade were the following probes and probe sets:

- BAC (bacterial artificial chromosome) clones of interest were identified through the Human Genome Browser Database of the Genome Bioinformatics Group at the University of California at Santa Cruz (http://genome.ucsc.edu/) and Ensembl Genome Data Resources of the Sanger Institute Genome Database (http://www.ensembl.org/). DNA probes (Table 1) obtained from Resources Center (Oakland, USA) were labeled by PCR with SpectrumGreen, SpectrumOrange or TexasRed-dUTP and applied for two- or three-color FISH-approaches.

- FISH-banding probe-sets as follows: genome wide multitude multicolor banding (mMCB) and chromosome specific high resolution array-proven multicolor-banding (aMCB) (14-16).

Additionally, commercially available probes were used: LSI *IGH* (14q32 Break probe, Abbott Molecular/Vysis, Mannheim, Germany), POSEIDON p16 (9p21 and 9q21 Control probe, Kreatech Diagnostics, Amsterdam, Netherland), SPEC ERG/TMPRSS2 TriCheck™ Probe (*ERG* in 21q12.13-q22.3, TMPRSS2 in 21q22.3 Zytovision, Bremerhaven, Germany), and subtelomeric probe for 14q (14q in D14S1420, Abbott Molecular/Vysis, Mannheim, Germany).

A total of 10-15 metaphase spreads were analyzed, using a fluorescence microscope (AxioImager.Z1 mot, Zeiss) equipped with appropriate filter sets to discriminate between a maximum of five fluorochromes and the counterstain DAPI (Diaminophenylindol). Image capturing and processing were carried out using an ISIS imaging system (MetaSystems, Altlussheim, Germany).

DNA isolation

Genomic DNA was extracted from cells fixed in acetic acid-methonal (1:3) by Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA). DNA concentration was determined by a Nanodrop spectrophotometer. The quality of DNA was checked using agarose gel electrophoresis. DNA-samples extracted from fixed cells of 2 healthy males and 2 healthy females by the same method were used as reference samples.

Multiplex ligation-dependent probe amplification (MLPA)

The P377-A1 Hematologic malignancies probemix and SALSA reagents were used for this study (MRC- Holland, Amsterdam, The Netherlands). Amplified probes and Genescan 500 ROX standard were separated by capillary electrophoresis using a 4-capillary ABI-PRISM 3130XL Genetic Analyzer (Applied Biosystems, Foster City, USA). Sizing of peaks and quantification of peak areas and heights were performed using GeneMarker v1.9 software (Applied Biosystems). A minimum of 4 healthy control samples were included in each run.

High resolution array-comparative genomic (aCGH)

aCGH was performed using Agilent SurePrint G3 Human Genome microarray 180 K (Agilent Technologies, Santa Clara, CA, USA), an oligonucleotide microarray containing approximately 180,000 probes 60-mer with a 17 kb average probe spacing. Genomic DNA

of patient was co-hybridized with a male control DNA (Agilent Technologies, Santa Clara, CA, USA). Labeling was performed using Agilent Genomic DNA enzymatic labeling kit (Agilent) according to the manufacturers' instructions. After hybridization, the aCGH slide was scanned on an Agilent scanner, processed with Feature Extraction software (v10.7) and results were analyzed using Cytogenomics (v2.9.1.3) using ADM2 as aberration algorithm.

Results

G-banding at a low resolution did not show any chromosomal aberrations. Retrospective application of mMCB revealed only one gross chromosomal alteration, an inverted duplication on a chromosome 14. To characterize the rearrangement in more detail further FISH experiments like aMCB for chromosome 14 (Fig. 1A) and locus-specific FISH probes (Tab. 1) were applied revealing a which der(14)(pter->q32.33::q32.33->q13::q32.33->qter).

Dual-color-FISH using a commercial available break apart rearrangement probe specific for *IGH*, both interphase nuclei and metaphases studies revealed splitting of IGH variable region (IGHV) and 3′ flanking region, both located downstream to the IGH locus (results not shown). MLPA analysis showed heterozygous deletion of *p16/INK4A*, *p15/INK4B* and *p14/ARF* and confirmed by interphase FISH (iFISH – results not shown).

aCGH revealed two large genomic imbalances: a gain of 70.6 Mb in the region of 14q13.2-q32.3 between the positions (GRCh37/hg19) 35,918,265 and 106,513,022 and loss of 3 Mb in the region of 9p21.3 between the positions 21,252,517 and 24,289,720. Both findings are compatible with FISH and MLPA result (Fig. 1C).

Besides, aCGH revealed five small genomic imbalances with loss of copy number variants in:

- 3q26.32 between the positions 176,825,586 and 177,697,157; 1 OMIM gene is located there: *TBL1XR1*;
- 10p15.3 between the positions 1,491,986 and 1,582,072; 2 OMIM genes are located there: *ADARB2 and NCRNA00168;*
- 16q13 between the positions 57,275,940 and 57,331,381; 2 OMIM genes are located there: *ARL2BP* and *PLLP*;
- 21q22.2 between the positions 39,764,621 and 39,865,171; 1 OMIM gene is located there: *ERG*;

- Xq13.3 between the positions 47,330,212 and 47,335,227; 1 OMIM gene in is located there: *ZNF41* (results not shown).

Discussion

Copy number variants of specific target genes are important in the development and progression of acute leukemia and may serve as potential biomarkers for prognosis and/or as targets for molecular therapy. Gene amplification is an important mechanism of oncogene activation in acute leukemia. However, it is difficult to identify or resolve genomic imbalances less than 10 Mb in size by banding cytogenetics due to poor quality of chromosomes being often not well-spread and clumsy or appearing as fuzzy with indistinct margins. Thus, molecular cyto(genetic) approaches such as FISH, MLPA, and aCGH have been shown to be potent means for detection of previous cryptic genomic imbalances (7; 17). Consequently, application of aforementioned approaches unraveled here a yet unreported genomic imbalance in a B-ALL case as 46,XX,del(9)(p21.3p21.3),der(14)(pter->q32.33::q32.33->q13::q32.33->qter). The characterization of that aberration revealed the involvement of the cancer-related oncogene *IGH@* at 14q32.33 being critical in leukaemogenic process (4).

Inversions (inv) within the long arm of a chromosome 14 are common karyotypic abnormalities in T-cell lymphoid malignancies like T-chronic lymphocytic leukemia (CLL) and adult T-cell leukemia. In contrast, in B-cell lineage ALL inv(14)(q11q32) involving *CEBPE* and *IGH@* is an exceedingly rare phenomenon associated with better prognosis and repeatedly reported with complete remission (4; 18-19). The good outcome of the present cases thus fits in that line.

To the best of our knowledge a derivative chromosome 14 like the here reported one has not been seen in ALL yet. In Fig. 1B a suggestion is depicted how the rearrangement might have happened. As it is a rearrangement involving an interstitial part of the long arm of chromosome 14, U-type exchange mechanisms as reported in comparable cases from clinical genetics (20) can be discarded.

Homozygous deletions of tumor suppressor genes *p16/INK4A*, *p15/INK4B* and *p14/ARF* at 9p21 represent a marker of unfavorable outcome. Thus the heterozygote deletion seen in the present case may be a hint for a careful follow-up of the patient, especially as there are hints that the prognosis is here closely linked to and depend on the treatment received (6-10).

Finally, the present patient showed copy number changes of five regions with yet unclear clinical significance. The identification of new copy number change can lead to identification of functional important genes in leukemogenesis:

- Deletion of *TBL1XR1* gene on 3q26.32 has been recently detected in *ETV6-RUNX1* positive ALL, primary central nervous system lymphomas and diffuses B large cell lymphoma. Remarkably, *TBL1XR1* is widely expressed in hematopoietic tissues and may play a key regulatory role in the NF-kappaB pathway, hence suggesting that *TBL1XR1* could have a potential biological role in ALL pathogenesis (21-22).
- *ADARB2* at 10p15.3 encodes a member of the double-stranded RNA adenosine deaminase family of RNA-editing enzymes and may play a regulatory role in RNA editing and function as tumor suppressor gene. Overall, reduction of RNA level of *ADARB2* due to a deletion may favor cancer development and progression (23-24).
- Also a recurrent deletion was found on 21q22.22 targeting exclusively *ERG*. *ERG* gene is a transcriptional factor which belongs to the erythroblast transformation-specific (ETS) family. The latter has a key regulatory role in hematopoietic differentiation during early T and B cell development. Overexpression of *ERG* gene was shown in acute myeloid leukemia and T-ALL and was associated with poor prognosis. Currently, deletion of *ERG* gene associated with a very good outcome in older children and young patient with BCP-ALL, as also seen in our case with complete remission and without MRD (25-26).
- Submicroscopic losses of *ARL2BP, PLLP and ZNF41* genes were reported here for the first time in ALL. *ARL2BP* is a member of ARF family of RAS-related GTPases and has an essential role in photoreceptor maintenance and function. Homozygous mutation in *ARL2BP* gene was identified in retinitis pigmentosa with or without situs inversus (27). Overexpression of *PLLP* gene has been detected in malignant pleural mesothelioma (28). *ZNF41* is a transcription factor belongs to a cluster of human zinc finger genes on chromosome Xp11.23. Mutations in *ZNF41* gene was identified in X-linked mental retardation (29).

Overall, we found unbalanced acquired gross and submicroscopic rearrangements in a case of B-ALL not reported before in this unique combination. The clinical consequences of the individual changes remain to be determined in detail. However, it is noteworthy that treatment according to PALG 5-ALL7-3 protocol achieved complete remission.

Conclusion

Molecular cyto(genetic) approaches are a helpful tool for identification of cryptic rearrangements and potential new target genes for leukemogenesis and progression of the disease as well as for clinical outcome and treatment options. Our results suggest that, the detection of submicroscopic alterations in B-ALL such as deletion of *TBL1XR1, CDKN2A/B* and *ERG* genes with a good outcome would be useful for diagnosis and risk stratification, especially in future protocols that include B-ALL patients.

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Table 1

Used probes, their location and obtained results are listed according to GRCh37/hg19.

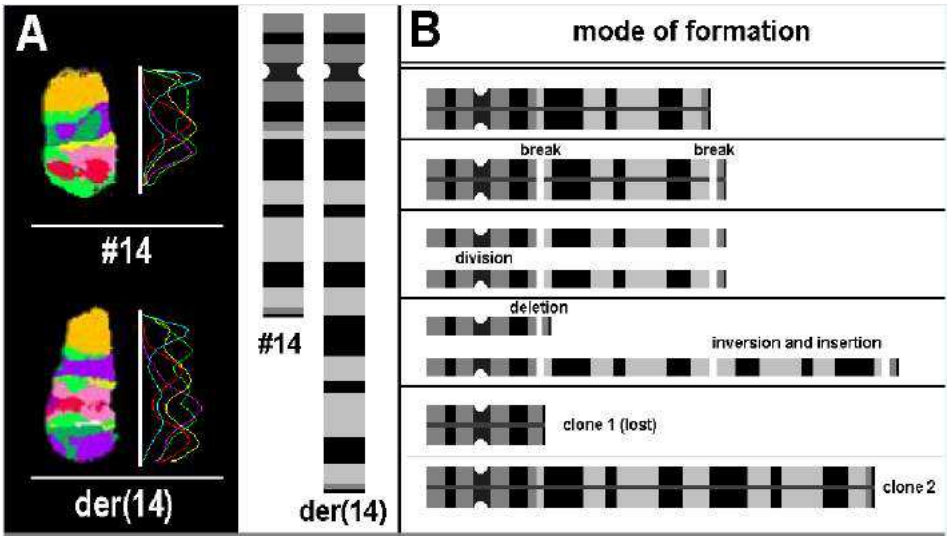
Cytoband	Location [GRCh37/hg19]	Probe	Result
3q26.32	chr3: 177,272,863-177,430,308	RP11-114M1	deletion on der(3) ish 3q26.3(RP11-114M1x1)[5]
3q26.32	chr3: 177,488,843-177,646,481	RP11-91K9	deletion on der(3) ish 3q26.3(RP11-91K9)[5]
9p21.3/ 9p11.1q11	chr9: 21,967,751-21,975,132 chr9: 47,300,001-50,700,000	SPEC p16/ CEN9	deletion on der(9) ish 9p21.3(p16x1)[8] nuc ish 9p21(p16x1)[147]/ 9p21(p16x2)[53]
14q11.2	chr14: 20,814,125-20,814,672	RP11-332N6	1 signal on der(14)
14q11.2	chr14: 20,940,682-21,103,092	RP11-14J7	1 signal on der(14)
14q12	chr14: 29,511,827-29,698,386	RP11-125A5	1 signal on der(14)
14q13.1	chr14: 32,299,162-32,460,130	RP11-501E21	1 signal on der(14)
14q13.2	chr14: 35,335,072-35,521,841	RP11-26M6	1 signal on der(14)
14q13.3	chr14: 36,683,813-36,704,814	RP11-259K15	2 signals on der(14)
14q21.1	chr14: 39,897,747-40,060,823	RP11-111A21	2 signals on der(14)
14q21.1	chr14: 40,408,068-40,537,355	RP11-34O18	2 signals on der(14)
14q21.3	chr14: 49,809,988-49,981,102	RP11-346L24	2 signals on der(14)
14q21.3	chr14: 50,148,020-50,148,604	RP11-831F12	2 signals on der(14)
14q23.1	chr14: 59,967,413-60,142,554	RP11-701B16	2 signals on der(14)
14q24.2	chr14: 70,701,212-70,701,81	RP11-486O13	2 signals on der(14)
14q31.1	chr14: 80,030,106-80,193,689	RP11-242P2	2 signals on der(14)
14q32.3	chr14: 106,053,226-106,518,932	LSI IGH	split signals on der(14)
14qter	chr14: 107,038,129-107,238,316	D14S1420	1 signal on der(14)

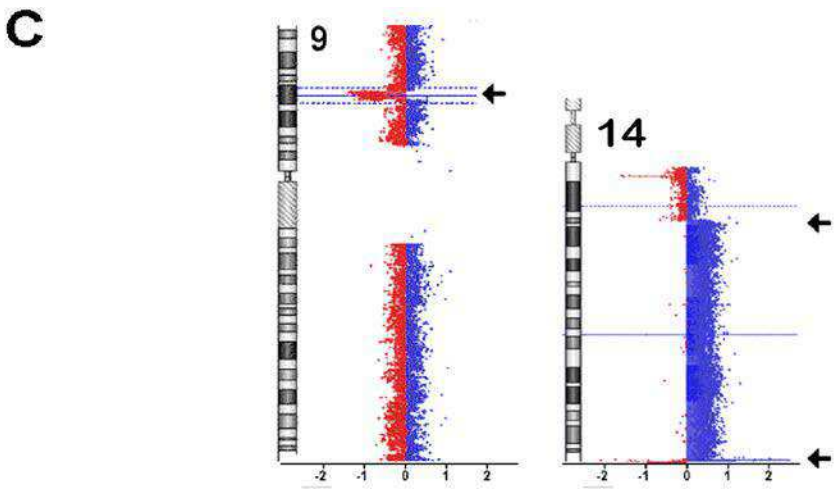
Figure 1

A) Result of aMCB 14 probe set suggested the breakpoints of der(14) as 14q13 and 14q32.33; those were confirmed by locus-specific FISH probes as detailed in Tab. 1. For aMCB the normal (#14) and the derivative chromosome 14 (der(14)) is shown in pseudocolor banding pattern and the corresponding underlying fluorochrome profiles. Schematic depiction of the der(14)(pter->q32.33::q32.33->q13::q32.33->qter) is also shown.

B) A mode of formation for the der(14) from Fig. 1A is suggested in this self-explaining schematic drawing.

C) aCGH revealed substantial genomic imbalances; loss in 9p21.3 detected initially by MLPA (result not shown) and gain of 14q13.2-q32.33 (arrows).





2.8. Article .7

Othman MA, Melo JB, Carreira IM, Rincic M, Glaser A, Grygalewicz B, Gruhn B, Wilhelm K, Rittscher K, Meyer B, Silva ML, Marques-Salles Tde J, Liehr T. **High rates of submicroscopic aberrations in karyotypically normal acute lymphoblastic leukemia.** Mol Cytogenet, 2015;8:65. (in press, MOCY-D-15-00065).

Note

The supplementary tables of this paper are in appendix

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MOLECULAR CYTOGENETICS

RESEARCH Open Access

High rates of submicroscopic aberrations in karyotypically normal acute lymphoblastic leukemia

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Abstract

Background: Acute lymphoblastic leukemia (ALL) is not a single uniform disease. It consists of several subgroups with different cytogenetic and molecular genetic aberrations, clinical presentations and outcomes. Banding cytogenetics plays a pivotal role in the detection of recurrent chromosomal rearrangements and is the starting point of genetic analysis in ALL, still. Nowadays, molecular (cyto)genetic tools provide substantially to identify previously non-detectable, so-called cryptic chromosomal aberrations in ALL. However, ALL according to banding cytogenetics with normal karyotype - in short cytogenetically normal ALL (CN-ALL) - represent up to ~50 % of all new diagnosed ALL cases. The overall goal of this study was to identify and characterize the rate of cryptic alterations in CN-ALL and to rule out if one single routine approach may be sufficient to detect most of the cryptic alterations present.

Results: Sixty-one ALL patients with CN-ALL were introduced in this study. All of them underwent high resolution fluorescence *in situ* hybridization (FISH) analysis. Also DNA could be extracted from 34 ALL samples. These DNA-samples were studied using a commercially available MLPA (multiplex ligation-dependent probe amplification) probe set directed against 37 loci in hematological malignancies and/or array-comparative genomic hybridization (aCGH). Chromosomal aberrations were detected in 21 of 61 samples (~34 %) applying FISH approaches: structural abnormalities were present in 15 cases and even numerical ones were identified in 6 cases. Applying molecular approaches copy number alterations (CNAs) were detected in 27/34 samples. Overall, 126 CNAs were identified and only 34 of them were detectable by MLPA (~27 %). Loss of CNs was identified in ~80 % while gain of CNs was present in ~20 % of the 126 CNAs. A maximum of 13 aberrations was detected per case; however, only one aberration per case was found in 8 of all in detail studied 34 cases. Of special interest among the detected CNAs are the following new findings: del(15)(q26.1q26.1) including *CHD2* gene was found in 20 % of the studied ALL cases, dup(18)(q21.2q21.2) with the *DCC* gene was present in 9 % of the cases, and the *CDK6* gene in 7q21.2 was deleted in 12 % of the here in detail studied ALL cases.

Conclusions: In conclusion, high resolution molecular cytogenetic tools and molecular approaches like MLPA and aCGH need to be combined in a cost-efficient way, to identify disease and progression causing alterations in ALL, as majority of them are cryptic in banding cytogenetic analyses.

Keywords: Multitude multicolor banding (mMCB), Acute lymphoblastic leukemia (ALL), Cryptic rearrangements, Fluorescence *in situ* hybridization (FISH), Multiplex ligation-dependent probe amplification (MLPA), Array-comparative genomic hybridization (aCGH)

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Background

Acute lymphoblastic leukemia (ALL) is a malignant disease of the hematological system with clonal proliferation of lymphoid progenitor cells. It arises from genetic alterations that block precursor B and T cell differentiation and predominantly affects children [1]. B-ALL constitutes 80-85 % of ALL cases and T-ALL the remainder ones. B-ALL patients have a favorable prognosis with an overall complete remission rate of 95 % for pediatric (children and adolescent between 1–15 years) but of only 60 % for adults. Adverse prognosis in T-ALL was correlated with presence of hyperleukocytosis, enhanced mediastinal mass, central neural system involvement, male gender and advanced age [1–5]. Cytogenetically detectable structural or numerical chromosomal abnormalities are detected in ~50 % of ALL cases. Such aberrations have prognostic significance [1, 6]. High hyperdiploidy (51–65 chromosomes) has been connected with good survival and excellent outcome in B-ALL, while hypodiploidy (<44 chromosomes) has an adverse prognosis [7–9]. Recurrent structural chromosomal abnormalities found in ALL can also be reciprocal translocations. ALLs with a translocation t(12;21)(p13;q22) leading to the *ETV6/RUNX1* gene-fusion are more likely to be cured, than those with a translocation t(9;22) or t(4;11), which tend to have unfavorable outcomes. Complex karyotypes, including three to five or more chromosomal abnormalities, are typically found in ~5 % of ALL cases and are also associated with an adverse outcome [10]. Finally, ALL cases with according to banding cytogenetics normal karyotype - in short cytogenetically normal ALL (CN-ALL) - are classified into intermediate risk group [6, 11, 12]. Malignant bone marrow of T-ALL patients shows a normal karyotype more frequently than those of B-ALL patients. Accordingly in those cases cytogenetic markers cannot be determined and therapeutic decisions may be hampered.

Based on the knowledge that chromosomes in ALL show a low banding resolution and that a good part of ALL cases present with a normal karyotype, it is not far to seek, that small aberration can easily be missed when analyzing ALL derived chromosomes by banding cytogenetics alone [6, 13]. Copy number alterations (CNAs) at the microscopic or submicroscopic level, i.e. focal deletions, but also duplications or sequence/point mutations in genes that primarily serve as transcriptional regulators of the lymphoid developmental pathway can nowadays be detected by approaches like multiplex ligation-dependent probe amplification (MLPA) or array-comparative genomic hybridization (aCGH) [12, 14, 15].

The present study includes 61 CN-ALL cases, which were retrospectively studied for the rate of cryptic (sub)-chromosomal changes to rule out if one single molecular (cyto)genetic routine approach may be sufficient to detect most if not all of the cryptic alterations present.

