Analysis and detection of cryptic chromosomal aberrations in chronic lymphocytic leukemia

Dissertation

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This work is dedicated…

to my parents

to my family

to my dear friends

Abbreviations

ABL	v-abl Abelson murine leukemia viral oncogene
ALL	acute lymphoblastic 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
CDKs	cyclin dependent kinases
CGH	comparative genomic hybridization
CLL	chronic lymphocytic leukemia
CML	chronic myelogenous leukemia
CNAs	copy number alterations
CNVs	copy number variations
COBRA-FISH	COmbined Binary Ratio labelling-FISH
del	deletion
DNA	deoxyribonucleic acid
FISH	fluorescence in situ hybridization
GTG	Giemsa banding, G-bands by trypsin using Giemsa
HSCs	haematopoietic stem cells
HSCT	hematopoietic stem cell transplantation
IGHV	immunoglobulin heavy chain variable
ISCN	international system for human cytogenetic nomenclature
Kb	kilobasepairs
LSP	locus-specific probe
Mb	megabasepaires
MCB	multicolor banding
MCL	mantle cell lymphoma
m-FISH	multicolor FISH
M-FISH	multiplex FISH
MLPA	multiplex ligation dependent probe amplification
NCI	National Cancer Institute
NGS	next generation sequencing
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
No.	number
NK	natural killer
PAC	P1-derived artificial chromosome
PB	peripheral blood
PCR	polymerase chain
PCP	partial chromosome paint
PFS	progression-free survival
PNA	purine nucleoside analogue
RB	retinoblastoma
SKY	spectral karyotyping
SNP	single nucleotide polymorphism
WBCs	white blood cells
WHG	whole human genome
t	translocation
TGF-β	transforming growth factor beta
T-PLL	T-cell prolymphocytic leukemia
TTFT	time to first treatment
UPD	uniparental disomy
ZAP-70	Zeta-chain-associated protein kinase 70

Contents

Summary:

Chronic lymphocytic leukemia (CLL) is the most frequent leukemia of adults in Western countries; also it is considered as a heterogeneous disease, as the overall survival of CLL patients is different according to the detected acquired genetic, especially chromosomal aberrations. Particularly important are the genes *TP53*, *ATM*, and *BIRC3*, which are associated with poor prognosis. Many techniques have been used for the detection of disease associated chromosomal abnormalities, such as banding cytogenetic (GTG-banding) or molecular cytogenetic analyses. However, especially GTG-banding is hampered in diagnostics of CLL due to the low mitotic index of the aberrant cells. Even after using a suitable mitogen such as 12-O-tetradecanoylphorbol-13-acetate (TPA), the detection rate reaches only approximately 48%. Interphase fluorescence in situ hybridization (iFISH) was introduced to overcome this limitation; however this leads to underestimation of the complexity in chromosomal rearrangements. Multiplex ligation dependent probe amplification (MLPA) can be a way out here, and it was introduced recently for the diagnosis of CLL giving the opportunity to detect multiple chromosomal aberrations simultaneously. The present work aimed to analyze and detect cryptic chromosomal aberrations in 150 CLL patients, by studying them comparatively for aberration detection rates using different approaches such as GTG-banding, iFISH and/or MLPA, in addition to array-based comparative genomic hybridization (array-CGH) in selected cases.

Overall 163 acquired aberrations in 67 of 85 samples (~79%) were identified; iFISH was superior to MLPA in the cases with low percentage of aberrant cells, but on the other hand MLPA revealed additional chromosomal abnormalities in 22 cases. Based on that data a cost efficient scheme was suggested combining the different techniques for better diagnosis and characterization of cryptic chromosomal aberrations in CLL. Additionally an assessment of *BIRC3* alterations, a gene recently found to play an important role in lymphatic leukemia, was performed on 117 CLL, and 45 B-ALL cases. *BIRC3* aberrations were detected in 23/117 (~20%) of CLL and 2/45 (~4%) of B-ALL cases. Based on these results *ATM* deletions may, but must not always be associated with *BIRC3* abnormalities. Thus *BIRC3* screening should be considered as independent diagnostic parameter of CLL in future. Finally, 150 CLL patients have been tested for their status of *TP53* deletion. Obviously cases with isochromosome 17q and deletion of *TP53* were associated with more complex karyotypic changes than such with deletion of *TP53* due to other chromosomal changes. This suggests that i(17q) presents an adverse prognostic marker, which should be considered more in future CLL-diagnostics.