Results

Standard cytogenetic analysis by G-banding revealed normal karyotypes in 61 ALL cases included in this study (Additional file 1: Table S1). In a first step all 61 cases were studied by the whole genome oriented fluorescence *in situ* hybridization (FISH)-banding based probe set multitude multicolor banding (mMCB) [16]. For further delineation of mMCB results appropriate FISH-probes and probe sets were applied (Additional file 1: Table S1). Based on these results 21/61 (34 %) cases were not cytogenetically normal but had gross acquired chromosomal aberrations: structural abnormalities were found in 15/61 cases (24 %) and even numerical ones were observed in 6/61 cases (10 %) (Table 1). Overall, in GTG-banding cryptic balanced and unbalanced translocations, derivative chromosomes, isochromosomes, interstitial deletions, inverted duplications and/or numerical aberrations were identified in 34 % of the studied CN-ALL cases by means of molecular cytogenetics. In Fig. 1 case P66 is exemplified with a three-way translocation between chromosomes #10, #11 and #14, inversion of second chromosome # 14 and insertion (11;10). The breakpoints of this P66 case were characterized as 10p12.3, 10q11.23, 11p15.3, 11q23.3, 14q11, 14q24.2, and 14q32.3.

34/61 studied CN-ALL cases (18 B-ALL, 8 T-ALL and 8 with undefined ALL) were studied further using MLPA and aCGH. Overall, 126 CNAs were detected by MLPA and aCGH in those cases. CNAs were identified in 27/34 (80 %) of the studied cases. 1 to 13 CNAs per case were detected (Table 1). The distribution of CNAs per chromosome and frequencies of gains and losses are summarized in Fig. 2; i.e. all chromosomes apart from 8 and Y were involved in CNAs in this study.

Deletions and duplications could be grouped according to their sizes as follows:

- focal CNAs (e.g. deletion of *CHD2* gene in 7 cases or duplication of *DCC* gene in 3 cases – Table 1);
- CNAs involving variable numbers of genes (e.g. deletion on 9p21.3 in 8 cases or amplification of 9q34.12q34.13 in one case – Table 1);
- CNAs involving large parts of whole chromosomal p and/or q arms (e.g. deletion on 4p16.3p14 in one case or duplication of 7p22.3p14.1 in one case – Table 1)
- CNAs of whole chromosomes (e.g. monosomy X in one case or trisomy #14 in one case – Table 1).

Most frequently observed deletion was 9p21.3 in 8/34 ALL cases (3x in B-ALL, 4x in T-ALL and 1x in undefined ALL); the *CDKN2A/B* genes were affected in all these eight cases. Furthermore, *PTEN* in 10q23.31 (6/34) and *IKZF1* in 7p12.2 (5/34) were the hit by deletions regularly. Besides, deletion in 15q26.1 (*CHD2* gene) was detected in 7/34 cases and duplication in 18q21.2 (*DCC* gene) in 3/34 cases.

Table 1 Summary of aberrations detected by metaphase directed FISH, interphase FISH to determine the percentage of specific aberrations, and aCGH in 34 ALL patients

Case number	Age [y]	Metaphase directed FISH	MLPA	LSPs for genes	aCGH – affected cytobands	Localization acc. to GRCH37/hg19	Size of imbalance [bp]
B-ALLs							
P1	1	46,XX	normal	normal	dup(11)(p15.5p15.4)	chr11:1,960,555-3,626,932	1,666,377
P8	30	47,XY,+21[5]/46,XY[2]	dup of 21q22.12	*RUNX1*: dup (72 %)	n.d.	n.d.	n.d.
P13	34	46,XY[8]	del of 10q23.3		del(10)(q23.2q23.31)	chr10:88,906,902-91,189,599	2,282,697
			del of 17p13.1	*TP53*: del (9 %)	del(17)(p13.1p13.1)	chr17:7,579,695-8,281,928	702,233
P17	27	46,XX[7]	n.d.	normal	normal	n.d.	n.d.
P23	59				del(3)(p25.3p25.3)	chr3:10,179,706-10,385,195	205,489
			del of 7p12.2		del(7)(p12.2p12.2)	chr7:50,337,405-50,482,274	144,869
					del(10)(q23.3q23.3)	chr10:89,570,600-89,676,741	106,141
					del(11)(q14.2q14.2)	chr11:85,683,188-85,944,362	261,174
		47,XX,+14[2]/		*IGH*: dup (58 %)	+14	+14	107,349,540
		46,XX[3]			del(15)(q26.1q26.1)	chr15:93,390,484-93,463,312	72,828
					del(17)(p13.1p13.1)	chr17:7,581,198-7,922,308	341,110
					del(17)(q11.2q11.2)	chr17:30,259,053-30,271,653	12,600
					del(18)(q21.32q21.32)	chr18:57,517,756-57,718,190	200,434
					del(21)(q22.3q22.3)	chr21:45,527,941-45,565,198	37,257
P28	84	46,XY,	del of 7p12.2		del(7)(p12.2p12.2)	chr7:50,353,062-50,444,269	91,207
		t(9;22)(q34;q11),	del of 9p21.3	*CDKN2A/B*: del (75 %)	del(9)(pterp11.2)	chr9:0–47,212,321	47,212,321
		del(11)(q13q25)[7]	del of 9p13.2		del(9)(q34.2qter)	chr9:136,917,580-141,213,431	4,295,851
					del(10)(q23.3q23.3)	chr10:89,619,806-89,731,258	111,452
			del of 11q22.3	*BIRC3*: del (75 %)	del(11)(q13.2qter)	chr11:67,773,863-135,006,516	67,232,653
				ATM: del (77 %)	del(15)(q26.1q26.1)	chr15:93,412,860-93,450,773	37,913
				MLL: del (80 %)	dup(20)(q11.23q12)	chr20:37,305,876-39,130,131	1,824,255
					del(20)(q12q13.12)	chr20:39,245,111-45,524,952	6,279,841
					dup(20)(q13.12q13.12)	chr20:45,524,953-45,780,811	255,858
					del(20)(q13.12q13.32)	chr20: 45,780,812-58,067,678	12,286,866
					del(21)(q22.2q22.2)	chr21:39,764,621-39,807,169	42,548
				BCR: del (94 %)	del(22)(q11.23q11.23)	chr22:23,584,037-23,592,537	8500
P43	69	46,XX,	normal	*TFG*: dup (15 %)	dup(3)(q12.2q12.2)	chr3:100,360,682-100,444,109	83,427
		der(4)(4pter- > 4q21.3::11q23.3-			del(7)(q21.2q21.2)	chr7:92,252,341-92,475,197	222,856
		>11q23.3::4q21.3- > 4qter),		*MLL*: ins (75 %)			

Table 1 Summary of aberrations detected by metaphase directed FISH, interphase FISH to determine the percentage of specific aberrations, and aCGH in 34 ALL patients *(Continued)*

		der(11)(11pter- > 11q23.3::11q23.3- > 11q24.2::11p15.4- > 11pter), der(11)(11qter- > 11q24.2::11p15.4- > 11qter)[5]					
P48	39	46,XY, t(6;11)(q15;p12), ins(6;11)(q22.1;q13q14), inv(6)(q15q25.3), del(11)(q21q23.2)[8]	n.d.	*ESR1*: del (89 %)	del(6)(q13q14.2)	chr6:73,331,571-84,140,938	10,809,367
					del(6)(q16.2q21)	chr6:99,282,580-109,703,762	10,421,182
					del(6)(q22.31q22.33)	chr6:124,125,069-128,841,870	4,716,801
					del(6)(q25.1q25.3)	chr6:151,725,897-157,531,913	5,806,016
					del(7)(p12.2p12.2)	chr7:49,991,954-51,207,236	1,215,282
					dup(11)(p15.5p15.4)	chr11:1,925,114-3,143,116	1,218,002
				WT1: del (91 %)	del(11)(p15.1p12)	chr11:20,546,133-37,403,781	16,857,648
				BIRC3: del (90 %)	del(11)(q14.1q14.3)	chr11:85,157,088-88,557,421	3,400,333
				ATM: del (77 %)	del(11)(q22.1q22.3)	chr11:100,992,179-114,667,959	13,675,780
					del(13)(q14.2q14.2)	chr13:48,980,623-49,148,073	167,450
P49	39	46,XX[10]	n.d.	normal	dup(11)(p15.5p15.4)	chr11:2,016,406-3,430,378	3,430,378
P51	59	46,XX[6]	normal	normal	del(10)(p12.1p12.1)	chr10:28,057,099-28,220,314	163,215
					del(15)(q26.1q26.1)	chr15:93,412,860-93,450,773	37,913
					del(X)(q21.1q21.1)	chrX:76,875,639-77,157,819	282,180
P52	21	46,XY[4]		normal	del(6)(p21.1p21.1)	chr6:45,395,872-45,409,919	14,047
					del(7)(q21.2q21.2)	chr7:92,149,393-92,495,958	346,565
			del of 10q23.3		del(10)(q23.3q23.3)	chr10:89,610,886-89,722,948	112,062
					del(11)(q14.2q14.2)	chr11:85,683,188-85,944,362	261,174
					del(15)(q26.1q26.1))	chr15:93,433,130-93,450,773	17,643
					del(17)(q23.1q23.1)	chr17:57,698,768-57,913,528	214,760
					del(20)(q13.2q13.2)	chr20:52,151,411-52,629,609	478,198
					del(X)(p22.33p22.33)	chrX:1,327,561-1,684,270	1,684,270
P53	34	46,XY[5]	normal	normal	dup(22)(q11.21q11.21)	chr22:18,706,001-21,561,514	2,855,514
P55	19	46,XY[6]	del of 17p13.1	*TP53*: del (100 %)	del(17)(pterp11.2)	chr17:0–20,219,464	20,219,464
					−20	−20	63,025,520
P56	47	45,XY,-21[2]/ 46,XY[4]	normal	normal	del(12)(pterp11.21)	chr12:0–31,260,891	31,260,891
P57	56	46,XY[3]	normal	normal	normal	n.d.	n.d.
P58	20	46,XX,		*TBL1XR1:* del (68 %)	del(3)(q26.32q26.32)	chr3:176,825,586-177,697,157	871,571

Table 1 Summary of aberrations detected by metaphase directed FISH, interphase FISH to determine the percentage of specific aberrations, and aCGH in 34 ALL patients *(Continued)*

		der(14)(pter- > q32::q32- > q13::q32- > qter)[10]	del of 9p21.3	*CDKN2A/B*: del (74 %)	del(9)(p21.3p21.3)	chr9:21,252,517-24,289,720	3,037,203
					del(10)(p15.3p15.3)	chr10:1,491,986-1,582,072	90,086
				IGH: split (78 %)	dup(14)(q13q32.33)	chr14:35,918,265-106,513,022	70,594,757
					del(16)(q13q13)	chr16:57,275,940-57,331,138	55,198
					del(21)(q22.2q22.2)	chr21:39,764,621-39,895,171	130,550
P64	5	46,XX,	n.d.		del(5)(q31.3q32)	chr5:142,096,863-145,891,069	3,794,206
		t(16;19)(p11.2;q13.3),					
		der(5)t(5;9)(q31;p13.2),		*CDKN2A/B*: del (86 %)	del(9)(p21.3p21.3)	chr9:21,218,548-23,002,377	1,783,829
		der(9)t(5;9)(q31;p13.2),					
		der(9)t(9;9)(q34;p13.2)[10]		*FUS*: split (75 %)			
P66	0.5	46,XX,	n.d.	*MLL*: split (70 %)	dup(11)(p15.5p15.4)	chr11:1,008,688-3,669,161	3,669,161
		der(10)(10pter- > 10p12.31::11q23.3- > 11q23.3::10p12.31- > 10q11.23::14q24.2- > 14qter),		*IGH*: inv (100 %)			
		der(11)(10qter- > 10q11.23::11p15.3- > 11q23.3::10p12.31- > 10p12.31::11q23.3- > 11qter),					
		der(14)t(11;14)(q15.3;q24.2), inv(14)(q11q23)[8]					
T-ALLs							
P5	22	46,XX[12]	normal	normal	normal	n.d.	n.d.
P6	16	47,XY,	normal	normal			
		+4,			+4	+4	191,154,276
		der(3)t(3;5)(p23;q31.1),					
		der(5)t(3;5)(p23;q35.3),					
		der(5)t(5;10)(q31.1;p12.3),					
		der(10)t(5;10)(q35.3;p12.3)[8]/					
		46,XY[13]					
P7	26	46,XY,	del of 9p21.3	*CDKN2A/B*: del (64 %)	del(9)(p21.3p21.3)	chr9:21,817,082-23,515,821	1,698,739
		t(2;9;18)(p23.2;p21.3;q21.33),	del of 13q14.2	*RB1*: del (25 %)	del(13)(q14.2q14.2)	chr13:48,982,463-49,062,316	79,853
		t(10;14)(q24;q11)[10]			del(16)(p13.3p13.3)	chr16:3,154,954-4,568,792	1,413,838
P18	36	46,XY[5]	dup of 18q21.2	*DCC*: dup (13 %)	n.d.	n.d.	n.d.
P32	27	47,XX,	del of 6q21		n.d.	n.d.	n.d.

Table 1 Summary of aberrations detected by metaphase directed FISH, interphase FISH to determine the percentage of specific aberrations, and aCGH in 34 ALL patients *(Continued)*

		+21,	del of 6q27				
		t(10;14)(q24;q11),	del of 9p21.3	*CDKN2A/B*: del (89 %)			
		del(6)(q15q27)[6]	del of 12p13.2	*ETV6*: del (78 %)			
			del of 13q14.3	*DLEU1*: del (15 %)			
			dup of 21q22.1	*RUNX1*: dup (78 %)			
P35	40	46,XY,i(9)(q21.11)[2]			del(2)(q34q34)	chr2:213,811,279-214,150,984	339,705
					dup(7)(pterp14.1)	chr7:0–38,218,586	38,218,586
					del(7)(q21.2q21.2)	chr7:92,252,341-92,460,773	208,432
					del(7)(q36.3qter)	chr7:156,881,580-159,138,663	2,257,083
			del of 9p21.3	*CDKN2A/B*: del (92 %)	del(9)(pterp11.2)	chr9:0–47,212,321	47,212,321
			del of 9p13.2		dup(9)(q21.11qter)	chr9:71,035,265-141,213,431	70,178,166
					del(10)(q23.2q23.31)	chr10:89,570,600-89,728,844	158,244
					del(11)(q22.2q22.2)	chr11:102,106,046-102,529,831	423,785
					del(13)(q14.2q14.2)	chr13:49,004,123-49,122,923	118,800
					del(15)(q26.1q26.1)	chr15:93,390,484-93,466,292	75,808
					del(16)(p13.3p13.3)	chr16:3,808,951-3,839,782	30,831
					del(18)(q21.32q21.32)	chr18:57,517,756-57,617,796	100,040
					del(20)(q13.2q13.2)	chr20:52,151,411-52,574,928	423,517
P38	22	46,XY[3]	normal	normal	normal	n.d.	n.d.
P61	18	46,XX,der(2)t(2;7)(q37.3;q34), t(7;10)(q34;q24.1 ~ 25.1) [4]/			del(1)(p36.31p36.23)	chr1:5,958,728-7,238,618	1,279,890
					del(4)(p16.3p14)	chr4:3,072,509-38,882,925	35,810,416
		46,XX[3]	dup of 6q23.3	*MYB*: amp (90 %)	dup(6)(q23.3q23.3)	chr6:134,245,761-136,118,354	1,872,593
			del of 9p21.3	*CDKN2A/B*: del (88 %)	del(9)(p21.3p21.3)	chr9:21,252,517-23,002,377	1,749,860
				ABL1: amp (95 %)	dup(9)(q34.1q34.1)	chr9:133,658,293-134,092,544	434,251
				FGFR2: del (57 %)	del(10)(q25.1q26.3)	chr10:112,392,101-135,534,737	23,124,636
B- or T ALLs (not clinically well defined)							
P11	26	46,XY[8]	n.d.	normal	normal	n.d.	n.d.
P16	17	46,XX[7]			del(1)(q25.3q31.1)	chr1:184,771,633-185,825,795	1,054,162
					del(4)(p15.33p15.31)	chr4:12,322,760-18,779,457	6,456,697
					del(4)(q21.22q24)	chr4:82,992,997-106,476,929	23,483,932
					del(7)(pterp14.2)	chr7:0–36,320,986	36,320,986
			dup of 7q22.1	*RELN*: dup (61 %)	dup(7)(q21.3q22.3)	chr7:96,048,870-106,348,693	10,299,823
					del(9)(p23p22.2)	chr9:12,656,733-17,466,907	4,810,174

Table 1 Summary of aberrations detected by metaphase directed FISH, interphase FISH to determine the percentage of specific aberrations, and aCGH in 34 ALL patients *(Continued)*

			del of 9p21.3	*CDKN2A/B*: del (81 %)	del(9)(p21.3p21.3)	chr9:20,279,653-22,555,566	2,275,913
					del(10)(p14p13)	chr10:6,889,266-12,484,159	5,594,893
			del of 12p13.2	*ETV6*: del (91 %)	del(12)(p13.2p13.1)	chr12:11,761,018-12,934,870	1,173,852
					del(18)(p11.32p11.31)	chr18:2,741,687-3,231,531	489,844
P21	62	46,XY[11]	n.d.	normal	normal	normal	normal
P24	23	46,XY[12]	dup of 18q21.2	*DCC*: dup (18 %)	n.d.	n.d.	n.d.
P30	46	46,XY[6]	normal	normal	n.d.	n.d.	n.d.
P33	76	45,X,-X[8]			del(4)(q24q24)	chr4:106,036,993-106,601,946	564,953
					del(7)(q21.2q21.2)	chr7:92,080,855-92,475,197	394,342
					dup(7)(q36.2q36.2)	chr7:153,039,830-154,467,634	1,427,804
			del of 10q23.3		del(10)(q23.3q23.3)	chr10:89,610,886-89,698,312	87,426
					del(15)(q21.2q21.2)	chr15:51,826,924-51,919,665	92,741
					del(15)(q26.1q26.1)	chr15:93,433,130-93,450,773	17,643
			del of 17p13.1	*TP53*: del (10 %)	del(17)(p13.1p13.1)	chr17:7,583,457-8,156,734	573,277
					del(17)(q11.2q11.2)	chr17:30,259,193-30,267,204	8011
			dup of 18q21.2	*DCC*: dup (10 %)	dup(18)(q21.2q21.2)	chr18:49,105,579-51,431,815	2,326,236
					del(20)(q13.2q13.2)	chr20:52,151,411-52,554,455	403,044
					del(21)(q22.12q22.12)	chr21:36,253,465-36,426,708	173,243
					-X	-X	155,270,560
P46	63	46,XY[8]		normal	dup(6)(q25.3q25.3)	chr6:157,944,961-158,033,908	88,947
			del of 7p12.2		del(7)(p12.2p12.2)	chr7:50,452,798-50,492,798	40,000
					dup(17)(q12q12)	chr17:36,046,040-36,095,204	49,164
P47	59	46,XX[6]		normal	dup(1)(p13.3p13.3)	chr1:107,921,895-107,970,781	48,886
			del of 7p12.2		del(7)(p12.2p12.2)	chr7:50,356,873-50,465,376	408,503
			del of 9p13.2		del(9)(p13.2p13.2)	chr9:37,006,073-37,320,759	314,686
					dup(9)(q31.1q31.1)	chr9:104,126,808-104,167,077	40,269
					del(15)(q26.1q26.1)	chr15:93,390,484-93,450,773	60,289
					del(18)(q21.32q21.32)	chr18:57,517,756-57,718,190	200,434
					del(19)(p13.3p13.3)	chr19:0–2,787,457	2,787,457

bp basepairs, *LSP* locus-specific probes as specified in Additional file 2: Table S2, *y* year

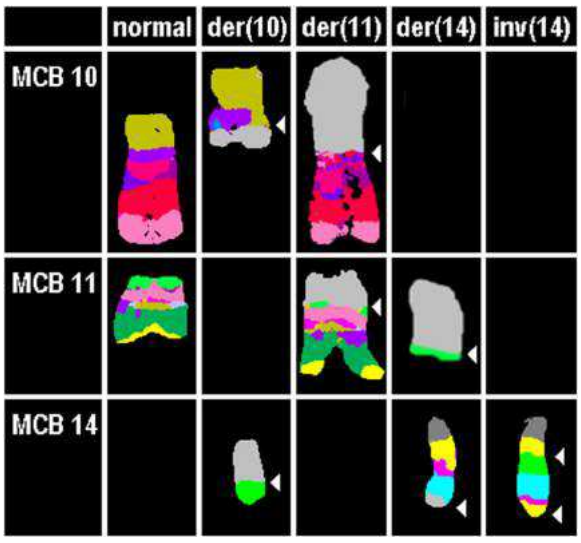

Figure 1 Result of aMCB probesets for chromosomes 10, 11, and 14 are shown, which characterized the breakpoints seen in case P66 as 10q11.23, 11p15.3, 14q11, 14q24.2, and 14q32.3. The final karyotype after application of all approaches as summarized in Additional file 1: Table S1 was 46,XX,der(10)(10pter- > 10p12.31::11q23.3- > 11q23.3::10p12.31- > 10q11.23::14q24.2- > 14qter),der(11)(10qter- > 10q11.23::11p15.3- > 11q23.3::10p12.31- > 10p12.31::11q23.3- > 11qter),der(14)t(11;14)(q15.3;q24.2),inv(14)(q11q23)

Conclusions

Cytogenetic analysis has been and still is the standard method for detection of diagnostically relevant recurrent chromosomal aberrations in ALL. It is well known that when using banding karyotyping cryptic chromosomal aberrations may be missed due to several reasons: (i) sensitivity of chromosomal banding techniques is limited, even in case of good chromosomal morphology, to aberrations being at least 10 Mb in size, (ii) aberrations may be cryptic or masked, i.e. they are not resolvable due to a similar or identical GTG-banding pattern and/or poor chromosome morphology, and (iii) metaphases may be difficult to obtain and to evaluated as chromosomes may not be well-spread, clumsy or appearing as fuzzy with indistinct margins; thus even numerical aberrations may be missed [6, 13, 17].

In the past molecular cytogenetic approaches have shown to be efficient to detect in banding cytogenetics cryptic chromosomal aberrations [6, 13, 17]. Besides in metaphase also interphase nuclei can be studied in case of low mitotic (non-dividing) cells and also alterations being at low mosaic level can be easily detected by that approach [12, 14, 18]. In this study, we detected previously cryptic aberrations in 21/61 (34 %) cases with ALL using metaphase directed FISH studies; even complex aberrations were identified in some of these cases (Table 1 and Additional file 1: Table S1).

For 34/61 cases DNA could be extracted from the cytogenetically worked up cell suspension. Thus, in those cases besides FISH also MLPA and aCGH could be applied additionally, i.e. approaches which have much higher resolution than FISH, but can only detect unbalanced aberrations and no low level mosaics. Using these approaches cryptic CNAs were detected in ~80 % of those ALL cases. All 126 CNAs detected by MLPA and aCGH have been checked by UCSC genome browser to exclude benign copy number variations (CNVs) (http://genome-euro.ucsc.edu/cgi-bin/hgGateway?redirect=auto&source=genome.ucsc.edu). Thus, all of them most likely are leukemia-related genetic changes, which were recognized in 27/34 ALL cases.

Of special interest may be a novel recurrent submicroscopic CNA expressed as loss of 15q26.1: focal deletion of *CHD2* gene located there was found in 7 of the 34 (20 %) studied ALL cases in this study. The *CHD2* gene is a member of the chromodomain helicase DNA-binding (CHD) protein family, which are all characterized by a chromatin-remodeling domain (the chromodomain) and an SNF2-related helicase/ATPase domain [19]. Thus, in future it may be of interest to study *CHD2* gene deletions also for presence of mutations in this gene and also to screen ALL patients in general for *CHD2* gene mutations.

Besides, duplication of *DCC* gene in 18q21.2 was present in 3 of the 34 (9 %) studied cases. *DCC* is a member of the immunoglobulin superfamily of cell adhesion

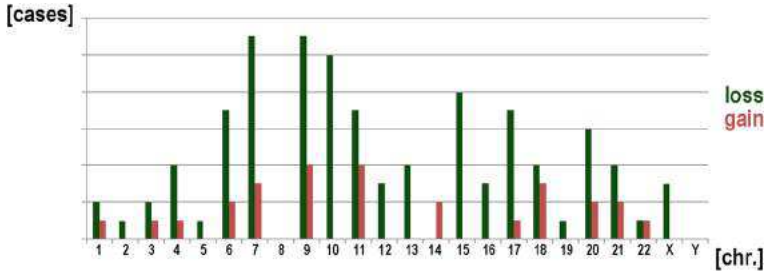

Figure 2 Distribution of CNAs as detected by aCGH in 27/34 studied cases. On X-axis the chromosome number is shown, while on Y-axis the total number of CNAs for each chromosome is depicted (scale 2).

molecules and acts as a transmembrane dependence receptor for netrins, key factors in the regulation of axon guidance during development of the central nerve system. Amplification of *DCC* gene was previously reported in chronic lymphocytic leukemia (CLL) [20, 21], however, this is the first report for *DCC* gene amplification in ALL. To evaluate the role of the *DCC* gene and to elaborate its potential as a molecular marker in ALL still needs more studies.

In general, submicroscopic CNAs were identified most frequently in chromosomes #7 and #9. CNAs in #7 involved deletion of *IKZF1* at 7p12.2 that encodes IKAROS protein and is required for the development of all lymphoid lineages in 5 of 34 (14 %) studied CN-ALL cases. According to the literature deletions and/or sequence mutations of *IKZF1* are present in 15 % of pediatric B-ALL, including ~70 % of BCR-ABL–positive ALL and with high-risk of relapse ~30 % of BCR-ABL–negative B-ALL [22]. However, deletions of *IKZF1* are predominantly monoallelic and involve the N-terminal zinc-finger domain of IKAROS protein and result in expression of dominant-negative isoforms with cytoplasmic localization and oncogenic activity as well as an association with very poor outcome [23, 24]. Thus, *IKZF1* has newly been considered as a prognostic marker for B-ALL and might be useful for risk stratification [24, 25].

Cyclin dependent kinase 6 (*CDK6*) at 7q21.2, is the catalytic subunit of a protein kinase complex that regulates cell cycle G1 phase progression and G1/S transition. Deletion of *CDK6* was identified in this study in 4 of 34 (12 %) of ALL cases. It has been shown recently that inhibition of CDK6 may lead to overcome the differentiation block seen in acute myelogenous leukemia (AML) with *MLL* translocations [26]. Further studied for this gene may also be recommended for better understanding of ALL biology.

The majority of #9 abnormalities is involving deletions of cell cycle regulatory genes at 9p21.3. The main target to deletions is *CDKN2A* which encodes for the two transcripts *p16/INK4A* and *p14/ARF* (alternative splicing), followed by *CDKN2B* gene (*p15/INK4B*); both are tumor suppressor genes. Deletions of *CDKN2A/B* can be found in 30 and 50 % of B-ALL and T-ALL cases, respectively [23, 25, 27]. In the present study such deletions were only found in 8/34 (24 %) of the studied ALL cases, which is most likely due to low case numbers. *CDKN2A*/B deletion can be detected at initial diagnosis or acquired at relapse, suggesting that *CDKN2A/B* deletion is a secondary genetic event. Also, the outcome of cases with *CDKN2A/B* deletion depends on the status of the second allele, as homozygous deletions are associated with poor outcome and heterozygous deletions represent markers for favorable outcomes [27, 28]. T-ALL-case P61 had such a prognostically adverse homozygous deletion in 9p21.3 together with amplification of 9q34.12 to 9q34.13; the latter contains the *ABL1* and *NUP214* genes (Fig. 3). *NUP214-ABL1* fusion gene amplification was previously mainly observed in T-ALL and associated with poor outcome [6].

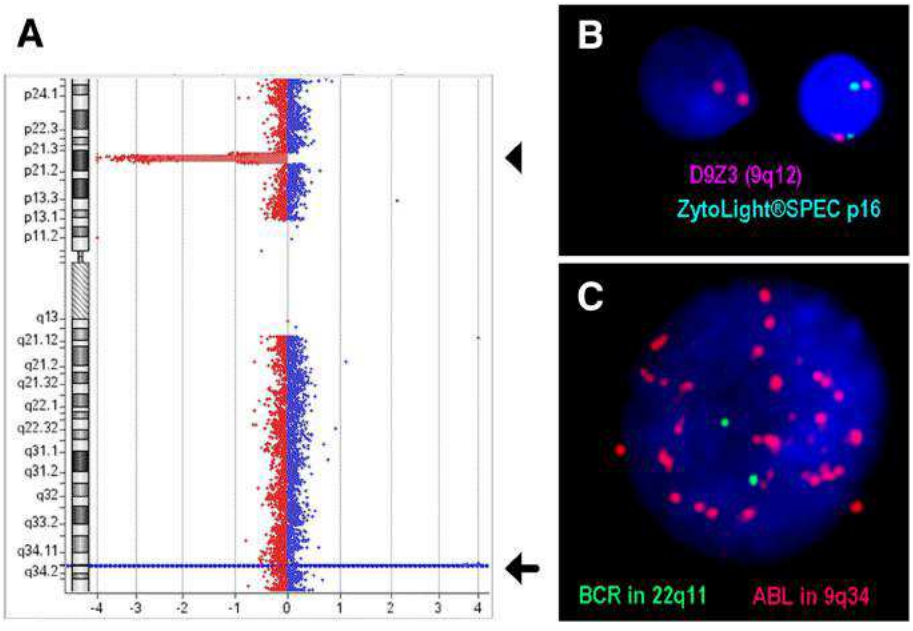

Figure 3 aCGH from case Nr. P61 showed two CNAs in chromosome 9; at 9p21.3 a homozygous deletion (arrowhead) and at 9q34.12 to 9q34.13 an amplification (arrow). **a** FISH confirmed presence of the homozygous deletion in 9p21.3 in interphase. **b** An amplification present as double minutes was confirmed using a probe specific for the *ABL*-gene

Another recurrent deletion in #9 in the studied ALL cases involved the *PAX5* gene located in 9p13.2, which encodes for a protein with key roles in lymphoid development. It was found to be deleted in B-ALL ($n = 2$) and T-ALL ($n = 1$ showed short arm 9p deleted) in this study. In the literature, deletion of *PAX5* was reported in 31.7 % of B-ALL and also it has been involved in several chromosomal translocations [29, 30]. In a recent report, *PAX5* deletion was observed in only 10 % and 18 % in children and adult B-ALL, respectively; notably *PAX5* deletion was frequently accompanied by deletion of *CDKN2A* (83.3 % of children and 100.0 % of adults) [28]. Also *PAX5* was found to be a common target in leukemogenesis of B-ALL, but not associated with adverse outcome [15]. In future, *PAX5* could be used as one of the molecular markers in diagnosis and monitoring of the disease, especially in B-ALL [28–30].

Besides, other CNAs have been identified here, encompassing single or few genes, only. Many of CN losses involve cell cycle regulatory and/or putative tumor suppressor genes like 10q23.3 (*PTEN*; $n = 6$), 13q14.2 (*RB1*; $n = 3$), and 17p13.1 (*TP53*; $n = 4$), or transcriptional regulators and co-activators like 3q26.32 (*TBL1XR1*; $n = 1$), 12p13.2 (*ETV6*; $n = 2$), 21q22.12 (*RUNX1*; n = 1) and 21q22.2 (*ERG*; $n = 2$), or regulators of chromatin structure and epigenetic regulators like 16p13.3 (*CREBBP*; $n = 2$). Although, oncogene overexpression resulting from gene duplication is infrequent in ALL, we found *MYB* duplication in one case, too. These observations of gene loss of function or overexpression being involved in leukemic transformation [15, 31] underline the heterogeneity of different ALL cases and the potential of molecular approaches to identify new subgroups of this disease.

The present study also highlights, that most likely all CN-ALL cases hold cryptic genomic alterations. DNA sequencing and single-nucleotide polymorphism (SNP) arrays have been used to detect mutations for a number of target genes that are known to key roles in lymphoid development. Thus, somatic mutations have been identified in both B and T-ALL patients [2]. For instance, mutations in *JAK2* were identified in 10 % of high-risk childhood B-ALL and shown to be associated frequently with other abnormalities, including deletions or mutations of *IKZF1* and overexpression the *CRLF2* gene [23]. In T-ALL, *NOTCH1*-activating gene mutation has been found in 60 % and *FBXW7*-inactivating gene mutation occurs in 20 % of pediatric T-ALL [32]. Less commonly, mutations in *PTEN*, *WT1*, amplification of *MYB* and sequence mutations in RAS signaling (*NRAS*, *KRAS*, and *NF1*) and tumor suppression (*TP53*) have been identified in ALL [8, 31].

Overall, sensitive methods to detect cryptic chromosomal aberrations in CN-ALL are useful and necessary for genetic risk–based classification and correct determination of treatment protocols. The present study highlights that molecular cytogenetic approaches together with molecular methods are suited to identify cryptic rearrangements and potential target genes that involved in leukemogenesis and progression of the disease. Also it could be demonstrated that aCGH is a highly efficient tool for detection of CNAs in CN-ALL. However, while aCGH (and MLPA) provide data on imbalanced genomic alterations, (molecular) cytogenetics additionally detects different leukemic subclones within one sample, as well as balanced translocations leading to tumor-specific fusion genes. It seems to be valid, that there is no leukemia clone without genetic alterations; we just have to use the appropriate techniques to identify them. In conclusion, to obtain a comprehensive picture of all relevant changes in each individual ALL case data from cytogenetics, FISH, MLPA and aCGH needs to be considered and included in diagnostics; however, sometimes such investigations may be hampered by lack of sufficient cellular material, as also in this study, where only 34/61 cases could also be studied on DNA level or other previous studies [16, 33].

Methods

Patients and sample preparation

Cell suspensions were obtained from bone marrow collected from 61 patients diagnosed with ALL (31 with B-ALL, 12 with T-ALL and 18 with undefined ALL; Additional file 1: Table S1). The samples were obtained under informed consent of the corresponding patients and according to institutional ethical committee guidelines (ethical commission of the university clinic Jena, Germany; code 1105-04/03).

GTG-banding

The bone marrow cells were unstimulated cultivated for 24 hours (with and without colchicin) and 48 h, and a standard cytogenetic cell preparation following air drying method was done [34]. GTG-banding was routinely done in each sample following standard procedures. Twenty metaphases were obtained for cytogenetic evolution on a banding level of 250–300 bands per haploid karyotype [35]. Apart from 4 all 61 studied cases had a normal karyotype of 46,XX or 46,XY. In one case the karyotype could not be determined due to low metaphase quality; one case just had (most likely age associated) loss of an X-chromosome in a subset of the cells, one case had a questionable der(19) in all cells, and another one a trisomy 14 in 6/20 studied cells.

Molecular cytogenetics

Fluorescence *in situ* hybridization was done according to standard procedures and/or according to manufacturer's instructions.

Homemade were the following probes and probe sets:

- 24-color-FISH using all human whole chromosome painting (WCP) probes [36];
- FISH-banding probe-sets as follows: genome wide multitude multicolor banding (mMCB) and chromosome specific high resolution array-proven multicolor-banding (aMCB) [16, 37, 38];
- WCP probes for all chromosomes were homemade [36].
- The following commercially available locus-specific probes (LSPs) (Additional file 2: Table S2) were used to validate and possibly confirm the breakpoints found in mMCB, aCGH and/or MLPA: from Abbott/Vysis (Wiesbaden, Germany), Kreatech Diagnostics (Amsterdam, Netherland), ZytoVision (Bremerhaven, Germany), and DNA from bacterial artificial chromosome (BACs) probes obtained from Resources Center (Oakland, USA) were labeled by PCR with SpectrumGreen, SpectrumOrange or TexasRed-dUTP and applied in two- or three-color FISH-approaches. For each interphase FISH analysis to determine the percentage of specific aberrations, at least 200 interphase nuclei were examined per sample and FISH-probe – the applied probes can be found in Additional file 2: Table S2.
- Homemade and previously reported chromosome-specific sub-CTM- (= subtelomere -/ subcentromere oriented) probe-sets were applied in selected cases [13] (Additional file 1: Table S1).

DNA isolation

Genomic DNA was extracted from cells fixed in acetic acid-methonal (1:3) by Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA). DNA concentration was determined by a Nanodrop spectrophotometer. The quality of DNA was checked using agarose gel electrophoresis. DNA-samples extracted from fixed cells of 2 healthy males and 2 healthy females by the same method were used as reference samples.

MLPA analysis

SALSA MLPA P377-A1 Hematologic malignancies probemix was used for this study (MRC- Holland, Amsterdam, The Netherlands). This probemix contains probes for 37 genes covered by 54 probes, which have diagnostic or prognostic significant role in hematologic malignancies. MLPA was performed according to the manufacturer's protocol, which includes three reaction phases: hybridization, ligation, and PCR amplification. Amplified probes and GeneScan LIZ 500 (Applied Biosystems, Foster City, USA) standard were separated by capillary electrophoresis using a ABI-PRISM 3130XL Genetic Analyzer (Applied Biosystems, Foster City, USA). GeneMarker (SoftGenetics, USA) was used to analyzeMLPA data. Detection threshold was set at 0.65-1.35; control samples of four healthy donors were included in each run.

Array-comparative genomic Hybridization (aCGH)

aCGH was performed using Agilent SurePrint G3 Human Genome microarray 180 K (Agilent Technologies, Santa Clara, CA, USA), an oligonucleotide microarray containing 170,334 probes 60-mer with a ~13 kb overall median probe spacing (11 kb in Refseq-genes). Genomic DNA of patients was co-hybridized with a sex-mismatched control DNA (G1471 or G1521; Promega, Mannheim, Germany). Labeling was performed using Agilent Genomic DNA enzymatic labeling kit (Agilent) according to the manufacturers' instructions. After hybridization and washing, the aCGH slide was scanned on an Agilent scanner, processed with Feature Extraction software (v12.0.2.2) and results were analyzed using Cytogenomics (v3.0) using ADM2 as aberration algorithm.

Additional files

Additional file 1: Table S1. All 61 CN-ALL cases studied; for each case age, gender and subtype of ALL is given. Also all FISH-probes, probe sets and approaches applied for each case are listed. Abbreviations: n.d. = not determined, y = year.

Additional file 2: Table S2. List of locus specific probes used in the present study for further characterization of acquired aberrations and/or determination of the percentage of deletions or duplications as determined by aCGH or MLPA.

Abbreviations

aCGH: Array-comparative genomic hybridization; ALL: Acute lymphoblastic leukemia; aMCB: Array-proven multicolor-banding; AML: Acute myelogenous leukemia; BAC: Bacterial artificial chromosome; B-ALL: B-cell ALL; Bp: Basepairs; CLL: Chronic lymphocytic leukemia; CN: Copy number; CNA: Copy number alteration; CN-ALL: ALL according to banding cytogenetics with normal karyotype; CNVs: Copy number variations; DNA: Deoxyribonucleic acid; FISH: Fluorescence *in situ* hybridization; GTG: G-banding with trypsin-Giemsa; LSPs: Locus-specific probes; MLPA: Multiplex ligation-dependent probe amplification; mMCB: Multitude multicolor banding; n.d.: Not determined; PCR: Polymerase chain reaction; SNP: Single-nucleotide polymorphism; sub-CTM: Subtelomere -/ subcentromere oriented; T-ALL: T-cell ALL; TPA: 2-O-tetradecanoylphorbol-13-acetate; WCP: Whole chromosome painting; Y: Year.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MAKO selected the cases, did parts of the FISH-studies and drafted the paper; JBM and IMC performed aCGH analyses and interpretation; MR and MAKO did MLPA analyses and interpretation; AG, BG, BG, KW, MLMS and TdJMS provided ALL-cases including clinical and banding cytogenetic data; KR and BM were involved in FISH-probe generation and application KR did also parts of the FISH-studies; TL planned and organized the study and did final drafting of the paper. All authors read and approved the paper.

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The supplementary tables are in appendix

2.9. Article .8

Othman MA, Vujić D, Zecević Z, Đurišić M, Slavković B, Meyer B, Liehr T. **A cryptic three-way translocation t(10;19;11)(p12.31;q13.31;q23.3) with a derivative Y-chromosome in an infant with acute myeloblastic leukemia (M5b).** Gene, 2015;563(2):115-119.

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Research paper

A cryptic three-way translocation t(10;19;11)(p12.31;q13.31;q23.3) with a derivative Y-chromosome in an infant with acute myeloblastic leukemia (M5b)

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ABSTRACT

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by the malignant transformation of hematopoietic precursors to a pathogenic cell clone. Chromosomal band 11q23 harboring *MLL* (=mixed lineage leukemia) gene is known to be involved in rearrangements with variety of genes as activating partners of *MLL* in different AML subtypes. Overall, an unfavorable prognosis is associated with *MLL* abnormalities. Here we investigated an 11-month-old male presenting with hyperleukocytosis being diagnosed with AML subtype FAB-M5b. In banding cytogenetics a der(19)t(19;?)(q13.3;?) and del(Y)(q11.23) were found as sole aberrations. Molecular cytogenetics revealed that the *MLL* gene was disrupted and even partially lost due to a t(10;19;11)(p12.31;q13.31;q23.3), an MLL/MLLT10 fusion appeared, and the der(Y) was an asymmetric inverted duplication with breakpoints in Yp11.2 and Yq11.23. The patient got hematopoietic stem cell transplantation from his haploidentical mother. Still three months afterwards 15% of blasts were detected in bone marrow and later the patient was lost during follow–up. The present case highlights the necessity to exclude *MLL* rearrangements, even when there seems to be no actual hint from banding cytogenetics.

1. Introduction

Infant acute leukemia (AL) is defined as malignancy of the blood occurring in the first years of life. Acute myeloid leukemia (AML) accounts 15%–20% of childhood AL cases, while AML is the most frequent form of adult AL providing ~80% of the cases (Rubnitz et al., 2010).

Chromosomal rearrangements involving the *MLL* (mixed lineage leukemia or myeloid/lymphoid leukemia, also called ALL1 for acute lymphoblastic leukemia 1 or *KMT2A* lysine (K)-specific methyltransferase 2A) gene located on chromosome 11 subband q23 are typically found in 35%–50% of childhood and in 5% of adult AMLs (Balgobind et al., 2011; Stasevich et al., 2006; De Braekeleer et al., 2005). The *MLL* gene has been found to be "promiscuous", being able to form fusion genes with more than 120 different translocation partners (Meyer et al., 2013). The t(9;11)(p22;q23) is the most frequent event involving the *MLL* gene in pediatric AML (50%), and the t(10;11)(p12;q23) is the second common one (13%) (Balgobind et al., 2011; Coenen et al., 2011; Meyer et al., 2013; DiNardo et al., 2015).