Zusammenfassung:

Chronische lymphatische Leukämie (CLL) ist die am häufigsten auftretende Leukämieform des Erwachsenenalters in den sog. „westlichen Ländern“. Die CLL wird es als eine heterogene Erkrankung angesehen, da Überlebensrate und -zeit (overall survival = OS) von CLL-Patienten durchaus unterschiedlich ist. Die OS hängt stark von den vorliegenden und entsprechend erfassten, erworbenen genetischen, insbesondere Chromosomenveränderungen ab. Von besonderer Bedeutung sind hierbei die Gene *TP53*, *ATM* und *BIRC3*, die allgemein mit einer schlechten Prognose der Krankheit assoziiert werden. Eine Vielzahl an Techniken wurden und werden zum Nachweis von CLL-assoziierten Chromosomenanomalien eingesetzt wie Bänderungs-Zytogenetik (GTG-Färbung) oder molekulare Zytogenetik. Hierbei ist anzumerken, dass insbesondere eine Karyotypanalyse durch den niedrigen mitotischen Index der aberranten CLL-Zellen nur eingeschränkt möglich ist. Dies gilt auch dann noch, nachdem geeignete Mitogene angewendet wurden, wie 12-O-Tetradecanoylphorbol-13-Acetate (TPA); hier sind Mitosen in nur etwa 48% der Fälle zu erwarten. Um solche Einschränkungen der Analyse zu umgehen wurde die Interphase Fluoreszenz in situ Hybridisierung (iFISH) eingeführt; jedoch kann der Einsatz dieser Methode zu einer Unterschätzung der vorliegenden Komplexität an Chromosomenaberrationen führen. Hier ist die Multiplex ligation dependent probe amplification (MLPA) als weiterer Fortschritt in der CLL-Diagnostik anzuführen, da diese Methode die Möglichkeit eröffnet gleichzeitig viele / viel mehr Chromosomenaberrationen zu erkennen. Die vorliegende Arbeit wurde mit dem Ziel durchgeführt zuvor kryptische chromosomale Aberrationen in 150 CLL Fällen nachzuweisen, und zwar durch den vergleichenden Einsatz von GTG-Bänderung, iFISH und MLPA, sowie in einzelnen Fällen ergänzt durch Mikro-array-Analyse (array-CGH). Insgesamt wurden 163 erworbene Aberrationen in 67 von 85 Fällen (~ 79%) nachgewiesen; iFISH hatte in solchen Fällen bessere Nachweisraten als die MLPA, welche nur einen geringen Anteil an anomalen Zellen hatten; im Gegensatz hierzu konnte die MLPA-Technik zusätzliche nicht mittels FISH erfassbare Chromosomenanomalien in 22/85 Fälle erfassen. Auf diesen Ergebnissen beruhend konnte hier ein kosteneffizientes Analyseschema entwickelt werden bei dem GTG-Bänderung, MLPA und iFISH so kombiniert, abgestimmt und gezielt eingesetzt werden, dass eine optimale Charakterisierung von kryptischen Chromosomenaberrationen bei CLL-Patienten möglich wird. Weiterhin wurden 117 CLL und 45 B-ALL Fälle auf das Vorliegen von *BIRC3* Veränderungen untersucht. Letztere wurden in 23/117 (~ 20%) der CLL und 2/45 (~ 4%) der B-ALL-Fälle nachgewiesen. Hieraus, und aus der gleichzeitigen Feststellung des *ATM*-Deletionsstatus der entsprechenden Patienten konnte erstmals gezeigt werden, dass *ATM* Deletionen zusammen mit *BIRC3* Genveränderung vorkommen können, aber nicht müssen. Ein Screening auf *BIRC3* für eine verbesserte Diagnosestellung bei der CLL wird vorgeschlagen. Abschließend wurden 150 CLL-Patienten auf ihren Isochromosom-17q-Status hin getestet. Offensichtlich waren Fälle mit Isochromosom-17q und Deletion von *TP53* mit komplexeren karyotypischen Veränderungen assoziiert als solche Fälle in denen Deletion von *TP53* aufgrund anderer chromosomaler Veränderungen vorlag. Das deutet darauf hin, dass Isochromosom-17q einen negativen prognostischen Marker darstellt, der künftig mehr Beachtung bei der CLL-Diagnostik finden sollte.