This t(10;11) is most often found in AML French–American–British (FAB) subtypes M4/M5; these patients present leukocytosis, extramedullary disease, poor long-term outcomes and high risk of relapse (Lillington et al., 1998; Balgobind et al., 2011; Meyer et al., 2013). In most cases, the t(10;11) leads to fusion of the 5′ end of *MLL* and 3′ of *MLLT10*. The mechanism of this rearrangement seems to be more complex than a simple reciprocal translocation because of an opposite orientation of both genes on chromosomes 10 and 11. This implicates that an inversion of one of the two genes is necessary to allow the formation of the *MLL–MLLT10* chimeric transcript (Stasevich et al., 2006; Matsuda et al., 2006). Besides, the *MLLT10* gene (previously *AF10*) can also form a fusion gene with *PICALM* (11q14) in AL (Brandimarte et al., 2013; Borel et al., 2012).

Overall, detection or exclusion of an *MLL* disruption or amplification is extremely necessary for treatment decisions, as well as for basic research enabling new insights into possible fusion genes involving *MLL*.

Abbreviations: AL, acute leukemia; aMCB, array-proven multicolor-banding; ALL, acute lymphatic leukemia; AML, acute myeloid leukemia; ANAE, alpha-naphthyl-acetate-esterase; BAC, bacterial artificial chromosome; BM, bone marrow; DAPI, Diaminophenylindol; FISH, fluorescence in situ hybridization; ISCN, International System for Human Cytogenetic Nomenclature; HSCT, hematopoietic stem cell transplantation; MLL, mixed lineage leukemia; mMCB, multitude multicolor banding; WBC, white blood cell; WCP, whole chromosome painting.

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Here we report a new case of childhood AML-M5b harboring a cytogenetically balanced translocation with break events in 10p12.31, 11q23.3, and 19q13.31 associated with a partial deletion of the *MLL* gene. Besides, an unusual rearrangement of the Y-chromosome was observed.

2. Material and methods

2.1. Clinical description

An 11-month-old male infant was presented in 2012 with hyperleukocytosis (white blood cell (WBC) count of 43.6×10^9/l). Bone marrow (BM) aspiration showed 95% blasts, being Sudan Black B staining negative and ANAE (alpha-naphthyl-acetate-esterase) stain positive. Immunophenotyping revealed positivity for MPO (myeloperoxidase), HLA-DR, CD4, CD33, CD45, CD15, CD11b and CD13 prompting a diagnosis of AML, and FAB classification as AML type M5b.

The infant was enrolled in protocol AML-BFM 98 and after induction therapy blasts in BM were only 5%. After one year of initial therapy the patient had medullary and extramedullary relapse, 82% blasts in BM being positive for HLA-DR, CD4, CD33, CD45, CD15, MPO, CD11b and CD13. The patient was further treated according to protocol AML-BFM 2004 and after induction therapy blasts in BM were again down to 5% but skin nodes being present. Hematopoietic stem cell transplantation (HSCT) from haploidentical mother was performed after conditioning with thiotepa, treosulfan and fludarabine. Three months after HSCT, 15% of blasts were again detected in BM, being CD45 positive; also skin biopsy showed extramedullary relapse. Unfortunately, later the patient was lost during follow-up.

2.2. Banding cytogenetic

Chromosome analyses were performed on unstimulated BM after direct chromosome preparation, as well as after 24 h culture. GTG-banding as well as C-banding were performed (Claussen et al., 2002).

A total of 30 metaphases were analyzed. Karyotype designation was done according to International System for Human Cytogenetic Nomenclature (ISCN, 2009). A chromosome analysis was possible on a level of 300 bands per haploid karyotype.

2.3. Molecular cytogenetics

Fluorescence in situ hybridization (FISH) was done according to standard procedures and according to manufacturers' instructions for the following commercially available probes: LSI *MLL* (11q23 Break probe, Abbott Molecular/Vysis, Mannheim, Germany), LSI *SRY* (Yp11.3, Abbott Molecular/Vysis, Mannheim, Germany), SPEC *ETV6*/*RUNX1* (*ETV6* in 12p13, *RUNX1* in 21q22, ZytoVision, Bremerhaven, Germany), SPEC 19q13/19p13 (ZytoVision, Bremerhaven, Germany), Centromere Y (CEPY (DYZ3): Yp11.1–q11.1 Alpha Satellite DNA; CEPY (DYZ1): Yq12 Satellite III DNA, Abbott Molecular/Vysis, Mannheim, Germany), and subtelomeric probes for Yp/Xp, and Yq/Xq (Yp in DXYS153, Xp in DXYS129; Yq in D11S1037, Abbott Molecular/Vysis, Mannheim, Germany).

Whole chromosome painting (WCP) probe for chromosomes 9, 10, 11, 19, and Y and BAC (bacterial artificial chromosome) clones of interest were identified through the Human Genome Browser Database of the Genome Bioinformatics Group at the University of California at Santa Cruz (http://genome.ucsc.edu/) and Ensembl Genome Data Resources of the Sanger Institute Genome Database (http://www.ensembl.org/). DNA probes (Table 1) obtained from BAC/PAC Resources Center (Oakland, USA) were labeled by PCR with SpectrumGreen, SpectrumOrange or TexasRed-dUTP and applied in two- or three-color FISH-approaches. The homemade multitude multicolor banding (mMCB) and chromosome specific high resolution array-proven multicolor-banding (aMCB) probe sets were also applied as previously reported (Weise et al., 2003, 2008).

A total of 10–15 metaphase spreads were analyzed, using a fluorescence microscope (Axio Imager.Z1 mot, Zeiss) equipped with appropriate filter sets to discriminate between a maximum of five fluorochromes and the counterstain DAPI (Diaminophenylindol). Image capturing and processing were carried out using an ISIS imaging system (MetaSystems, Altlussheim, Germany).

3. Results

Cytogenetic study performed at diagnosis on a bone marrow cell culture revealed a 46,X,del(Y)(q11.23),der(19)t(19;?)(q13.3;?)[26]/46,XY[4] without evidence for 11q23 rearrangement (Fig. 1). FISH analysis using WCP probes for chromosomes 19 and Y revealed a balanced translocation of 19q to another chromosome. mMCB probeset showed that the rearrangement indeed was more complex: 46,X,der(Y)(Ypter→Yq11.23::Yp11.2→Ypter),t(10;19;11)(p12;q13;q23). Chromosome specific aMCB probesets for #10, #11, #19, and Y confirmed the mMCB result (Fig. 2A). Locus specific probes narrowed down the breakpoints as shown in Table 1 to 10p12.31, 11q23.3, 19q13.31, Yp11.2 and Yq11.23.

The LSI *MLL* break apart probe gave the following result (Fig. 2B): the 5′ *MLL* probe was given a green split signal on derivative chromosomes 10 and 11. This probe includes exons 1 to 6 of the *MLL* gene, according to http://www.vysis.com and based on *MLL* gene nomenclature available from http://www.ensembl.org; transcript ID ENSG00000118058. Also

Table 1
Used probes, their location and obtained results are listed.

Cytoband	Positions [hg18]	Probe	Result on derivative chromosomes
Yp11.31	chrY: 264,089–264,253	CTC-839D20	2 signals on der(Y)
Yp11.31	chrY: 2,714,896–2,715,792	LSI SRY	2 signals on der(Y)
Yp11.32	chrY: 317,555–517,715	DXYS153	2 signals on der(Y)
Yp11.2	chrY: 6,752,454–6,919,727	RP11-115H13	2 signals on der(Y)
Yp11.1–q11.1	chrY: 11,200,001–12,500,000	DYZ3	1 signal on der(Y)
Yq11.221	chrY: 15,173,440–15,173,599	RP11-71M14	1 signal on der(Y)
Yq11.221	chrY: 15,688,562–15,841,531	RP11-59K8	1 signal on der(Y)
Yq12	chrY: 27,200,001–57,772,954	DYZ1	Deletion on der(Y)
Yqter	chrY: 57,719,381–57,727,828	EST Cdy 16c07 for SYBL1	Deletion on der(Y)
10p12.31	chr10: 20,782,567–20,938,614	RP11-51E20	Signal on der(19)
10p12.31	chr10: 21,321,413–21,495,264	RP11-165O3	Signal on der(19)
10p12.31	chr10: 22,399,352–22,575,929	RP11-108B14	Signal on der(10)
11q23.3	chr11: 117,812,415–117,901,146	LSI MLL	Split signal on der(10) and der(11) and deletion of 3′ part of MLL
19q13.2	chr19: 47,022,914–47,206,527	RP11-688M4	Signal on der(19)
19q13.31	chr19: 48,171,290–48,356,279	RP11-313K22	Signal on der(19)
19q13.31	chr19: 49,097,834–49,247,766	RP11-143F10	Split signal on der(19) and der(11)
19q13.31	chr19: 49,726,602–49,900,222	RP11-21J15	Signal on der(11)
19q13.32	chr19: 52,803,265–53,038,398	SPEC GLTSCR1/R2/CRX	Signal on der(11)

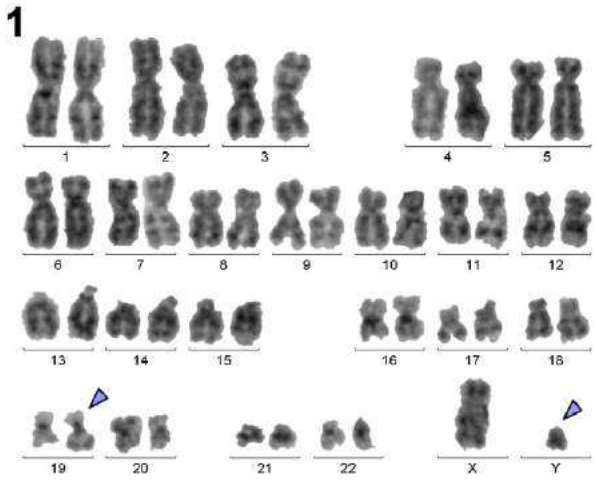

Fig. 1. G-banded karyogram from bone marrow cells at diagnosis, showing mos 46,X,del(Y)(q11.23),der(19)t(19;?)(q13.3;?)[26]/46,XY[4].

the 3′ *MLL* region of ~190 kb in size (red signal in Fig. 2B) was deleted due to the rearrangement.

Breakpoints on chromosomes 10 and 19 were narrowed down using the BAC-probes listed in Table 1. The breakpoint in 10p12.31 was mapped between positions 21,495,264 and 22,399,352, where the *MLLT10* gene has been mapped to 21,863,580–22,072,560. The breakpoint in 19q13.31 was mapped between positions 49,097,834 and 49,247,766; 2 OMIM genes are located there: ZNF45, and ZNF155 (Fig. 2C). The positions are given according to NCBI36/hg18, as numerous of the used BAC-probes could not be found in later genomic browser versions. The hybridization signals of the subtelomeric (Yp and Yq) probes were revealed: duplication in Yp subtelomeric region and deletion in Yq region (Fig. 3).

4. Discussion

In the present case two independent rearrangements were observed, one involving three autosomes and one of a gonosome. Both were already partially visible after GTG-banding analyses, however, their real nature could only be resolved by molecular cytogenetics.

Structural abnormalities involving the Y-chromosome are rare events in hematological malignancies. A der(Y)t(Y;1)(q12;q21) is

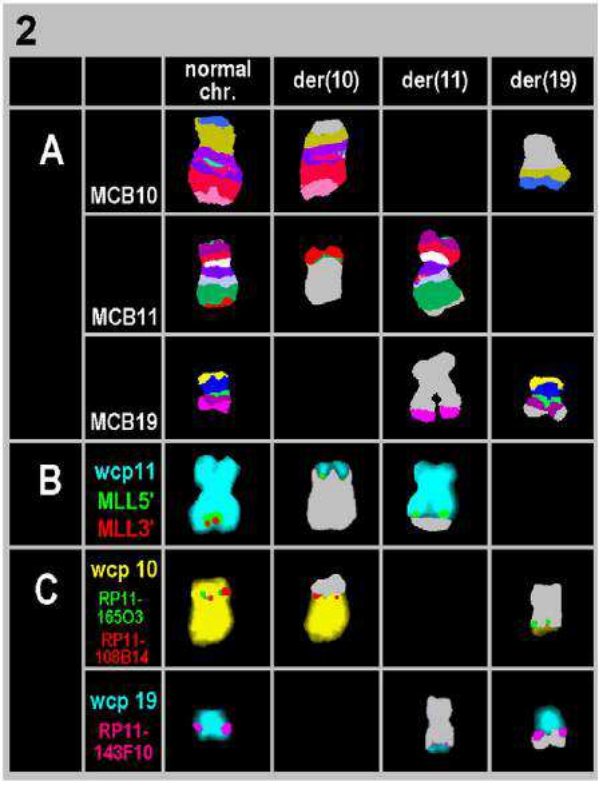

Fig. 2. A) Results for aMCB probesets for chromosomes 10, 11, and 19 are shown, which characterized the breakpoints as 10p12, 11q23 and 19q13 after mMCB (results not shown). B) LSI MLL break apart probe revealed a fusion signal on normal chromosome 11 and one green signal each on der(10) and der(11); still red signal was absent in whole metaphase spread. The breakpoint in 11q23 could be narrowed down to 11q23.3. C) Further characterization of the breakpoints in derivative chromosomes 10 and 19 by BAC-probes revealed breakpoints as 10p12.31 and 19q13.31.

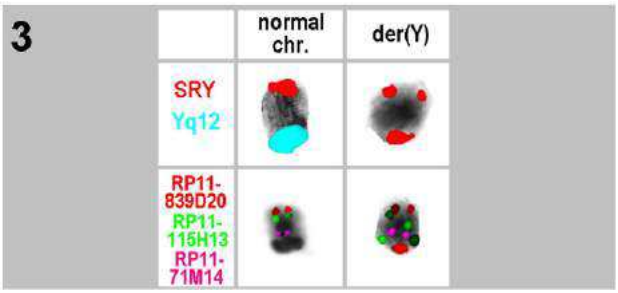

Fig. 3. FISH-probes as specified in the figure and detailed in Table 1 enabled confirmation of mMCB result for derivative Y-chromosome as der(Y)(Ypter→Yq11.23::Yp11.2→Ypter).

described to be a recurrent but uncommon chromosomal rearrangement in AML (Manabe et al., 2013); also a t(Y;11) involving the MLL gene was reported once (Bernasconi et al., 1999). Besides inverted duplication shaped derivative Y-chromosomes have not been reported in AML yet. The observed der(Y)(Ypter → Yq11.23::Yp11.2 → Ypter) here was only present in the malignant cell clone and a normal Y-chromosome was present in cells with normal male karyotype. Thus, it is unlikely that the patient originally had a mosaic karyotype 46, der(Y)(Ypter → Yq11.23::Yp11.2 → Ypter)/46,XY even though such cases are reported in the literature (for overview see (Liehr, 2014)). Thus, this is to the best of our knowledge the first asymmetric inverted duplication shaped derivative Y-chromosome ever reported. It remains to be determined if gain of Yp11.32–p11.2 and loss of Yq12, might be implicated in leukemogenesis due to gene dosage effects.

Gene fusion, as a result of chromosomal translocation, is an important event in leukemogenesis. *MLL* rearrangements are strongly associated with AML M4/M5 and characterized by extreme leukocytosis, skin involvement, and central nervous system disease (Coenen et al., 2011; DiNardo et al., 2015). Two clinical subgroups of patients have a high frequency of 11q23 aberration and M5 subtypes: one is AML in infants (<1 year) with *MLL* rearrangement in about 50% of cases; the other group is adult "secondary leukemia" potentially after treatment with DNA topoisomerase II inhibitors. However, the patients with M5a subtype are more likely to have a t(9;11) than patients with M5b, while other translocations are more frequent in M5b patients (Flandrin, 2002; De Braekeleer et al., 2005; Balgobind et al., 2011).

In the present childhood AML-M5b case a yet unreported (Mitelman et al., 2014) cytogenetically balanced but molecular proven unbalanced translocation t(10;19;11)(p12.31;q13.31;q23.3) was described. Only by molecular cytogenetics resolvable findings were (i) the fusion of 5′ *MLL* (11q23.3) to *MLLT10* (10p12.31) and (ii) the deletion of 3′ *MLL*. The fusion of 10p12 with 19q13.31 and that of 11q23.3 and 19q13.31 could involve the following genes: *MLLT10*, *ZNF155*, *and MLL*.

Only four AML cases were reported with three way translocations before involving the same three chromosomes 10, 11 and 19, still all of them involving other chromosomal breakpoints especially in chromosome 19, than the present case (Pui et al., 1994; La Starza et al., 2006; Mulaw et al., 2012; Petković et al., 1992). Also a t(11;19)(q23;q13) *MLL–ACTN4* fusion was previously seen (Burmeister et al., 2009). *ACTN4* on chromosome 19q13 is an actin-filament cross-linking protein. Mutations in *ACTN4* or *ACTN4* deficiency lead to focal and segmental glomerulosclerosis.

MLLT10 gene rearrangements have been identified to a high percentage in pediatric AML cases; it encodes for a leucine zipper protein that functions as a transcription factor (Dreyling et al., 1998). The t(10;11) is a recurrent reciprocal translocation in AL and has two common variants; t(10;11)(p12;q21) and t(10;11)(p12;q23), the latter tending to be more frequently observed in young children AML (Lillington et al., 1998) and rarely seen in acute lymphatic leukemia (ALL) (Coenen et al., 2011); the second variant is t(10;11)(p12;q21) identified mainly in T-ALL patients, as well as reported in AML and myeloid sarcoma (Bohlander et al., 2000; Mulaw et al., 2012).

Such t(10;11) rearrangements are often described as "cryptic" because in 10% of AML cases they are not detectable by banding cytogenetics. As patients with t(10;11) are associated with unfavorable outcome due to the less response to therapy their identification is of high importance for therapy planning (DiMartino et al., 2002; Caudell and Aplan, 2008; Coenen et al., 2011). *MLL* and *MLLT10* fusion may form due to translocations, insertions, deletions or due to more complex rearrangements (Stasevich et al., 2006; Matsuda et al., 2006). As in the present case observed, translocations involving band 11q23 usually lead to a breakage in the *MLL* gene where the 5′ part of the gene is retained on the derivative chromosome 11. Therefore, the active fusion gene (5′ *MLL*–3′ partner) is almost always located on the der(11), except in rare cases of insertion of the 5′ *MLL* to another chromosome. The breakpoints within the *MLL* gene cluster in the 8.5 kb region, called the breakpoint cluster region (bcr) are located between exons 5 and 11. *MLL* partner plays a critical role in determining the disease phenotype; for example: *MLL–MLLT7* in T-ALL, *MLL–MLLT2* in B lineage ALL, *MLL–MLLT3* and *MLL–MLLT10* in AML-M5, *MLL–MLLT1* in ALL/AML. This suggests that the fusion protein affects the differentiation of the hematopoietic pluripotent stem cells or the lymphoid or myeloid committed stem cells (De Braekeleer et al., 2005; Stasevich et al., 2006; Chaplin et al., 2001). However, a deletion of 3′ *MLL* in combination with a translocation is observed in approximately 20% of the cases with t(4;11) and t(9;11), which leads to worse course of disease compared to those without deletion (Corral et al., 1993; Kobayashi et al., 1993).

11q23 abnormalities occur predominantly in pediatric AML (FAB type M5) and *MLL* rearrangements are frequently associated with monoblastic leukemias. Abnormalities in this region can occur very early in hematopoietic stem cell development. Due to strong prognostic impact patients without known recurrent translocations, such as t(8;21) and inv(16), should be investigated by FISH for *MLL* rearrangements. We would also like to highlight that immunophenotyping is as important as molecular (cyto)genetic analyses as both can complete each other. The translocation partners for 11q23 are numerous and markedly heterogeneous, thus, additional molecular methods may be needed to further assess the partner genes for *MLL*. Also RT-PCR might be suitable to detect the most frequently observed *MLL* fusion transcripts.

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MOLECULAR CYTOGENETICS

CASE REPORT Open Access

Molecular characterization of the rare translocation t(3;10)(q26;q21) in an acute myeloid leukemia patient

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Abstract

Background: In acute myeloid leukemia (AML), the MDS1 and EVI1 complex locus - *MECOM*, also known as the ecotropic virus integration site 1 - *EVI1*, located in band 3q26, can be rearranged with a variety of partner chromosomes and partner genes. Here we report on a 57-year-old female with AML who presented with the rare translocation t(3;10)(q26;q21) involving the *MECOM* gene. Our aim was to identify the fusion partner on chromosome 10q21 and to characterize the precise nucleotide sequence of the chromosomal breakpoint.

Methods: Cytogenetic and molecular-cytogenetic techniques, chromosome microdissection, next generation sequencing, long-range PCR and direct Sanger sequencing were used to map the chromosomal translocation.

Results: Using a combination of cytogenetic and molecular approaches, we mapped the t(3;10)(q26;q21) to the single nucleotide level, revealing a fusion of the *MECOM* gene (3q26.2) and *C10orf107* (10q21.2).

Conclusions: The approach described here opens up new possibilities in characterizing acquired as well as congenital chromosomal aberrations. In addition, DNA sequences of chromosomal breakpoints may be a useful tool for unique molecular minimal residual disease target identification in acute leukemia patients.

Keywords: AML, *MECOM*, Chromosomal microdissection, Next-generation sequencing, Molecular marker

Background

EVI1 is one of several protein isoforms encoded by the *MECOM* locus at human chromosome 3q26 that also yields the MDS1 and MDS1-EVI1 protein isoform [1]. The role of MDS1 and MDS1-EVI1 in malignancy is still unclear, though the EVI1 transcription factor plays an essential role in the proliferation and maintenance of hematopoietic stem cells [2]. Aberrant EVI1 expression occurs in approximately 8% of patients with *de novo* acute myeloid leukemia (AML) [3]. The overexpression of *EVI1* can be achieved not only through rearrangements of band 3q26 but also without the presence of 3q26 abnormalities, therefore indicating that other mechanisms can lead to *EVI1* activation [4-6]. Moreover, a substantial number of patients with 3q26 rearrangements do not express *EVI1* [7]. In approximately 2% of AML cases, inv(3)(q21q26)/t(3;3)(q21;q26) is observed, where it has been suggested that the promoter of the house-keeping *RPN1* gene could be responsible for the activation of *EVI1* [8]. Other *EVI1* rearrangements include, e.g. 7q21 (*CDK6*), 7q34 (*TCRB*), 12p13 (*ETV6*) and 21q22 (*RUNX1*) [6,9]. Even though partner chromosomes and molecular consequences differ between various types of *EVI1* rearrangements, elevated expression predicts poor prognosis for the affected patients [4,10,11].

Here we report the rare case of chromosomal translocation t(3;10)(q26;q21) involving *MECOM*. Using modern cytogenetic and molecular biological techniques we were able to characterize the nucleotide sequence of this breakpoint and thus identify the fusion partner on chromosome 10.

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Case presentation

A 57-year old female was diagnosed with AML (FAB M2) after a blood cell count and bone marrow examination was initiated in June 2013. Hematologic parameters were as follows: hemoglobin 6,2 g/dl, platelets 44 × 10^9/l, and white blood cells (WBC) 3,34 × 10^9/l with 7,8% neutrophils, 62,9% lymphocytes and 28,7% monocytes, 0% eosinophils and 0,3% basophils. A bone marrow aspirate revealed slightly hypercellular marrow with normocellular particles. Megakaryocytes were found in reduced density. There was significant hiatus leucaemicus with evidence of medium-sized blasts with poor basophilic cytoplasm and distinct granulation. Flow cytometry performed on the bone marrow revealed 31% myeloid-appearing blasts with expression of CD34 and CD117, and confirmed the diagnosis of AML.

Conventional cytogenetic analysis of a 24-h culture, performed on bone marrow cells by standard techniques and evaluated by G-banding, revealed a balanced t(3;10)(q26;q21) in 20/22 metaphases. Involvement of the *MECOM* gene was confirmed by FISH with the use of a commercially available probe set.

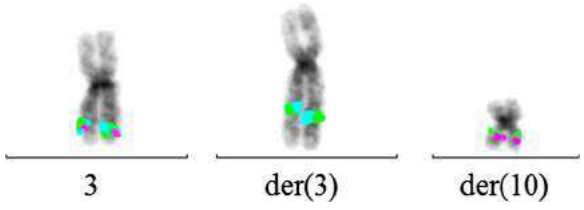

Figure 2 FISH analysis. Metaphase-FISH analysis using EVI1 break-apart probe shows normal fusion signal on chromosome 3 (green, purple, blue) and split-signal on der(3) (green, blue) and der(10) (green, purple) indicating rearrangements of 3q26 region.

Results

Cytogenetic and molecular-cytogenetic analyses of bone marrow cultures revealed an aberrant karyotype 46,XX,t(3;10)(q26;q21) – Figure 1. A commercial EVI1 break-apart probe yielded a split signal in all dividing and 80% of the interphase bone marrow cells, demonstrating the rearrangement of the 3q26 chromosomal region (Figure 2).

Ten derivative chromosome 10 breakpoint regions were dissected, amplified and sequenced. In total, 81 753 reads were obtained and aligned to reference sequences of chromosomes 3 and 10 (NCBI build 37.3). Long-range PCR primer design resulted in a product that was then subjected to Sanger sequencing. The nucleotide sequence of the der(10) breakpoint (Figure 3) revealed a fusion of the *MECOM* gene on 3q26 to *C10orf107* on 10q21.

Additionally, the bone marrow sample was subjected to reverse transcription real-time PCR analysis to determine the expression levels of cEVI1 (i.e., the sum of all *EVI1* mRNA variants) relative to those of the internal reference gene *ABL*. We found that *EVI1* expression was 26-fold higher when compared with healthy control (data not shown).

Discussion

In the present report we describe a rare case of acute myeloid leukemia with a t(3;10)(q26;q21) translocation involving *MECOM*. To our knowledge [12], only one case with this translocation has been reported [9], but the fusion partner on chromosome 10 was not characterized. Using a novel technical approach we were able to identify the fusion partner and precise nucleotide sequence of the breakpoint, which may serve as a patient-specific molecular target for subsequent real-time PCR-based minimal

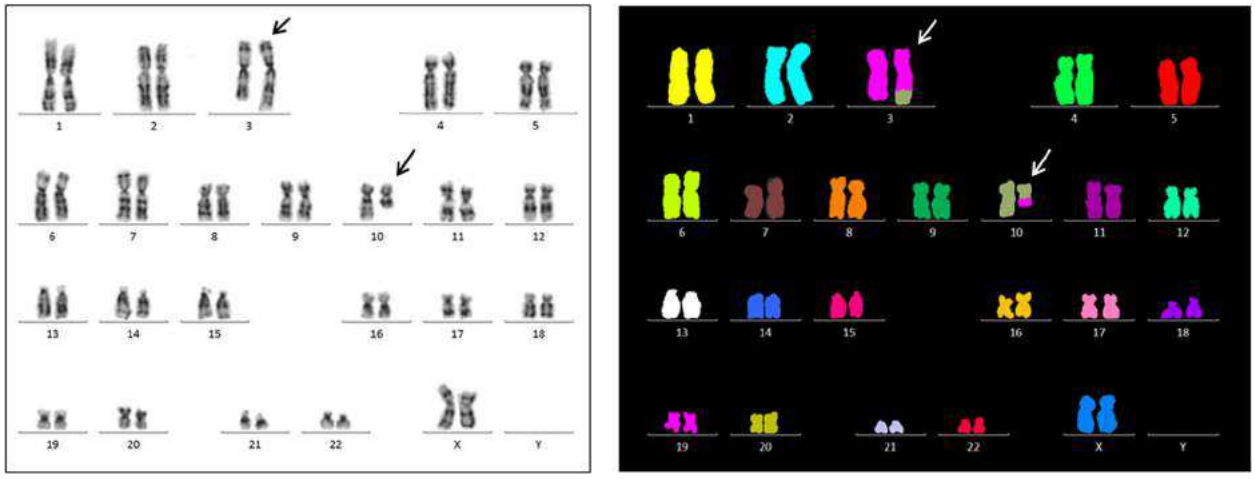

Figure 1 Karyotype analyses. G-banding (left part) and multicolor FISH (mFISH) (right part) analyses showed aberrant karyotype 46,XX,t(3;10)(q26;q21). The arrows indicate the derivative chromosomes.

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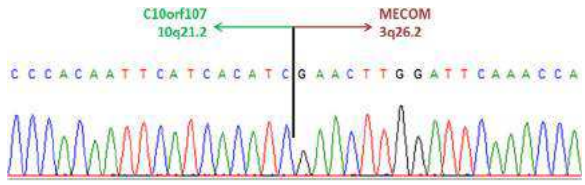

Figure 3 Derivative chromosome 10 breakpoint sequence. The electropherogram shows the result of direct sequencing of long-range PCR product which revealed fusion of *MECOM* gene on chromosome 3q26.2 and *C10orf107* on chromosome 10q21.2.

residual disease (MRD) monitoring. We further demonstrated by real-time quantitative reverse transcription PCR that the t(3;10)(q26;q21) results in *EVI1* over-expression.

Deregulated expression of *EVI1* and other genes (e.g. *BAALC*, *WT1*) involved in cell proliferation, survival and differentiation have been used as alternative MRD targets [13-16]. However, the sensitivity of expression assays is dependent on the level of initial expression; therefore, these assays are suitable only in AML cases with a high initial expression level of a specific target normalized to an endogenous control gene at diagnosis. Even in those cases, the sensitivity is usually not sufficient for subsequent MRD monitoring. Therefore, in patients presenting with a fusion transcript and/or gene mutation, a specific PCR assay is preferred. These PCR-based methods are currently the most sensitive techniques for MRD follow-up, reaching sensitivities of $10^{-4} - 10^{-5}$ [17,18].

Real-time PCR-based MRD assays allow the highly accurate quantification of residual leukemic cells and evaluations of treatment outcome in AML patients. The merit of MRD monitoring during patient's treatment and prognostic relevance has been confirmed by various studies [17,19,20]. Common targets for MRD detection include fusion transcripts (e.g. *RUNX1-RUNX1T1, PML-RARα, DEK-NUP214, CBFβ-MYH11*) [21] and mutations of clinically relevant genes (e.g. *NPM1, CEBPα, FLT3, c-KIT*) [17-22]. Unfortunately, approximately half of AML patients lack a molecular target suitable for MRD monitoring [23]. Therefore, introducing novel approaches for the identification of unique clone-specific markers is highly desirable. The procedure described here is based on characterizing nucleotide sequences of unique chromosomal breakpoints, allowing the design of a specific real-time PCR assay for MRD assessment. In this way, AML patients could benefit from accurate and sensitive MRD monitoring, even in the absence of other well-introduced molecular marker [24].

Mapping chromosome breakpoints is a conventional method for identifying specific genes in leukemic patients, as well as patients with solid tumors and individuals with balanced translocations [25-27]. A fundamental requirement is the ability to karyotype and precisely identify derivative chromosomes using classic karyotyping or molecular cytogenetic tools such as mFISH and mBAND analyses. Hybridization with even higher resolution, such as BAC-FISH (Bacterial Artificial Chromosome FISH) can help to narrow-down the chromosomal breakpoints further, though it is still not subtle enough to allow subsequent molecular methods to be used and to identify nucleotide sequence. There have been a number of methods proposed to address this issue, with varying strengths and weaknesses. Array-CGH has improved in resolution, allowing deletions, amplifications, and non-balanced translocations to be more precisely characterized, but array-CGH in principle cannot detect targets arising from balanced chromosomal translocations [28].

Conclusion

The combination of cytogenetic and molecular methods described here enabled us to proceed from the chromosomal level (cytogenetically identified abnormality) to the molecular level (unique DNA sequence) in a case of the novel t(3;10)(q26;q21) translocation. Using this procedure, acquired as well as congenital chromosomal aberrations can be characterized. In contrast to other mapping methods (e.g. BAC-FISH, array CGH) our technique allows the rapid mapping of chromosomal breakpoints down to the DNA sequence level and immediate elucidation of possible genes involved. This can be invaluable for studying such aberrations in a wide variety of fields, including the evolution of diseases or the genetic basis of inherited syndromes.

Methods

Cytogenetic and molecular cytogenetic analyses

The heparinized bone marrow sample was cultivated for 24 h in RPMI 1640 media supplemented with 10% fetal calf serum, penicillin/streptomycin and L-glutamine (PAA Laboratories, Austria) at 37°C/5% CO_2. Karyotype was investigated by G-banding and multiplex fluorescence *in situ* hybridization (mFISH) with the 24XCyte probe kit (MetaSystems, Germany). ISCN 2013 nomenclature was used to describe chromosome abnormalities [29]. Interphase fluorescence *in situ* hybridization (FISH) analysis was performed using a commercially available EVI1 break-apart probe (MetaSystems, Germany).

DNA/RNA isolation, reverse transcription

DNA and RNA were isolated from the mononuclear fraction of bone marrow samples at diagnosis. DNA was isolated using the MagNA Pure automatic isolator (Roche, Germany) according to the manufacturer's instructions. RNA was extracted by TRI Reagent (Molecular Research Center, USA) according to the manufacturer's recommendations. Reverse transcription was performed using the

Verso cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions.

Real-time quantitative reverse transcriptase PCR

Primers and probes to amplify and quantify *EVI1*-expression were forward: 5′ ACCCACTCCTTTCTTTA TGGACC 3′, reverse: 5′ TGATCAGGCAGTTGGAATT GTG 3′, probe: FAM - 5′ TGAGGCCTTCTCCAGGAT TCTTGTTTCAC 3′ - BHQ1. Expression was normalized against the expression of the control gene ABL. Primers and probe to quantify ABL gene were as follows: forward: 5′ TCCTCCAGCTGTTATCTGGAAGA 3′, reverse: 5′ T GGGTCCAGCGAGAAGGTT 3′, probe: FAM-5′ CCAG TAGCATCTGACTTTGAGCCTCAGGG 3′ - BHQ1. PCR conditions started with a denaturation at 95°C for 8 minutes, followed by 45 cycles of denaturation at 95°C for 20 s, annealing at 57°C for 30 s and elongation at 72°C for 30 s.

Chromosomal breakpoint identification

The cell suspension and DNA sample were treated and analyzed as previously described [24]. Briefly, regions around the breakpoints of derivative chromosomes were dissected by glass microneedles manipulated by micromanipulator using an inverted microscope (Axiovert 10, Zeiss, Germany). The microdissected fragments were directly subjected to amplification by degenerate oligonucleotide-primed (DOP) PCR and then sequenced on the GS Junior platform (Roche, Germany) for next generation sequencing. Obtained reads were aligned to reference sequences of chromosomes 3 and 10, using in-house developed software. The last mapped reads from both chromosomes were used as docking sites for primers for long-range PCR to amplify the putative breakpoint. Primers for long-range PCR were designed in Vector NTI Advance (v. 11.5, Invitrogen, USA). PCR amplification was done using the Expand Long Range dNTPack kit (Roche, Germany). The long-range PCR product was directly sequenced using Sanger sequencing to reveal the precise nucleotide sequence of the breakpoint.

Consent

Written informed consent was obtained from the patient for publication of this Case Report. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TJ, RP, LZ, DWH, LZ participated in the design of the study and carried out molecular cytogenetic and molecular genetic studies; RP designed the computer software and performed the biostatistical analysis; JS carried out the next-generation sequencing; IP, K-AK, AR performed flow cytometry analysis, collected and provided the clinical data; OAKM, NK performed chromosomal microdissection; SP designed and coordinated the study. All authors read and approved the final manuscript.

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2.11. Article .10

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MOLECULAR CYTOGENETICS

CASE REPORT Open Access

A de novo acute myeloid leukemia (AML-M4) case with a complex karyotype and yet unreported breakpoints

Walid Al-achkar[1*], Abdulmunim Aljapawe[2], Moneeb Abdullah Kassem Othman[3] and Abdulsamad Wafa[1]

Abstract

Background: Acute myelogeneous leukemia (AML) is a malignancy of the hematopoietic stem cells, for which cytogenetic analysis is still one of the most important diagnostic and prognostic tools. Still, we are far away from having seen and described all possible genetic changes associated with this kind of acquired disease.

Results: Bone marrow cells of a female patient with clinical diagnoses of AML and immunophenotypically confirmed AML-M4 were studied by GTG-banding. The later was not able to resolve all karyotypic changes and the complex karyotype was characterized in more detail by fluorescence in situ hybridization (FISH) and array-proven multicolor banding (aMCB). To the best of our knowledge, the present case is the only one ever seen with a del(5)(q14q34), a der(17)t(4;17)(p13;p13), a del(2)(p23), a der(4)t(4;7)(p13;q11.23), a der(22)t(11;22)(q23;q11.2) and two complex rearranged chromosomes 11 involving chromosomes 7 and 22 as well as 2.

Conclusions: The yet unreported breakpoints observed in this case seem to be correlated with an adverse prognosis. Overall, molecular cytogenetic studies are suited best for identification and characterization of chromosomal rearrangements in acute leukemia and single case reports as well as large scale studies are necessary to provide further insides in karyotypic changes taking place in human malignancies.

Keywords: Acute myeloid leukemia (AML), Chromosomal abnormalities, Fluorescence in situ hybridization (FISH), Array-proven multicolor banding (aMCB)

Background

Acute myelogeneous leukemia (AML) is a disease of the myeloid compartment of the hematopoietic system and is characterized by the accumulation of undifferentiated blast cells in the peripheral blood and bone marrow [1]. Cytogenetics is considered the most important independent prognostic parameter in AML [2,3]. Chromosomal abnormalities also provide useful information for monitoring residual disease [4]. Most of chromosomal abnormalities are detectable by banding cytogenetic analysis, and they occur in 55% of de novo AML in adults [5,6]. Some chromosomal aberrations in AML are recurrent and closely associated with specific cytomorphological subtypes according to French-American-British (FAB) criteria [7-10]. However, 5-10% of AML patients present with multiple chromosomal rearrangements involving three or more chromosomes. These patients usually have a poor prognosis, and it is likely that some of these rearrangements contribute to their disease progression [2].

We present a primary AML-M4 case with yet unreported translocation events including seven different chromosomes.

Results

Prior to chemotherapy treatment banding cytogenetics revealed a karyotype 46,XX,del(5q)[8]/46,XX,del(5q),der(17)t(4;17)[5]/45,XX,der(2)t(2;11),der(4)t(4;7),del(5q),-7,der(11)t(11;7;22),der(17)t(4;17),der(22)t(11;22)[9]/46,XX[1] (Figure 1) which was further specified by molecular cytogenetic studies (Figures 2 and 3). Dual-color FISH using a probe specific for BCR and ABL revealed two signals of ABL on both normal chromosome 9, one BCR signal was located on chromosome 22 and the other BCR

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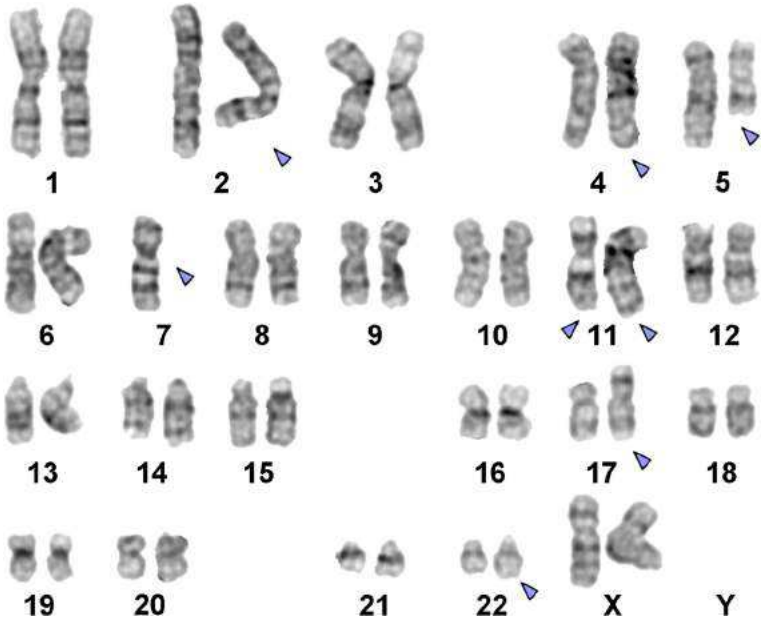

Figure 1 GTG-banding revealed a complex karyotype involving six chromosomes and monosomy 7. All derivative or clonally missing chromosomes are highlighted by arrowheads.

gene was observed on a der(11) (Figure 2A). Three-color FISH using BCR and ABL mixed with MLL probes revealed the MLL gene signal was located on the short arm of der(11), the other MLL gene signal was observed on der(22), BCR gene signal was located on der(22) and the two ABL gene signals were on the both normal chromosome 9 (Figure 2B). Dual-color FISH using WCP and CEP-specific probes were performed to confirm the rearrangement (data not shown). The locus-specific probe 17p13 (p53) confirmed the presence of TP53 on the normal position in short arm of chromosome 17 (data not shown). Finally, aMCB using probes for the corresponding chromosomes was performed as previously reported [11] (Figure 3). Thus, the following final karyotype was determined:

46,XX,del(5)(q14q34)[8]/46,XX,del(5)(q14q34),der(17)t(4;17)(p13;p13)[5]/45,XX,del(2)(p23),der(4)t(4;7)(p13;

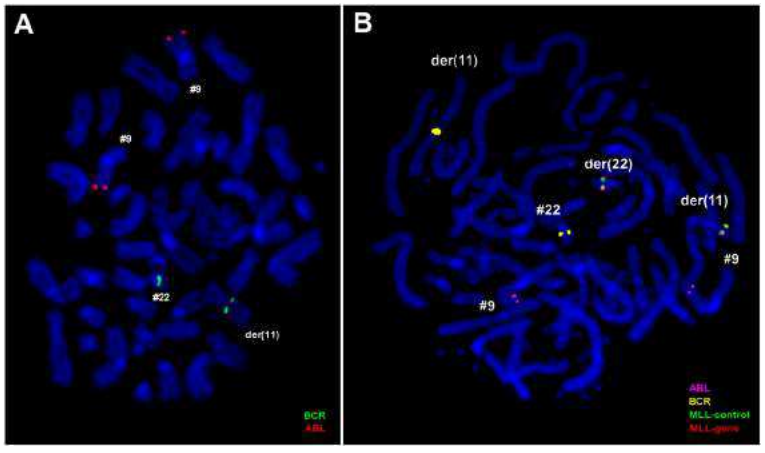

Figure 2 FISH-results using locus-specific probes. (**A**) Metaphase FISH using probes for BCR (green) and ABL (orange) showed two orange signals on the two chromosomes 9, one green on the chromosome 22 and the other green signal was observed on der(11). (**B**) Metaphase FISH using probes for BCR (yellow) and ABL (red) mixed with MLL break-apart probe showed one fusion signal was located on the short arm of der (11), the second fusion signal was observed on der(22), two orange signals on the two chromosomes 9, one green on the chromosome 22 and the other green signal was observed on der(11). Abbreviations: # = chromosome; der = derivative chromosome.