1. Introduction

1.1. Cytogenetic and molecular (cyto)genetics

The beginning of diagnostic cytogenetics arose in the mid of 19th century. Then the normal number of chromosomes in the human somatic cell was finally accurately identified in 1956 by Tjio and Levan (Tjio and Levan 1956), which was independently confirmed in the same year by Ford and Hamerton (Ford and Hamerton 1956).

The continued technical improvements in the cytogenetic field enabled the researchers to identify chromosomal abnormalities, which are correlated with specific disorders, such as, in 1959 Lejeune and colleagues found the trisomy for chromosome 21 in fibroblast cultures from patients with Down syndrome (Lejeune et al. 1959).

In 1960, Peter Nowell and David Hungerford identified a minute chromosome in the white blood cells (WBCs) of patients with chronic myelogenous leukemia (CML), which was called Philadelphia chromosome (Nowell and Hungerford 1960).

Thirteen years later, it was discovered that, this chromosome is a product of balanced translocation between the long arms of chromosomes 9 and chromosome 22; specifically a t(9;22)(q34;q11) (Rowley 1973).

The development of chromosome preparations and banding techniques in the end of the 1960s allowed the more precise identification and characterization of inherited and acquired alterations in human malignancies (Caspersson et al. 1968).

RB1 (retinoblastoma) gene, which is located in 13q14, was the first identified tumor suppressor gene. As in 1983 Cavenee et al. introduced the proof of Knudson's "two-hit" hypothesis. They reported that cancer may originate from the (functional) loss of both alleles of the *RB1* gene, when a germline mutation is present on one allele and a subsequent mutation of the other allele takes place (Cavenee et al.1983, Knudson et al. 1971).

1.1.1. Chromosome banding

The development of banding and staining protocols between 1968 and 1980s facilitated the identification of many recurrent numerical and structural chromosomal abnormalities. Also chromosome analysis has been used widely in diagnosis of leukemia and lymphoma (Lawler 1977).

Giemsa (G-) banding was introduced after the development of Q-banding. Giemsa stain is used in G-banding after proteolytic enzyme treatment of the chromosome preparations. This type of staining produced highly reproducible dark and light bands along each chromosome, which could be seen by standard light microscopy. G-banding technique is still considered as gold standard for the detection of both numerical (gain or loss of a chromosome) and structural aberrations (e.g. translocation, deletion, inversion, fragile sites, etc.), as it provides a whole-genome perspective. In spite of that the resolution of G-banding is limited (approximately 400-550 bands per haploid tumor cytogenetic genome), thus chromosomal aberrations can be missed and complex aberrations are too difficult to be resolved even by skilled cytogeneticists (Wang and Fedoroff 1972, Yunis 1976, Othman et al. 2014).
Based on the banding patterns for each chromosome a system of nomenclature was introduced, and this international system for human cytogenetic nomenclature (ISCN) is still in place and actualized regularly (Shaffer et al. 2013).

1.1.2. Molecular cytogenetics

Molecular cytogenetics involves the combination of both molecular and cellular levels in microscopic analyses (Speicher and Carter 2005). The identification of particular chromosomal rearrangements such as too small or too complex aberrations could be achieved through the application of more sensitive and sophisticated techniques than G-banding (Li and Pinkel 2006).

Thus, fluorescence in situ hybridization (FISH) has been introduced as a suitable method for the characterization of both constitutional and acquired chromosomal abnormalities by application of chromosome-specific probes and probe sets (Pinkel et al. 1986).
For further identification of new biomarkers and potential therapeutic targets in leukemia and/or cancer, other techniques were developed such as comparative genomic hybridization (CGH), array-based CGH (array-CGH) and single nucleotide polymorphism (SNP array-CGH), and multiplex ligation dependent probe amplification (MLPA) (Glassman and Hayes 2005, Le Scouarnec and Gribble 2012, Hömig-Hölzel and Savola 2012).

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

The hybridization of radioactively labeled DNA and RNA probes to tissue squashes has been described since 1969 by Gall and Pardue (Gall and Pardue 1969), but the major advanced in this field has been accomplished in 1986 by using non-radioactively labeled probes (Pinkel et al.1986, Cremer et al. 1986).