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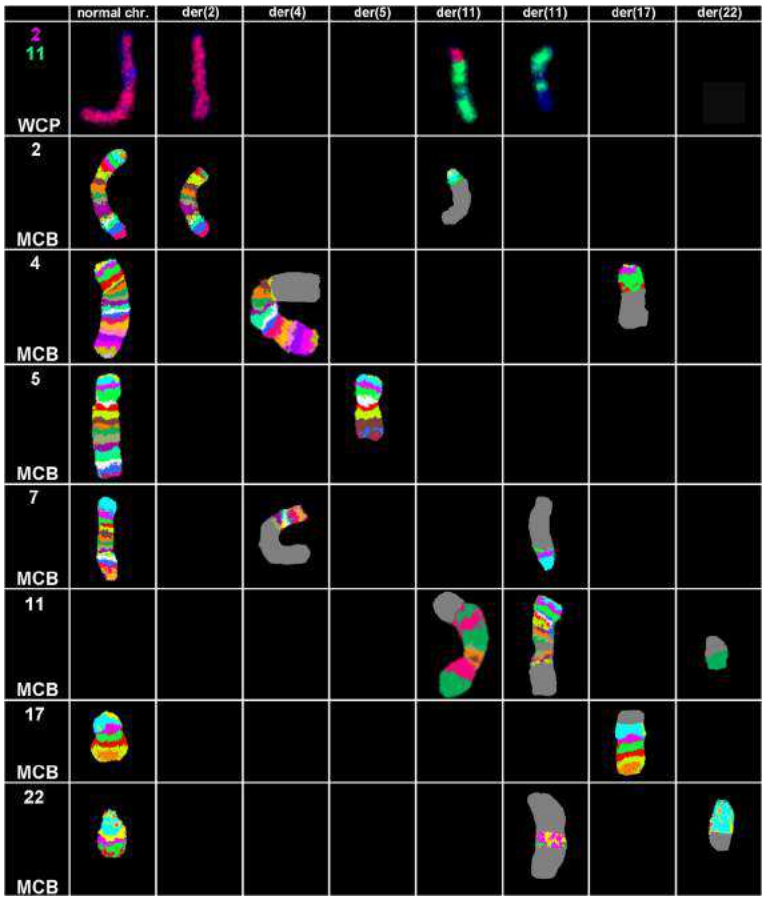

Figure 3 Array-proven multicolor banding (aMCB) was applied to determine the involved in this complex rearrangement. In each lane the results of aMCB analysis using probe-sets for chromosomes 2, 4, 5, 7, 11, 17 and 22 are shown. The normal chromosomes are shown in the first column, the derivative of all five chromosomes in the following ones. In the light gray by aMCB-probes unstained regions on the derivative chromosomes are depicted. Abbreviations: # = chromosome; der = derivative chromosome.

q11.23),del(5)(q14q34),-7,der(11)(11qter->11p11.2::11p11.2->11q23::2p23->2pter),der(11)(11pter->11q13::22q11.2->22q13.3::11q13->11q21::7p12->7pter),der(17)t(4;17)(p13;p13),der(22)t(11;22)(q23;q11.2)[9]/46,XX[1].

The abnormal cell population (57%) showed the following immunophenotype: $CD45^{+dim}$(90.4%), $HLADr^{+}$(86%), $CD117^{+}$(57%), $CD34^{+}$(57%), $CD18^{+}$(60%), $CD38^{+}$(83%) and expressed CD2 (50%), CD7(24.2%), CD13 (39%), CD33 (20%), CD123 (65%), CD15 (44%) and CD11c (52%) heterogeneously. The abnormal cells negatively reacted with antibodies to CD10, CD64, CD14, CD16, CD5 and CD19. This immunophenotype was consistent with AML-M4 according to FAB classifications.

Conclusions

We described a primary AML-M4 case with cytogenetic rearrangements involving seven different chromosomes. According to the literature, not a single case of AML showed a der(4)t(4;7)(p13;q11.23), a der(11)(11qter->11p11.2::11p11.2->11q23::2p23->2pter), a der(17)t(4;17)(p13;p13), or a der(11)(11pter->11q13::22q11.2->22q13.3::11q13->11q21::7p12->7pter) [12]. However, a t(2;11)(p23;q23) was observed in one case of refractory anemia with excess blasts-1 [12]. To the best of our knowledge, the present case is the only one ever seen case of AML with these cytogenetic aberrations [12].

The common chromosomal abnormalities in the AML-M4 include monosomy 5 or del(5q), monosomy 7 or del (7q), trisomy 8, t(6;9) (p23;q34), and rearrangements involving the MLL gene mapped at 11q23 [del(11)(q23); t(9;11)(p22;q23), t(11;19)(q23;p13)], and Core Binding Factor B (CBFβ) mapped at 16q22 [del(16)(q22), inv(16)(p13q22), t(16;16)(p13;q22)] [13]. However, in the present case both MLL genes were intact.

In general, a complex karyotype in MDS or AML is associated with a median survival of less than 1 year [11,14]. Furthermore, the adverse prognostic effect of monosomal karyotype was evident both in the presence and absence of monosomy 5 and/or 7, which

suggests that tumor suppressor or other critical genes are not necessarily clustered in specific chromosomes but are instead distributed across several chromosomes [15].

Monosomy 7 is a valuable prognostic marker in AML, and chromosome 7 defects are prominent cytogenetic lesions in primary myelofibrosis, associated with unfavorable prognosis; they present with high incidences after leukemic transformation [16]. Similarly, deletions on 7p12 of *IKZF1* gene (which encodes the transcription factor Ikaros) are associated with a very poor outcome and high relapse rate in B-cell acute lymphocytic leukemia [17]. Monosomy 7 is known as a recurrent cytogenetic aberration in approximately 10% of adult and 5% of childhood AML cases [18]. Jäger et al. [19] found two of seven myeloproliferative neoplasms patients with loss of IKZF1 had monosomy 7. This result suggests that IKZF1 may represent an important tumor-suppressor gene affected by monosomy 7 [19].

The International Prognostic Scoring System (IPSS) classifies cytogenetic and molecular genetic data in AML with clinical data into four risk groups: favorable, intermediate-I, intermediate-II and adverse [20]. The adverse prognostic groups included inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1; t(6;9)(p23;q34); DEK-NUP214; t(v;11)(v;q23); MLL rearranged; -5 or del(5q); -7; abnl (17p); complex karyotype [20].

Complex karyotypes, which occur in 10-12% of AML patients, have consistently been associated with a very poor outcome [21]. A complex karyotype has been defined as the presence of 3 or more (in some studies ≥ 5) chromosome abnormalities. For AML it turned out that the presence of t(8;21), inv(16) or t(16;16), and t(15;17) ameliorates the adverse effect of increase karyotypic complexity [20]. As indicated in the new WHO classification, cases with other recurring genetic abnormalities, such as t(9;11) or t(v;11), inv(3) or t(3;3), and t(6;9) should also be excluded from complex rearranged karyotype patient group [22], because these groups constitute separate entities. One striking observation is the increasing incidence of adverse versus favorable cytogenetic abnormalities with increasing age. This, at least in part, contributes to the poorer outcome of AML in older adults [23].

In conclusion, we reported a de novo case of AML-M4 with yet unreported translocation events involving seven different chromosomes. Taken together all findings an adverse prognosis for this specific AML-case must be considered.

Materials and methods

Case report

A 65-year-old woman was diagnosed as suffering from AML in September 2011. Anemia, thrombocytopenia, fever, fatigue and weight loss were the indicative symptoms. Her hematologic parameters were: white blood cells (WBC) of 34.2×10^9/l with 25.5% neutrophils, 36.2% lymphocytes, and 38.3% immature cells, red blood cell (RBC) count was $1.86\times10^6/mm^3$, hemoglobin level was 6.7 g/dl and the platelet count was 19×10^9/l. No treatment had been administered prior to the tests mentioned below. All human studies have been approved by the ethics committee of the Atomic Energy Commission, Damascus, Syria and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The patient gave his informed consent prior to its inclusion in this study. Later the patient was lost during follow-up.

Chromosome analysis

Chromosome analysis using GTG-banding was performed according to standard procedures [24]. A minimum of 20 metaphase cells derived from unstimulated bone marrow culture were analyzed. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature [25].

Molecular cytogenetics

Fluorescence in situ hybridization (FISH) using LSI BCR/ABL dual color dual fusion translocation probe (Abbott Molecular/Vysis, Des Plaines, IL, USA), MLL break-apart probe (Q-Biogene, USA) mixed with LSI BCR/ABL dual color dual fusion translocation probe chromosome enumeration probe (CEP) for chromosomes 9 and 11 (Abbott Molecular /Vysis) and 17p13 (p53), dual color probe (Q-Biogene, USA) were applied according to manufacturer's instructions. Whole chromosome painting (WCP) probes for chromosomes 2, 4, 5, 7, 11, 17 and 22 were also applied (MetaSystems, Altlussheim, Germany) [24]. FISH using the corresponding chromosome specific array-proven multicolor banding (aMCB) probe sets based on microdissection derived region-specific libraries was performed as previously reported [26]. A minimum of 20 metaphase spreads were analyzed, using a fluorescence microscope (AxioImager.Z1 mot, Carl Zeiss Ltd., Hertfordshir, UK) equipped with appropriate filter sets to discriminate between a maximum of five fluorochromes plus the counterstain DAPI (4′,6- diamino-2-phenylindole). Image capture and processing were performed using an ISIS imaging system (MetaSystems).

Flow cytometric immunophenotype

Flow cytometric analysis was performed using a general panel of fluorescent antibodies against the following antigens typical for different cell lineages and cell types: CD1a, CD2, CD3, CD4, CD5, CD8, CD10, CD11b, CD11c, CD13, CD14, CD15, CD16, CD19, CD20, CD22, CD23, CD32, CD33, CD34, CD38, CD41a, CD45, CD56,

CD57, CD64, CD103, CD117, CD123, CD138, CD209, CD235a and CD243; In addition to antibodies to Kappa and Lambda light Chains, IgD, sIgM, and HLADr. All antibodies purchased from BD Biosciences. Samples analyzed on a BD FACSCalibur™ flow cytometer. Autofluorescence, viability, and isotype controls were included. Flow cytometric data acquisition and analysis were conducted by BD Cellquest™ Pro software.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
WA-A, AA and AW provided the case and/or did primary cytogenetic and main part of the FISH-tests; MAKO did detailed FISH studies. WA drafted the paper and all authors read and approved the final manuscript.

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3. Discussion

As mentioned in the introduction: normal karyotypes are found in 40-50% of all AL cases exclusively studied by routine GTG-banding analysis (Mrózek et al. 2009, Walker and Marcucci 2012, Ilyas et al. 2015). However, analyses using high resolution molecular (cyto)genetic techniques lead to detection of cryptic chromosomal abnormalities (Karst et al. 2006, Tyybäkinoja et al. 2007, Gross et al. 2009, Haferlach et al. 2014). Thus, the first phase of the present work was dedicated to identify cryptic chromosomal aberrations in 103 CN-AL cases using FISH-banding technique. In the second phase a detailed characterization of newly identified tumor-associated breakpoints was done. The third part of this thesis was to study submicroscopic CNAs in AL. The fourth and final step evaluated the newly identified tumor-associated rearrangements with regard to their potential clinical relevance.

3.1. Cytogenetic analysis in the diagnosis of AL

Cytogenetic banding analysis has still been the standard method for detection of diagnostically relevant recurrent chromosomal aberrations in AL and the karyotype alone or together with other parameters is used to stratify patients into three prognostic groups: favorable, intermediate and unfavorable. For instance, APL-patients with a favorable prognosis due to presence of a translocation t(15;17) with the well-known PML-RARA rearrangement are treated by ATRA- and anthracycline- or ATRA and arsenic trioxide-based protocols. Other AL-patients may have an unfavorable prognosis in connection with a Ph-translocation t(9;22), 11q23 alterations, monosomic and/or complex karyotypes; such patients need intensive protocols and/or allogeneic bone marrow transplantation during their first remission (Grimwade et al. 2010, Kayser et al. 2012, Ferrara and Schiffer 2013). As it is well known that, when using banding karyotyping, about 40-50% of AL patients show a cytogenetically normal karyotype, such patients are categorized as having an intermediate prognosis (Mrózek et al. 2009, Walker and Marcucci 2012, Ilyas et al. 2015). This takes into consideration that cryptic chromosomal aberrations may be missed due to: (i) limited sensitivity of chromosomal banding techniques, even in case of good chromosomal morphology, the aberrations have at least 10Mb in size to be visible, (ii) cryptic or masked aberrations, i.e. they are not resolvable due to a similar or identical GTG-banding pattern and/or poor chromosome morphology, and (iii) `bad metaphases', which may be difficult to obtain and to be evaluated, as chromosomes may not be well-spread, clumsy or appearing as fuzzy with indistinct margins; thus even numerical aberrations may be missed (**articles 2, 3, 5, 6,** Karst et al. 2006, Mrózek et al. 2009). In cases according to banding cytogenetics normal

karyotype, repeated chromosomal analysis is obviously not suited for disease monitoring (Murphy and Bustin 2009, Polampalli et al. 2011).

3.2. Molecular cytogenetics studies of CN-AL cases

Molecular cytogenetic approaches have shown their ability to uncover and detect cryptic chromosomal aberrations since more than 2 decades and also in this work (**articles 2, 3, 5, 6, 7, 8**, Karst et al. 2006, Mrózek et al. 2009). Besides metaphases also interphase nuclei can be useful for diagnostics. In case of low mitotic index, alterations can also be detected and/or monitored in non-dividing cells and low mosaics level can be easily detected by that approach (Inaba et al. 2013, Woo et al. 2014). Such studies were also used successfully for determination of mosaic levels in the present work (**article 7**).

Nowadays, FISH using locus-specific and chromosome enumeration probes is a routine technique for classification, risk stratification and predication of therapy. In (**article 1**) we reviewed the effectiveness of FISH technique in cancer diagnosis and particularly in leukemia. FISH approaches are especially suited to characterize chromosomal breakpoints, submicroscopic copy number changes and fusion genes due to translocations or other rearrangements. All these features are characteristically found as acquired aberrations in AL.

3.2.1. Detection of new chromosomal aberrations

To identify yet unreported acquired chromosomal aberrations in 61 CN-ALL and 42 CN-AML cases were studied by the whole genome oriented FISH-banding based probe set mMCB (Weise et al. 2003); results are summarized in **articles 2, 3, 5, 6, 7, 8 plus yet unpublished data**. Overall, balanced and unbalanced translocations, derivative chromosomes, isochromosomes, insertions, interstitial deletions, inverted duplications and/or numerical aberrations were identified in CN-ALL and CN-AML cases. It could be confirmed that mMCB probe set provides an optimal possibility to detect and characterize simultaneously all subregions in each human chromosome and for the analyses of inter-and intra-chromosomal rearrangements of the whole human karyotype in one single experiment with a resolution between 3-10 Mb. Still the sensitivity of mMCB is dependent on sizes of rearranged fragments and labeling of the underlying partial chromosome painting probes. Based on the aforementioned range of resolution it is logical to state that mMCB is not suited to detect submicroscopic aberrations smaller than 3 Mb. Thus, iFISH probes, LSPs, MLPA and array-CGH were applied additionally in the studied AL-cases. A major restriction of this kind of comprehensive analyses is the large amount of routine material needed. Nonetheless,

this kind of problem is well-known in tumor cytogenetic studies (Weise et al. 2003, Liehr 2009, Heller et al. 2004).

In the retrospectively studied of 61 CN-ALL cases, chromosomal abnormalities were identified in 34% (21/61) and new clonal cryptic rearrangements were found in 9/21 (43%) cases. Interestingly most of those originally considered as patients with a CN-AL had in reality complex karyotypes. Chromosomes 2, 3, 4, 5, 6, 7, 9, 10, 11 and 14 were most frequently involved in structural abnormalities. Data published in **article 2** revealed a single cryptic and complex rearrangement for chromosome 11 involving a reciprocal translocation and an inversion, in **article 3** a complex four-way translocation involving the chromosomes 3, 5 and 10, in **article 5** a balance three-way translocation including chromosomes 2, 9 and 18 and in **article 6** an inverted duplication on a chromosome 14 leading to an *IGH@* locus splitting and rearrangement were reported. To the best of our knowledge these rearrangements have not been seen in ALL before (Cancer Genome Anatomy Project (CGAP); Atlas of Genetics and Cytogenetics in Oncology and Hematology; **article 7**, Table 1). Still, the majority of CN-ALL cases (66%) presented with a normal karyotype after mMCB-analysis (**article 7**). To data, only few comparable studies are available using FISH-banding techniques to screen for cryptic chromosomal aberrations and to define the novel chromosomal rearrangements. A study conducted by Karst et al. (2006) used mMCB probe and detected acquired cryptic chromosomal aberrations after G-banding analysis in 57% of ALL cases. Recently, a few similar studies have focused exclusively on the analysis of cases presenting with complex karyotypes (Al-Achkar et al. 2010, Ney Garcia et al. 2015).

Several groups have applied mFISH such as SKY/M-FISH to clarify the karyotypes and characterize the composition of marker chromosomes or incomplete identified karyotypes (Rowley et al. 1999, Mathew et al. 2001, Elghezal et al. 2001, Lu et al. 2002, Nordgren et al. 2002, Poppe et al. 2005, Mkrtchyan et al. 2006). Rowley et al. (1999) did not find any cryptic abnormalities in 5 T-ALL cases with normal karyotype and clarified already known chromosomal rearrangements in 3 cases. Additionally, Nordgren et al. (2002) demonstrated that, SKY and LSPs could identify chromosomal aberrations in up to ~80% of ALL cases. Altogether, SKY/ M-FISH failed to detect any cryptic chromosomal abnormalities of CN-ALL cases but, of course, could refine the result of most known chromosomal rearrangements. Still, mFISH technique has limitations because they enable to detect most of intra-chromosomal abnormalities such as interstitial deletions and inversions and inter-chromosomal anomalies >5 Mb (Rowley et al. 1999, Mathew et al. 2001, Elghezal et al. 2001, Lu et al. 2002, Nordgren et al. 2002, Karst et al. 2006).

More specifically analyzing the data obtained here for CN-AML cases, chromosomes 9 and 11 were found most frequently involved in structural abnormalities, and chromosome 7 in numerical abnormality (**article 8 and yet unpublished data**). This kind of chromosomal aberrations was recognized in 12/42 (28%) and rare clonal rearrangements were observed in 2/42 (5%). This is in agreement with the study done by Gross et al (2009) who applied FISH-banding technique and identified cryptic chromosomal abnormalities in 2/26 CN-AML cases. As well, Zhang et al. (2000) used SKY and found clonal abnormalities in 2/28 of CN-AML cases. In **article 8** a rare cryptic three way translocation between chromosomes 10, 19 and 11 and deletion of the 3' *MLL* gene was reported by us.

However, in the present work, there is one additional limitation of mMCB technique to be discussed here. Due to florescence interference of labeled subregions in each chromosome with same/ similar colors ambiguously identification of submicroscopic translocations, cryptic deletion, small amplification and cryptic insertion may happen (**articles 2, 3, 5, 6, 7, 8 plus yet unpublished data,** Karst et al. 2006, Gross et al, 2009). To overcome this problem, the overwhelming majority of aberrations detected here were confirmed using MCB and/or LSP analysis e.g. to clearly distinguish unbalanced translocations from balanced ones. Additionally, aCGH and MLPA, which use genomic DNA, are powerful tools in the analysis of unbalanced chromosomal rearrangements such as CNA gains and losses particularly in leukemia. Overall, iFISH, MLPA and aCGH could be the methods of choice when the mitotic index is low and the quality of metaphases is suboptimal (Usvasalo et al. 2009, Yasar et al. 2010).

Summary: The present work detected new clonal abnormalities using high resolution FISH-banding technique in 103 AL cases reported previously to have a normal karyotype according to G-banding.

3.2.2. Further characterization of newly identified breakpoints

Delineation of mMCB results and definition of the breakpoints, either balanced or imbalanced ones (losses or gains), was done using either MCB and/or (to do in more detail) large numbers of LSPs (BACs and commercially available probes) for the target sequences; for more details see **articles 1-10.** As the whole human genome has been sequenced and human sequences are harbored in BAC clones they can easily be used as FISH probes. Thus, breakpoints could be narrowed down and candidate genes could be determined on the molecular level using genome browsers. In this work, besides the CN-AL cases, seven AML and one ALL with

complex karyotypes were also studied in detail to characterize their breakpoints (**articles 4, 9, 10 and yet unpolished data).** Interestingly, in the case published in **article 9** a rare translocation t(3;10)(q26;q21) was detected. Other technical approaches including NGS, long-range PCR and direct Sanger sequencing were used to map this chromosomal translocation in detail through co-work with a partner laboratory in Prague (Czech Republic). Thus, nucleotide sequence of the breakpoint revealed a fusion of the *MECOM* gene on 3q26 to C10orf107 on 10q21. Aberrant expression of *MECOM* gene results in disturbance of the normal proliferation and differentiation of HSC and finally leads to maturation arrest (Balgobind et al. 2010). In **yet unpublished data** *MECOM* gene rearrangements were also identified in two further AML cases. One case had an unbalanced translocation der(3)t(3;7)(q26.2q21.2). LSPs and aCGH revealed *MECOM-CDK6* fusion gene. The cyclin dependent kinase 6 (*CDK6*) is disrupted or overexpressed by translocation in hematological malignancies, particularly in T-ALL and T cell lymphoblastic lymphoma, whereas the variant translocation t(3;7)(q26;q21) is less frequently reported in myeloid leukemia (Lien et al. 2000, Raffini et al. 2002, Storlazzi et al. 2004). The second case had a balanced translocation t(3;8)(q26.2;q24.2). Few such cases have been reported in the literature with *PVT1-MECOM* fusion gene and associated with loss of chromosome #7 as also observed in our CN-AML case (Mitelman et al. 2015). *PVT1* is an oncogene and contains a long non-coding RNA. The role of *PVT1* in leukemogenesis still is unclear, thus, aberrations in *EVI1* may leads to deregulated expression, similar to other balanced or unbalanced chromosomal translocations involving chromosome 3q26 (Tseng et al. 2014, Lennon et al. 2007). However, overexpression of *MECOM* indicates for unfavorable prognosis in AML (Haferlach et al. 2012).

MLL gene (11q23) was identified most frequently rearrangements in the present work; in AML, the *MLL* partner genes were *MLLT3* (9p21.3), *MLLT4* (6q27) and *MLLT10* (10p12.3) while in ALL the partner genes were *MLLT2* (4q21) and *MLLT10*. In **article 8** (Table 1) chromosomal breakpoints were narrowed down for a rare three-way translocation as 10p12.31, 11q23.3 and 19q13.31, and the breakpoints of the altered Y-chromosome as Yp11.2 and Yq11.23. Additionally, 3'MLL was deleted and aCGH confirmed the deletion between 118,394,728-118,952,688 according to GRCH37/hg19. Commonly, the cryptic insertion of *MLL* gene within partner genes cannot be detected by G-banding and rarely identified by mMCB. In **article 2** (Table 1) the corresponding breakpoints were narrowed down and defined to be 11p15.4 and 11q24.2 on both homologous chromosomes 11. Besides, cryptic insertion of 5'MLL gene into the *AFF1* gene in chromosome 4q21 was detected only

by a dual-color break apart probe. In **yet unpublished data** two further cases were identified with cryptic *MLL*-gene insertions; an infant with B-ALL had an ins(10;11)(p12.3;q23.3) and in this case *3'MLLT10* was inserted into *MLL* gene. This variant translocation t(10;11)(p12;q23) has been frequently observed in young children with AML and very rarely with ALL (Lillington et al.1998, Coenen et al. 2011). The second case was an adult AML subtype M5 and identified insertion ins(6;11)(q27;q23). The translocation t(6;11)(q27;q23) is frequently seem in AML and could be detected by G-banding. To best our knowledge, yet only one case has been reported with an insertion of chromosome 11q13q23 into chromosome 6 in an adult AML subtype M4 (Mitelman et al. 2015, Martineau et al. 1998). Overall, *MLL* gene plays an essential role in normal hematopoietic growth and differentiation. Abnormalities in this region can occur very early in HSC development (Ansari and Mandal 2010, Ferrando et al. 2003) and *MLL* is important as molecular marker to be investigated in the early diagnosis of AL.

In the present work, MCB and LSPs were proven to be highly useful for refining of conventional banding karyotypes and elucidating composition of marker chromosomes or incompletely identified rearrangements. All normal and complex karyotypes fall into two main groups: such with common and such with unique breakpoints. Thus, the potential pathogenic impact of the identified breakpoints is suspected to be due to non-random chromosomal translocations, insertions, and low or high gene dosages. The consequences of these abnormalities lead to identify the gene(s) which are important for leukemia transformation in the past (Aplan 2006) and also in the present work.

Summary: Characterization of chromosomal breakpoints is required in the diagnosis of acute leukemia, to help in classification, risk stratification and prediction of therapy of the disease.

3.3. Identifications of acquired CNAs in AL

The better understanding of leukemogenesis and providing entries to therapy development, different molecular techniques for diagnostic purposes could be applied. Besides FISH, MLPA and aCGH are useful, i.e. approaches which have much higher resolution than FISH, but can only detect unbalanced aberrations and no low level mosaics. Few studies have applied MLPA and aCGH to identify CNAs in AL (Haferlach et al. 2014, Schwab et al. 2013, Strefford et al. 2007, Tyybäkinoja et al. 2007). In the present work, DNA was isolated from the cytogenetically worked up cell suspensions of 34/61 CN-ALL and 27/47 AML (42 normal and 5 complex karyotypes) cases. Cryptic CNAs were detected in ~80% and in ~63% of

those CN-ALL and AML cases respectively. This is in agreement with the study conducted by Haferlach et al. (2014) who used aCGH and, detected CNAs in 80.3% of CN-ALL cases. As well, Strefford et al. (2007) also used aCGH and demonstrated that, 83% of ALL cases had CNAs. A study performed by Tyybäkinoja et al. (2007) also applied aCGH for 26 CN-AML cases and found cryptic CNAs only in 4/26 (15%) of CN-AML. Additionally, a large study performed by Schwab et al. (2013) who used MLPA to screen for the most frequently deleted genes in high risk BCP-ALL found deletion of IKZF1, PAX5, CDKN2A/B and RB1 as also reported here in **article 7**.

All here reported CNAs have been checked by UCSC genome browser to exclude benign copy number variations (CNVs): http://genome-euro.ucsc.edu/cgi-bin/hgGateway?redirect=auto&source=genome.ucsc.edu. Thus, all of them most likely are leukemia-related genetic changes, which were recognized in 27/34 of CN-ALL and in 17/27 of AML cases. According to the result of **article 7 and yet unpublished data**, the CNAs in CN-ALL cases were identified most frequently in chromosomes #7, #9, #10, #11, #13, #15, #17, #18, #20 and #21, i.e. 8-15 CNAs per chromosomes, while in AML often in chromosomes #7, #11, and #15, i.e. 2-3 CNAs per chromosomes (Table 6.1). One of the known shortcuts of aCGH is the inability to detect reliably acquired CNAs less than ~20 Kb in size particularly in AML. A suggestion to overcome this problem is to used high-resolution SNP-array-CGH analysis (**article 7 plus yet unpublished data,** Le Scouarnec and Gribble 2012, Bullinger and Fröhling 2012).

3.3.1. CNAs expressed as losses

According to results shown in **article 7**, significant losses of CNs in CN-ALL were observed more frequently for chromosomes #7, #9, #10, #11, #13, #15, #17, #20 and #21. Furthermore, CNAs have been identified here, encompassing single or few genes, only. Chromosome 7 involved deletion of *IKZF1* at 7p12.2 in 5 of 34 (14%) studied CN-ALL cases. *IKZF1* encodes IKAROS protein that required for the development of all lymphoid lineages. Deletions and/or sequence mutations of *IKZF1* were present in 15% of pediatric B-ALL (Mullighan et al. 2009b). Besides, deletion of 7q21.2 region was observed in the present work in 4 of 34 (12%) of CN-ALL cases and mapped for *CDK6* gene. The majority of chromosome 9 abnormalities was expressed as deletions of cell cycle regulatory genes at 9p21.3 in 8/34 (24%) of the studied CN-ALL cases. *CDKN2A*/B genes deletion can be detected at initial diagnosis or acquired at relapse, suggesting that *CDKN2A/B* gene deletion is a secondary genetic event (Schwab et al. 2013, Kim et al. 2011, Sulong et al. 2009).

Table 6.1. Summary of the most common recurrent CNAs in AL detected by aCGH and MLPA in the present work

CN-AL	type of CNA	chromosome	band	gene	no. of cases
CN-ALL	Loss	7	p12.2	*IKZF1*	5
			q21.2	*CDK6*	4
		9	p21.3	*CDKN2A/B*	8
			p13.2	*PAX5*	3
		10	q23.31	*PTEN*	6
		11	q14.2	*PICALM*	3
		13	q14.2	*RB1*	3
		15	q26.1	*CHD2*	7
		17	p13.1	*TP53*	4
		20	q13.2	*ZNF217*	4
		21	q22.2	*ERG*	2
	Gain	18	q21.2	*DCC*	3
AML	Loss	7	-7/del(7q)	-----	4
		11	q23.3	*MLL*	2
		15	q26.1	*CHD2*	2
	Gain	8	q24.2	*MYC*	1

Additionally, deletion of *PAX5* gene located in 9p13.2 was found in 3/34 (9%). Deletion of *PAX5* was reported in 31.7% of B-ALL and also it has been involved in several chromosomal translocations. In future, *PAX5* could be used as one of the molecular markers in diagnosis and monitoring of the disease, especially in B-ALL (Schwab et al. 2013, Nebral et al. 2009, Mullighan et al. 2007). Deletion of *PTEN* gene at 10q23.31 was detected in 6/34 (17%) of the studied CN-ALL cases. Deletion of the tumor suppressor gene *PTEN* leads to activation of the PI3K/AKT pathway and in subsequent increase in protein synthesis, cell cycle progression, migration, and survival. Consequently, deletion of *PTEN* trends to poor outcome. Recently, numerous targeting drugs for the PI3K/AKT pathway for the therapy of cancer have entered in clinical trials (Zhao et al. 2013, Ciuffreda et al. 2014, Mendes et al. 2014). In chromosome 13 was found deletions involving *RB1* gene at 13q14.2 in 3/34 (9%) of the studied CN-ALL cases. Deletion of *RB1* gene is highly frequent observed in B-CLL but rarely seen in ALL. Thus, *RB1* pathway was identified as potential targets for therapy of ALL (Schwab et al. 2013, Cavé et al. 2001).

Interestingly, a novel recurrent submicroscopic CNA expressed as loss of 15q26.1: focal deletion of *CHD2* gene located there was found in 7 of the 34 (20%) CN-ALL and in 2 of the

27 (7%) AML studied cases. In chromosome 17 deletion of TP53 gene at 17p13.1 was identified in 4/34 (12%) of studied CN-ALL cases. Deletions and sequence mutations of *TP53* gene associated with non-response of chemotherapy and unfavorable outcome in ALL (Hof et al. 2011, Stengel et al. 2014). Recurrent deletion was found also in 21q22.22 targeting exclusively *ERG* in 2/34 of studied CN-ALL cases. *ERG* gene is a transcription factor which belongs to the erythroblast transformation-specific (ETS) family. It has a key regulatory role in hematopoietic differentiation during early T and B cell development. Overexpression of *ERG* gene was shown in AML and T-ALL and was associated with poor prognosis. Currently, deletion of *ERG* gene is associated with a very good outcome in older children and young patient with BCP-ALL (Clappier et al. 2014, Marcucci et al. 2005).

Besides, many of CNAs losses involving transcriptional regulators and co-activators genes like 3q26.32 (*TBL1XR1*; n = 1), 12p13.2 (*ETV6*; n = 2), and 21q22.12 (*RUNX1*; n= 1), or regulators of chromatin structure and epigenetic regulators genes like 16p13.3 (*CREBBP*; n = 2) were identified - for more information refer to **article 7**.

In AML losses of CNAs were observed less frequent than in CN-ALL (**yet unpublished data**). Overall, CNAs were found most often in chromosomes #7 and #11. Recurrent loss of the chromosome 7 and 7q was recognized in 4/27 (15%) of studied AML cases. Loss of -7/del(7q) leads to leukemic transformation due to loss of function of such putative tumor suppressor gene in these regions that regulates myeloid growth and differentiation and associated with adverse outcome (Hosono et al. 2014, Braoudaki and Tzortzatou-Stathopoulou 2012). In chromosome 11 deletion of 11q23.3 including 3' *MLL* gene was detected in 2/27 (7%) of the studied AML cases; one case had a translocation t(10;19;11)(p12;q13;q23) and the second had a translocation t(9;11)(p21.3;q23.3). The fact that both patients died in short time after HSCT might be due to presence of *MLL* gene rearrangements **(article 8 and yet unpublished data)**.

3.3.2. Gains

Gains of CNAs in CN-ALL and AML were found less frequent than losses. Gains of CNAs according to **article 7** were seen in ~20% of CN-ALL studied cases. Interestingly, duplication of *DCC* gene in 18q21.2 was present in 3 of the 34 (9%). However, oncogene overexpression resulting from gene duplication is infrequent in ALL. Still, we found *MYB* and *ABL1* amplification in one case. In **yet unpublished data** of AML studied cases, gain of CNs was found in ~28% of the cases. Remarkable, amplification of *MYC* oncogene was detected in one of studied AML cases. *MYC* gene amplification was previously observed in approximately

1% of the AML and MDS cases and outcome still is unclear (Storlazzi et al. 2006, Tyybäkinoja et al. 2007, Mrózek 2008).

3.3.3. New candidate genes

Besides the confirmation of involvement of yet rarely reported genes in AL also three new candidate genes were identified in the present study.

CDK6 gene at 7q21.2 is the catalytic subunit of a protein kinase complex that regulates cell cycle G1 phase progression and G1/S transition. Deletion of *CDK6* was identified in this study in 4 of 34 (12%) of CN-ALL cases. It has been shown recently that inhibition of CDK6 may lead to overcome the differentiation block seen in AML with *MLL* translocations (Placke et al. 2014). Thus, further studies for this gene may also be recommended for better understanding of ALL biology.

CHD2 gene was found to be heterozygously deleted in 7 of CN-ALL and 2 of AML cases. The *CHD2* gene is a member of the chromodomain helicase DNA-binding (CHD) protein family, which are all characterized by a chromatin-remodeling domain (the chromodomain) and an SNF2-related helicase/ATPase domain (Carvill et al. 2013). Thus, in future it may be of interest to study *CHD2* gene deletions also for presence of mutations in this gene and also to screen ALL patients in general for *CHD2* gene mutations.

The *DCC* gene is a member of the immunoglobulin superfamily of cell adhesion molecules and acts as a transmembrane dependence receptor for netrins, key factors in the regulation of axon guidance during development of the central nerve system. Amplification of *DCC* gene was previously reported only in CLL (Derks et al. 2010, Alhourani et al. 2014), however, this is the first report for *DCC* gene amplification in ALL. To evaluate the role of the *DCC* gene and to elaborate its potential as a molecular marker in ALL still needs more studies.

Overall, combination of molecular cyto(genetic) techniques are necessary to provide comprehensive details for each clinical case (Roberts and Mullighan 2015, Ilyas et al. 2015).

Summary: High rates of CNAs were detected in CN-ALL, that mean all cases hold detectable cryptic genomic aberrations, whereas AML cases showed lower rate of CNAs and most likely hold more point mutations or epigenetic changes in relevant genes. Thus, besides here used approaches DNA sequencing and SNP-array-CGH may be necessary to be used for mutation detection in AL.

3.4. Correlations with clinical data of patients

Chromosomal alterations and breakpoints in CN-AL and/or in complex rearranged AL could be assessed in this work for 58% of the cases. As well known from literature, at diagnosis of AL different prognostic factors besides cytogenetics need to be investigated quickly and cost-effectively such as age, gender, WBC count, cytomorphology of leukemic cells and immunophenotype (Pui et al. 2003, Burmeister et al. 2009, Döhner et al. 2010, Vardiman et al. 2009). Thus, the best way to evaluate the prognostic significant of each cytogenetic abnormality may be different. For instance, one of the well characterized recurrent chromosomal abnormalities in AL is the *MLL* (11q23) gene rearrangements which occurred in 10% of ALL cases overall, the majority being infant B-ALL (<1 year of age). Up to 93% of affected infants under the age of 90 days harbor *MLL* rearrangements such as translocations t(4;11), t(11;19), or t(1;11), and most of these children cannot be rescued with the currently available therapies. On the other hand *MLL* is involved in 30%–50% of childhood and in 5% of adult AMLs. However, cytogenetic abnormality of *MLL* gene predicts a different outcome depending on the disease phenotype (**articles 2, 8,** Balgobind et al. 2011, Chowdhury and Brady 2008). It has been proposed that infant leukemia with and without *MLL* gene rearrangements are different diseases with different clinical characteristics and different responses to therapy (Tuborgh et al. 2013). The first indication on *MLL*-gene involvement in infant leukemia (<10%) is the presence of skin lesions which so called leukemia cutis. Thus, a skin biopsy can be the first screen for the presence of leukemic blast cells. BM aspiration revealed either lymphoblasts or monoblasts and the immunophenotyping of routinely processed BM specimens is very helpful in establishing the diagnosis of AL (Cho-Vega et al. 2008, Vardiman et al. 2009), however, 11q23 abnormalities occur predominantly in AML (FAB types M5 and M4), high WBCs count and frequently associated with monoblastic cells, whereas in ALL highly associated with BCP-ALL, high WBC counts and CD10 negative and CD15 positive. All of these clinical parameters still to be considered at the diagnosis of AL because *MLL* rearrangements are easily to missed by G-banding, associated with unfavorable outcome in most cases and need correct therapeutic decision (**article 2, 8,** Pui et al. 2003, Burmeister et al. 2009, Döhner et al. 2010, Vardiman et al. 2009).

In the present work, three BCP-ALL cases with normal karyotype and four AML-M5 cases were studied; one normal and three complex karyotypes were identified including *MLL* gene rearrangements according to the above criteria (**articles 2, 8 and yet unpublished data**). Overall, detection or exclusion of *MLL* disruption or amplification is extremely necessary for treatment decisions, as well as for basic research enabling new insights into possible fusion

genes involving *MLL*. According to FISH the translocation partners for 11q23 are numerous and markedly heterogeneous. Thus, to detect the lower level of clonal abnormality, an additional molecular method may be needed such as RT-PCR to further evaluate the MLL partner fusion genes.

Detection of specific recurrent chromosomal abnormalities such as deletion of CDKN2A/B can be evaluated. Deletions of CDKN2A/B (9p21.3) can be found in 30-50% of ALL as also were only found in the present work in 24% of studied CN-ALL cases. Deletions of CDKN2A/B result in inactivation of genes in this locus, mainly p16 and p15, suggesting in inactivation of these genes that contribute to leukemogenesis. The outcome of cases with CDKN2A/B deletion depends on the status of the second allele, as homozygous deletions are associated with poor outcome and heterozygous deletions represent markers for favorable outcomes (**article 5, 6, 7,** Schwab et al. 2013, Zhao et al. 2013, Kim et al. 2011, Sulong et al. 2009).

All cases were described here in this work with complex karyotypes in **article 3, 4, 5, 8, 10 and yet unpublished date,** were associated with adverse prognosis and with maximal overall survival rate less than 2 years. This is in agreement with the study conducted by Moorman et al. (2007) who observed patients with complex karyotype had an unfavorable outcome and relapses occurring in the first 2 years after diagnosis. Actually, this subgroup does not appear to be associated with age, gender, or WBC count, as well with immunophenotype. Most of the abnormal chromosomes in complex karyotypes were identified as unbalanced and balanced translocations (**article 3, 4, 5, 8, 10 and yet unpublished date),** the unbalance translocations that was reported in **article 3,** suggesting that, activation of such oncogenes in 5q31.1 (IL3) and in 10p12.3 (MLLT10) are important in leukemogensis and associated with poor outcome. Thus, in AL cases with complex karyotypes, patients require intensive chemotherapy and allogeneic bone marrow transplantation during their first remission (Moorman et al. 2007, Mrózek 2008, Kayser et al. 2012, Ferrara and Schiffer 2013). Only, one case presented here in **article 6** was observed with a good prognosis and with overall survival till date after first diagnosis for 4 years with CR and without signs for MRD.

Summary: *MLL* gene rearrangements should be considered and tested by molecular approaches in case of a normal cytogenetic particularly, BCP-ALL with CD10-negative and high WBC count as well in AML with subtypes M5 and M4.

4. Conclusions and outlook

The present work highlights that most if not all of CN-AL cases hold cryptic genomic alterations. Overall, sensitive methods to detect cryptic chromosomal / genetic aberrations in CN-AL are useful and necessary for genetic risk-based classification and correct determination of treatment protocols.

Molecular cytogenetic approaches together with molecular methods are suited to identify cryptic rearrangements and potential target genes that involved in leukemogenesis and progression of the disease. The present work demonstrated that aCGH is a highly efficient tool for detection of CNAs in CN-ALL. However, while aCGH (and MLPA) provide data on imbalanced genomic alterations, (molecular) cytogenetics additionally detects different leukemic subclones within one sample, as well as balanced translocations leading to tumor-specific fusion genes. In CN-AML, DNA sequencing and SNP-array-CGH have been used to detect mutations for a number of target genes that are known to key roles in myeloid development. It seems to be valid, there is no leukemia clone without genetic alterations; we just have to use the appropriate techniques to identify them. In conclusion, to obtain a comprehensive picture of all relevant changes in each individual acute leukemia case, data from cytogenetics, FISH, MLPA, aCGH, SNP-array-CGH and DNA sequencing would need to be considered and included in diagnostics; however, sometimes such investigations may be hampered by lack of sufficient cellular material and or by financial restrictions.

Overall the questions studied in this thesis could be answered as follows:

1 How many cryptic chromosomal rearrangements were present in the 103 studied CN-AL-patients?

  In 21/61 CN-ALL and 12/42 CN-AML cases previously overlooked cryptic chromosomal rearrangements could be detected; new clonal cryptic rearrangements were found in 9/21 (43%) of CN-ALL and in 2/42 (4%) of CN-AML cases.

2 Can the (new) identified tumor-associated acquired chromosomal breakpoints in CN-AL be characterized in detail?

  83% of the overall 124 cryptic chromosomal breakpoints could be characterized in detail here by FISH alone; those include 11 new breakpoints in CN-ALL and 3 in CN-AML.

3 Can the (new) identified tumor-associated acquired chromosomal breakpoints in complex-AL cases be characterized in detail?

80% of the overall 35 chromosomal breakpoints could be characterized in detail here by FISH alone; those included 2 breakpoints in one ALL and 11 breakpoints in seven corresponding AML cases.

4 How many of the cryptic changes were submicroscopic structural CNAs detectable by MLPA and array-CGH?

79% of the overall 155 cryptic chromosomal breakpoints could be characterized in detail here by MLPA and or aCGH; those include 3 new candidate genes for ALL and 1 for AML: *CDK6* (7q12.2), *CHD2* (15q26.2) and *DCC* (18q21.2).