The principle of FISH technique is based on the hybridization of labelled DNA probes to its complementary target DNA sequence. Then hybridized probes are detected via fluorochromes by epifluorescence microscopy, via colorimetric enzyme assays by transmission light microscopy or via metallic compounds in the electron microscope (Joos et al. 1994).

The targeted DNA could be metaphase chromosomes, interphase nuclei, or tissue sections fixed to a glass slide (Fig. 1.1). Interphase directed FISH technique can detect chromosomal abnormalities e.g. in those types of cancers which have low mitotic index of aberrant cells including certain subtypes of leukemia (Liehr 2009, Bishop 2010).

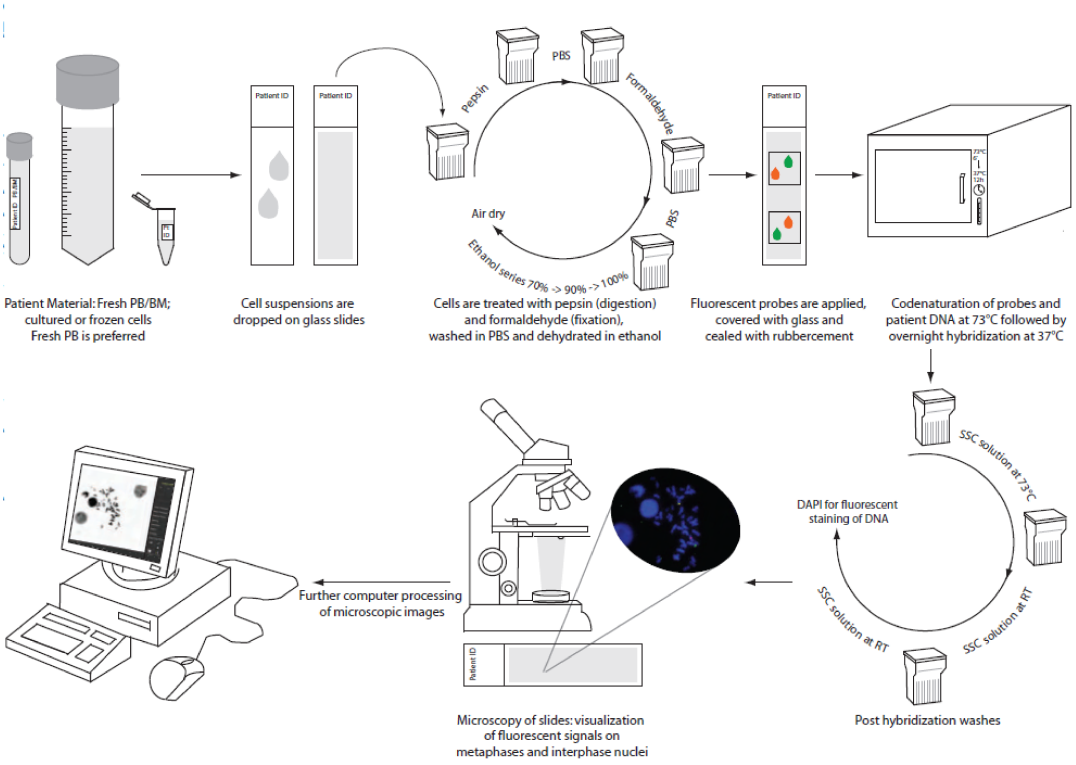

Figure 1.1. Principle of a FISH experiment performed on metaphases or interphase nuclei. Fluorescence labeled DNA probe complementary to a chromosomal region of interest is used together with the target DNA which is fixed onto the slide surface. DNA probes and target DNA are denatured and hybridized together. After washing the slides they can be visualized under a fluorescence microscope. If the DNA complementary to the probe is present in the sample a signal with the color of the emission wavelength of the fluorochrome of the probe is visible (figure adapted from Put et al. 2012).

1.1.2.2. Probes used for FISH

The accuracy and reliability of FISH analysis depend particularly on the specificity and sensitivity of the applied probes and the hybridization detection efficiency (Divane et al. 1994). There are many different types of DNA probes that can be used in FISH, which have been grouped as outlined below.

1.1.2.2.1. Locus-specific probes (LSP)

LSP bind to a particular region of a chromosome or locus of 0.1 to several megabase pairs (Mb) in size.

They can detect amplified oncogenes, deletion of tumor suppressor genes, fusion and/or translocations of genetic regions involved in cancer; they are applied in (leukemia) diagnostics and research (Liehr et al. 2015).