5 Could the new tumor-associated acquired rearrangements aligned with diagnostic, prognostic and therapeutic relevance?

All here included cases were retrospectively studied, but not for all cases the clinical data was available. Still the new three aforementioned candidate genes *CDK6*, *CHD2* and *DCC* were found only in patients with poor therapeutic response.

Even though during the last years and the present study already major progress was achieved for ALL and AML patients, still lots of work is necessary for better understanding the biology of these malignant disorders. Candidate genes need to be correlated with clinical outcomes, and it can be expected that future studies will provide more insights into mechanisms of leukemiogensis, identify novel molecular markers, lead to the development of new diagnostic tools and to new entries of therapy development.

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6. Appendix

6.1. Tables

Table 1.1 Immunological classifications of ALL

lymphoid-lineage in ALL	Expression (CD)
pro-B-ALL	CD19, CD22, CD72, CD74, CD79a, HLA-DR, TdT
common ALL	CD10, CD19, CD20, CD22, CD72, CD74, CD79a, HLA-DR
pre-B-ALL	CD10, CD19, CD20, CD22, CD72, CD74, CD79a, HLA-DR, IgM, Pax5
mature-B-ALL	CD5, CD19, CD20, CD21, CD22, CD24,CD72, CD79a, HLA-DR, IgD, IgG, IgM, TdT
pro-T-ALL	cyCD3, CD7, CD10, CD34, TdT
pre-T-ALL	CD2, cyCD3, CD5, CD7, CD10, CD34, TdT
cortical-T-ALL	CD1a, CD2, cyCD3, CD4, CD5, CD7, CD8, CD10, TdT
mature -T-ALL	CD2, cyCD3, mCD3, CD4, CD5, CD7, CD8, CD10, TCRβ

Table 1.2 Cytogenetic prognostic markers in ALL subtypes.

ALL subtypes	cytogenetic abnormality	outcome
pre-B-cell ALL	t(12;21) hyperdiploid (>50 chromosomes) *ERG* deletion	favorable
	t(1;19)	intermediate
	t(9;22) t(17;19) t(v;11)(v;q23); MLL rearranged complex karyotype hypodiploidy (<44 chromosomes) *CRLF2* rearrangements *iAMP21*	poor
	PAX5 rearrangements *ABL1* rearrangements *PDGFRB* rearrangements *JAK2* rearrangements	unkown
B-cell ALL	t(8;14) t(8;22) t(2;8)	intermediate
T-cell ALL	7q34 or 14q11 rearrangements	intermediate
all	normal karyotype	intermediate

Table 1.3 Recurrent structural chromosomal aberrations in ALL

ALL Subtypes	Aberrations	Fusion genes
Pre-B-cell ALL	t(1;19)(q23;p13)	*PBX1/E2A*
	t(4;11)(q21;q23)	*MLL/AF4*
	t(5;14)(q31;q32)	*IL3/IGH*
	t(6;11) (q27;q23)	*MLL/AF6*
	t(6;14)(q32;p22).	*ID4/IGH*
	t(9;11)(p22q23)	*MLL/MLLT3(AF9)*
	dic(9;12)(p13;p13)	*PAX5/ETV6*
	t(9;22)(q34;q11)	*BCR/ABL*
	t(10;11)(p13-14;q14-21)	*MLL/MLLT10(AF10)*
	t(12;21)(p13;q22)	*TEL/AML1*
	t(11;19)(q23;13.3)	*MLL/ENL*
	t(14;19)(q32;q13)	*IGH/ CEBPA*
	t(17;19)(q22;p13)	*HLF/E2A*
	t(X;14)(p22;q32)/t(Y;14)(p11.2;q32)	*CRLF2/IGH*
	ins(4;11)(q21;q23	*MLL /AFF1*
	ins(5;11)(q31;q13q23)	*MLL /AFF4*
	inv(11)(q13q23)	*MLL/ BTBD18*
	inv(14)(q11q32)	*CEBPE/IGH*
	inv(19)(p13q13)	*TCF3/TFPT*
T-cell ALL	t(1;7)(p34;q34)	*LCK/TCRβ*
	t(1;7)(p32;q34)	*TAL1/TCRβ*
	t(1;14)(p32;q11)	*TAL1/TCRδ*
	t(5;14)(q35;q32)	*TLX3/ BCL11B*
	t(6;7)(q23;q34)	*MYB/ TCRβ*
	t(7;9)(q34;q32)	*TAL2/TCRβ*
	t(7;9)(q34;q34)	*TAN1/TCRβ*
	t(7;10)(q24;q24)	*HOX11/TCRβ*
	t(7;11)(q34;p13)	*RHOM2/TCRδ*
	t(7;12)(q34;p12)	*TCRβ/ LMO3*
	t(7;19)(q34;p13)	*TCRβ/LYL1*
	t(8;14)(q24;q11)	*MYC/TCRα/δ*
	t(10;11)(p13;q14)	*MLLT10(AF10)/HOXA*
	t(10;14)(q24;q11)	*HOX11/TCRδ*
	t(11;14)(p15;q11)	*LMO1/TCRδ*
	t(11;14)(p13;q11)	*LMO2/TCRδ*
	t(11;19)(q23;p13)	*MLL/ENL*
	t(12;14)(p13;q11)	*CCND2/ TCRδ*
	inv(7)(p15q34)	*HOXA/TCRβ*
	inv(14)(q13q32.33)	*NKX2.1*
Mmature B-cell ALL	t(8;14)(q24;q32)	*MYC/IGH*
	t(8;22)(q24;q11)	*MYC/IgL*
	t(2;8)(p12;q24)	*MYC/Igk*

Table 1.4 Common DNA CNAs detected in ALL

gene/CNA detected	Location / Frequency	ALL subtype
del *TAL1*	1p32/20%–30%	T-ALL
del *EBF1*	5q33.3 / 2%	B-ALL
amp *MYB*	6q23.3/ 8%	T-ALL
del 6q (*TSG*)	6q16 / 20-30%	B-ALL / T-ALL
del *IKZF1*	7p12.2 / 15%	B-ALL
amp *NUP214–ABL1*	9q34.12-9q34.13 /4%	T-ALL
del *CDKN2A/B & MTAP*	9p21.3 /30%	B-ALL / T-ALL
del *PAX5*	9p13.3 /30%	B-ALL
del *PTEN*	10q23.3 /10%	T-ALL
del *ATM*	11q22.3 / 15%	B-ALL / T-ALL
del *ETV6*	12p13.2 / 13%	B-ALL / T-ALL
del *RB1*	13q14.2 / 4%	B-ALL / T-ALL
del *CREBBP*	16p13.3 / 19%	Relapsed B-ALL
del *TP53*	17p13.1 3%	B-ALL / T-ALL
amp *iAMP21* (*RUNX1*)	21q22.12 / 2%	B-ALL
del *ERG*	21q22.2 / 4%	B-ALL
del *CRLF2*	Xp22.2 / 5%	B-ALL

Table 1.5 Immunological classifications of AML

myeloid-lineage in AML	Expression (CD)
M0	CD7, CD13, CD33, CD34, CD38, CD45, CD117, HLA-DR
M1	CD7, CD13, CD33, CD34, CD38, CD45, CD117, HLA-DR, MPO
M2	CD7, CD11b, CD13, CD14, CD15, CD19, CD33, CD34, CD38, CD45, CD56, CD64, CD65, CD117, HLA-DR, MPO
M3	CD9, CD13, CD33, CD45, CD65, CD68, CD117, HLA-DR, MPO
M4	CD2, CD4, CD7, CD11c, CD13, CD14, CD15, CD33, CD34, CD45, CD56, CD64, CD65, CD117, HLA-DR, lyzozome, MPO
M5	CD2, CD4, CD7, CD11b, CD11c, CD13, CD14, CD15, CD33, CD36, CD45, CD64, CD65, CD68, CD117, HLA-DR, Llyzozome, MPO
M6	CD34, CD36, CD45, CD117, CD235a, GPHA, HLA-DR,
M7	CD4, CD33, CD34, CD36, CD41, CD42a, CD45, CD61, CD117, HLA-DR, VWF
acute basophilic leukemia	CD11b, CD13, CD33, CD45, CD123, CD203c, HLA-DR,

Table 1.6 WHO classification of AML

AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22), RUNX1-RUNX1T1 AML with inv(16)(p13q22) or t(16;16)(p13;q22), CEFB-MYH11 acute promyelocytic leukemia with t(15;17)(q22;q12), PML-RARA AML with t(9;11)(p22;q23); MLLT3-MLL AML with t(6;9)(p23;q34); DEK-NUP214 AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1 AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1 AML with mutated NPM1 (provisional entity) AML with mutated CEBPA (provisional entity)
AML with myelodysplasia-related features
Secondary, therapy related AML and MDS
AML, not otherwise specified
AML with minimal differentiation AML without maturation AML with maturation Acute myelomonocytic leukemia Acute monoblastic/acute monocytic leukemia Acute erythroid leukemia (erythroid/myeloid and pure erythroleukemia variants) Acute megakaryoblastic leukemia Acute basophilic leukemia Acute panmyelosis with myelofibrosis
myeloid sarcoma
myeloid proliferations related to Down syndrome
transient abnormal myelopoiesis myeloid leukemia associated with Down syndrome
blastic plasmacytoid dendritic cell neoplasm

Table 1.7 Common chromosomal abnormalities in AML subtypes

AML subtypes	cytogenetic abnormality	affected genes
M0	complex Karyotype, +4, +11, +13, +21	?
M1	t(9;22)(q34;q11) -3, -5, del(5q),-7, del(7q), +11, +13, +21	*BCR/ABL*
M2	t(8;21)(q22;q22) t(2;4)(p23;q35) t(7;11)(p15;p15) t(6;9)(p23;q34) t(11;19)(q23,p13) t(11;20)(p15;q11) del(2p), +4, -5, del(5q), -7, del(7q), +8, del(9q),+11, +21, -Y	*RUNX1T1/RUNX1* *?* *HOX9/ NUP98* *DEK/NUP214* *MLL/ELL* *NUP98/TOP1*
M3	t(15;17)(q22;q12-21) t(11;17)(q23;q12) del(7q), i(17q), +21	*PML/RARA* *PLZF/RARA*
M4	inv(16)(p13q22),t(16;16)(p13;q22) t(1;7)(q10;p10) t(6;9)(p23;q34) t(8;16)(p11;p13) t(10;11)(p13;q23) t(11;17)(q23.q25) t(11;19)(q23,p13) t(12;22)(p13;q21) t(16;21)(p11.2;q22) del(16)(q22), +4, -5, del(5q), -7, del(7q), +8, del(9q), del(11)(q23q24), +22	*CBFA/MYH11* *?* *DEK/NUP214* *KAT6A/CREBBP* *MLL-MLLT10* *MLL/SEPT9* *MLL/ELL* *ETV6/RUNX1* *FUS/ERG*
M5	t(6;11)(q27;q23), t(9;11)(p21;q23), t(10;11)(p13;q23), ins(10;11)(p11;q23q24), t(11;17)(q23;q25) t(11;19)(q23;p13) t(8;16)(p11;p13) abn11q23 +8	*MLL/MLLT4* *MLL/MLLT3* *MLL/MLLT10* *MLL/SEPT9* *MLL/ELL* *KAT6A/CREBBP* *MLL*
M6	inv(3)(q21;q26), ins(3;3)(q26;q21q26), t(3;3)(q21;q26) t(3;5)(q25.1;q35) dup(1q), -5,-7, del(7p), del(9q), del(20)(q11), i(21q)	*RPN1/MECOM* *NPM/MLF1*
M7	t(1;22)(p13;q13) del(20q11), +21	*RBM15/MKL1*

Table 1.8 Cytogenetic prognostic markers in AML

outcome	cytogenetic abnormality
favorable	t(8;21)(q22;q22); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* t(15;17)(q24.1;q21.1)/PML-RARA Mutated *NPM1* without *FLT3*-ITD (normal karyotype) Mutated *CEBPA* (normal karyotype)
intermediate	t(9;11)(p22;q23); *MLLT3-MLL* Mutated *NPM1* and *FLT3*-ITD (normal karyotype) del(7q), del(9q), del(11q), abn(12p). del(20q) -Y, +8, +11, +13, +21, normal karyotype
adverse	t(v;11)(v;q23); MLL rearranged inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* t(6;9)(p23;q34); *DEK-NUP214* t(1;22)(p13;q13)/RBM15-MKL1 complex karyotype ≥3 abnormalities –5 or del(5q); –7; abnl(17p), mutations in *IDH1* and/ or *IDH2*, *BAALC* overexpression

Supplementary Table 1 (Article 7)

case number	age [y]	gender	banding cytogenetic result	FISH probes	molecular approaches
B-ALLs					
P1	1	F	46,XX[7]	mMCB	MLPA aCGH
P8	30	M	46,XY[8]	mMCB LSPs #21	MLPA
P13	34	M	46,XY[8]	mMCB LSPs #10, #17	MLPA aCGH
P14	18	M	46,XY[20]	mMCB	n.d.
P17	27		46,XX[7]	mMCB	aCGH
P22	42	F	46,XX[20]	mMCB	n.d.
P23	59	F	46,XX[14]/47,XX,+14[6]	mMCB MCB#14 LSPs #14	MLPA aCGH
P25	71	F	46,XX[5]	mMCB	n.d.
P28	84	M	46,XY[5]	mMCB MCB#11 LSPs #9, #11	MLPA aCGH
P29	59	M	46,XY[5]	mMCB	n.d.
P37	52	M	46,XY[5]	mMCB	n.d.
P40	57	F	46,XX[6]	mMCB MCB #11	n.d.
P41	31	M	46,XY[4]	mMCB MCB #15 WCP #8	n.d.
P43	69	F	46,XX[20]	mMCB MCB #11 CEP #4 LSPs #11	MLPA aCGH
P44	24	M	46,XY[3]	mMCB WCP #4, #10	n.d.
P48	39	M	46,XY[20]	mMCB M-FISH MCB #6, #11 WCP #6, #11 subCTM11 LSPs #6, #11	aCGH
P49	39	F	46,XX[10]	mMCB	aCGH
P50	21	F	46,XX[2]	mMCB	n.d.
P51	59	F	46,XX[6]	mMCB	MLPA aCGH
P52	21	M	46,XY[4]	mMCB	MLPA aCGH
P53	34	M	46,XY[5]	mMCB	MLPA aCGH
P55	19	M	46,XY[6]	mMCB	MLPA aCGH
P56	47	M	46,XY[20]	mMCB	MLPA aCGH
P57	56	M	46,XY[3]	mMCB	MLPA aCGH
P58	20	F	46,XX[20]	mMCB MCB #14 WCP #8, #14 LSPs #9, #14	MLPA aCGH
P59	25	M	46,XY[2]	mMCB	n.d.
P62	34	F	46,XX[3]	mMCB	n.d.
P64	4	F	46,XX,?der(19)[20]	mMCB MCB #5, #9, #16, #19 WCP#5, #9, #16, #19, X LSPs #5, #9, #16, #19	aCGH
P65	18	M	46,XY[10]	mMCB MCB #8, #14, LSPs #8, #14	n.d.
P66	0.5	F	n.d.	M-FISH MCB #10, #11; #14; WCP #10, #11, #14, LSPs #10, #11, #14	aCGH
P67	12	M	46,XY[15]	M-FISH	n.d.

				MCB #1, #7 LSPs #1, #7, #11	
T-ALLs					
P3	19	M	46,XY[8]	mMCB	n.d.
P5	22	F	46,XX[12]	mMCB	MLPA aCGH
P6	16	M	46,XY[9]	mMCB M-FISH MCB #3, #5, #10 WCP #4	MLPA aCGH
P7	26	M	46,XY[7]	mMCB M-FISH MCB #2, #9, #11, #18 WCP #10, #14 subCTM #11 LSPs #2, #9, #18	MLPA aCGH
P15	44	F	46,XX[5]	mMCB	n.d.
P18	36	M	46,XY[5]	mMCB MCB5 LSPs #18	MLPA
P26	28	F	46,XX[5]	mMCB	n.d.
P32	27	M	46,XX[17]	mMCB MCB #6, #10, #14 subCTM #6 LSPs #9, #12, #13	MLPA
P35	40	M	46,XY[10]	mMCB LSPs #9	MLPA aCGH
P36	58	M	46,XY[20]	mMCB	n.d.
P38	22	M	46,XY[3]	mMCB	MLPA aCGH
P61	18	F	46,XX[20]	mMCB M-FISH MCB #2, #4;#7, #10 WCP #2, #7, #10 LSPs #2;#7, #10	MLPA aCGH
B- or T ALLs (not clinically well defined)					
P2	23	F	46,XX[11]	mMCB	n.d.
P4	18	F	46,XX[2]	mMCB	n.d.
P9	4	F	46,XX[2]	mMCB	n.d.
P10	15	F	46,XX[5]	mMCB	n.d.
P11	26	M	46,XY[8]	mMCB WCP #11, #22	aCGH
P12	24	F	46,XX [5]	mMCB	n.d.
P16	17	F	46,XX[7]	mMCB LSPs #9; #12	MLPA aCGH
P19	9	M	46,XY[5]	mMCB	n.d.
P21	62	M	46,XY[11]	mMCB	aCGH
P24	23	M	46,XY[12]	mMCB LSPs #18	MLPA
P27	71	F	46,XX[5]	mMCB	n.d.
P30	46	M	46,XY[6]	mMCB MCB #9	MLPA
P31	58	M	46,XY[5]	mMCB	n.d.
P33	76	F	46,XX[4]/45,X,-X[14]	mMCB LSPs #9, #12, #18	MLPA aCGH
P34	61	M	46,XY[7]	mMCB MCB5 LSPs #5	n.d.
P39	52	F	46,XX[5]	mMCB	n.d
P46	63	M	46,XY[8]	mMCB CEP #7 WCP#5, #10	MLPA aCGH
P47	59	M	46,XX[6]	mMCB	MLPA aCGH

Supplementary Table 2 (Article 7)

probe	locus
CEB108/T7 (Abbott/Vysis)	1p36.3
ZytoLight®SPEC *ALK* (ZytoVision)	2p23.2~23.1
D2S447 (Abbott/Vysis)	2q37.3
ZytoLight®SPEC *TFG* (ZytoVision)	3q12.2
RP11-114M1 and RP11-91K9 (*TBL1XR1*)	3q26.32
D3S4559 (Abbott/Vysis)	3p26.3
CEP4 = D4Z1 (Abbott/Vysis)	4p11-q11
C84c11/T3 (Abbott/Vysis)	5p15.33
LSI D5S721 (Abbott/Vysis)	5p15.2
LSI *EGR1*/D5S23 (Abbott/Vysis)	5q31
POSEIDON *PDGFRB* (Kreatech)	5q33
D5S2907 (Abbott/Vysis)	5q35.3
ZytoLight®SPEC *MYB* (ZytoVision)	6q23.2~q23.3
ZytoLight®SPEC CEN6 = D6Z1 (ZytoVision)	6p11.1-q11.1
ZytoLight®SPEC *ESR1* (ZytoVision)	6q25.1
RP11-112P10 (*RELN*)	7q22.1
VIJyRM2000 (Abbott/Vysis)	7q36.3
ZytoLight®SPEC *CDKN2A* (ZytoVision)	9p21.3
ZytoLight®SPEC CEN9 = D9Z3 (ZytoVision)	9q12
LSI *ABL* (Abbott/Vysis)	9q34
Z96139 (Abbott/Vysis)	10p15.3
ZytoLight®SPEC *WT1* (ZytoVision)	10p13
ZytoLight®SPEC CEN 10 = D10Z1 (ZytoVision)	10p11.1-q11.1
ZytoLight®SPEC *PTEN* (ZytoVision)	10q23.3
ZytoLight®SPEC *FGFR2* (ZytoVision)	10q26.13
D10S2290 (Abbott/Vysis)	10q26.3
D11S2071 (Abbott/Vysis)	11p15.5
POSEIDON NUP98 (Kreatech)	11p15.4
ZytoLight®SPEC *BIRC3* (ZytoVision)	11q22.2
ZytoLight®SPEC *ATM* (ZytoVision)	11q22.3
LSI *MLL* (Abbott/Vysis) or POSEIDON *MLL* (Kreatech)	11q23.3
D11S1037 (Abbott/Vysis)	11q25
8M16/SP6 (Abbott/Vysis)	12p13.3
ZytoLight®SPEC ETV6 (ZytoVision)	12p13.2
LSI 13 (*RB1*) (Abbott/Vysis)	13q14.2
LSI D13S25 (Abbott/Vysis)	13q14.3
LSI *IGH* (Abbott/Vysis)	14q32.33
D14S1420 (Abbott/Vysis)	14q32.33
ZytoLight®SPEC *FUS* (ZytoVision)	16p11.2
ZytoLight®SPEC TP53 (ZytoVision) or LSI p53 (Abbott/Vysis)	17p13.1
CEP 18 = D18Z1 (Abbott/Vysis)	18p11.1-q11.1
LSI *BCL2* (Abbott/Vysis)	18q21
RP11-346H17 (*DCC*)	18q21.2
ZytoLight®SPEC *MALT1* (ZytoVision)	18q21.32
ZytoLight®SPEC 19q13 (ZytoVision)	19q13.3
POSEIDON *MLLT1* (Kreatech)	19p13.3
ZytoLight®SPEC 19p13 (ZytoVision)	19q13.43
ZytoLight®SPEC *RUNX1* (ZytoVision)	21q22.12
ZytoLight®SPEC *ERG* (ZytoVision)	21q12.13
LSI BCR (Abbott/Vysis)	22q11.2

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6.4. Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

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mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: PD Dr. rer. nat./ med. habil., h.c. (YSU) Thomas Liehr.

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Jena, 28. 08. 2015 _____.

Moneeb AK Othman