1.1.2.2.2. Chromosome painting probes

Whole chromosome painting (WCP) probes, which are generated by flow sorting or whole chromosome microdissection, hybridize the entire length of the chromosome. In addition to that partial chromosome painting (PCP) probes, which are generateable only by microdissection, could be used to label the short and long arm of a particular chromosome or chromosomal subregions. PCPs and WCPs are particularly useful for examining both structural and numerical chromosomal abnormalities in leukemia (Cremer et al. 1988, Pinkel et al. 1988, Guan et al. 1994).

1.1.2.2.3. Centromeric probes

Chromosome-specific centromeric probes (CEP) are generated from repetitive sequences found in the centromeric regions of the human chromosomes, and commercially available CEP probes are used to determine whether an individual has the correct number of chromosomes in both interphase and metaphase, such as in Down syndrome cases (Liehr et al. 2015).

1.1.2.2.4. Multicolor FISH probe (mFISH)

Multicolor FISH (mFISH) has been reported for the first time in 1989 by Nederlof and coworkers (Nederlof et al. 1989). Several approaches have been developed, that permit the simultaneous painting of all 24 human chromosomes in specific color combinations: 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). In all of these techniques a series of four to seven different fluorochromes in a combinatorial labeling and/or ratio-labeling is used (Riegel 2014, Liehr et al. 2004, Liehr 2009). Nowadays, SKY and M-FISH are the most commonly applied WCP-based FISH approaches. Each of these techniques provides a precisely tool for characterization of complex chromosomal abnormalities in a single hybridization, and in one metaphase spread (Liehr 2015).

1.1.2.2.5. FISH-banding approaches

Although multiple FISH-banding approaches were reported, the only routinely used one is multicolor chromosome banding (MCB). It is available as a chromosome-specific and a whole genomic variant; the latter is called multitude multicolor banding (mMCB). MCB and mMCB is widely applied to describe marker and/or derivative chromosomes in clinical and tumor cytogenetics (Weise et al. 2003, Liehr et al. 2002a, 2002b, Liehr 2009).

1.1.2.3. Array comparative genomic hybridization (array-CGH)

Comparative genomic hybridization (CGH) has been introduced in 1992 for the comprehensive analysis of the entire genome and characterization of genetic imbalances in tumors, which could not be karyotyped (Kallioniemi et al. 1992).

Subsequently array-based CGH (array-CGH) technique was established, which has much higher resolution than CGH [i.e. ~50-100 kilobases (kb)]. In this approach 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) have been used as hybridization targets instead of the metaphase chromosomes (Solinas-Toldo et al. 1997, Pinkel et al. 1998).

Array-CGH is suited to identify chromosomal imbalances particularly in leukemia and lymphoma, but balanced aberrations such as recurrent balanced translocations, inversions or insertions cannot be detected by this approach (Riegel 2014, Le Scouarnec and Gribble 2012).

In contrast with array-CGH, SNP-array-CGH based approaches have the additional advantage of detecting copy number neutral loss of heterozygosity, which may be hints on deletions or

acquired uniparental disomy (UPD). Also this approach has higher resolutions (down to ~1kb) (Le Scouarnec and Gribble 2012).

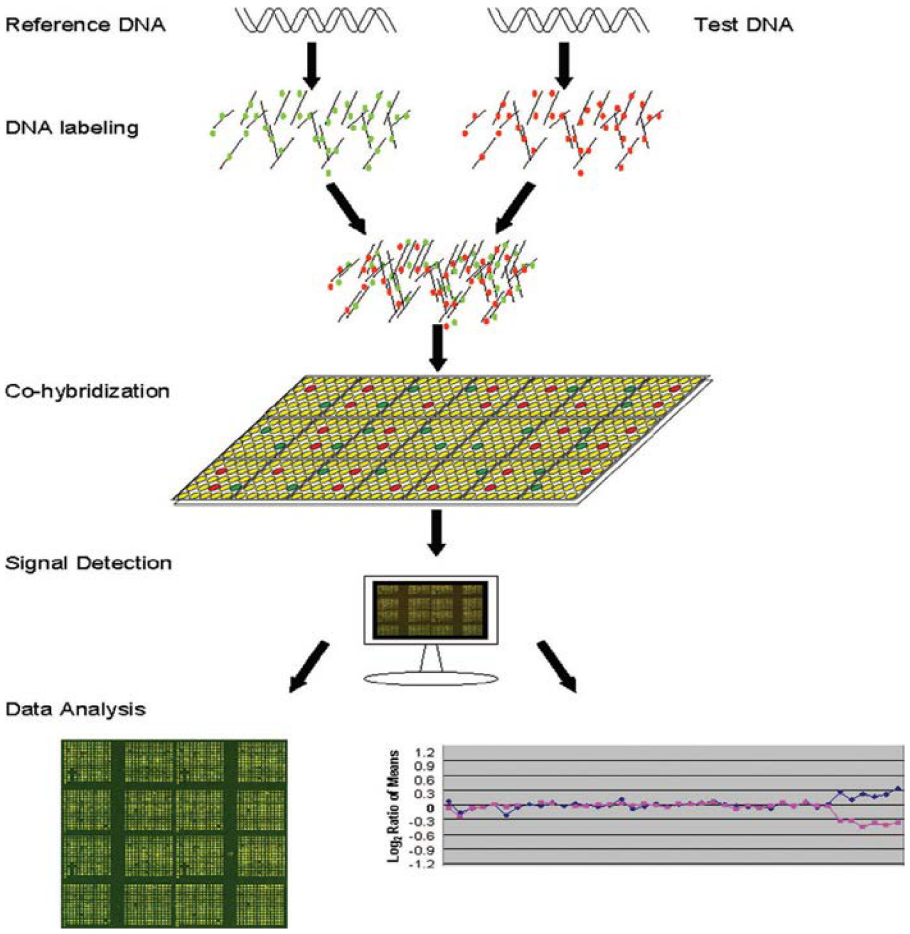

Figure 1.2. Principle of array-CGH. Test DNA and control DNA are differentially labeled (figure adapted from Bejjani BA et al. 2006).

1.1.2. Molecular genetics

Molecular genetics increased our understanding of the mechanisms, pathophysiology and the progression of leukemogenesis through the study of the structure and function of genes at a molecular level.

The major breakthrough leading to the identification of the *BCR-ABL* gene was the cloning of the genes involved in the CML-specific translocation t(9;22), revealing that the *5′ABL* gene on chromosomal region 9q34 fused to the *3′BCR* gene which is located on 22q11 (Heisterkamp et al. 1983, Groffen et al. 1984, Rowley 1999).

During the past decade, there has been tremendous progress in molecular genetics approaches (Murphy and Bustin 2009, Kohlmann et al. 2013).

In the following emphases is given only to a few selected developments that are of special interest for this work.

1.1.3.1. Multiplex ligation-dependant probe amplification (MLPA)

MLPA is one of the variations of polymerase chain (PCR) reaction based techniques. MLPA can detect simultaneously the copy number changes, DNA methylation, and point mutations of up to 50 genomic DNA sequences in one single experiment (Fig 1.3), as it depends on specifically bound probes which are amplified by universal primers.

MLPA was first described for the detection of exon alterations in the human *BRCA1*, *MSH2* and *MLH1* genes, and the detection of trisomies such as present in Down syndrome (Hömig-Hölzel and Savola 2012, Schouten et al. 2002).

Lately, MLPA has been applied in both molecular diagnosis of tumors and in cancer research such as glioma, uveal melanoma, acute lymphoblastic leukemia (ALL), and breast cancer (Hömig-Hölzel and Savola 2012).

Despite the advantages of MLPA as a fast, reliable, cost-effective technique, and e.g. method of choice for routine diagnostic of chronic lymphocytic leukemia (CLL), there are many limitations connected with this technique. MLPA is not suitable for the detection of balanced translocations, inversions, and unknown point mutations. Also in tumor cases which have low percentage of aberrant cells alterations may be missed by MLPA. Still in these situations sensitivity of MLPA could be increased by multiple target probes for the same chromosomal region or gene. Heterozygous deletions and/or duplications can be detected reliably by MLPA only if the sample contains at least 20% to 30% of the tumor cells in the case of deletion, and 40% of aberrant cells in duplication (Hömig-Hölzel and Savola 2012, Alhourani et al. 2014).

In spite of the limitation of MLPA technique, it is a powerful tool for diagnosis and progression of cancer, considering all possible shortcuts adequately (Hömig-Hölzel and Savola 2012).

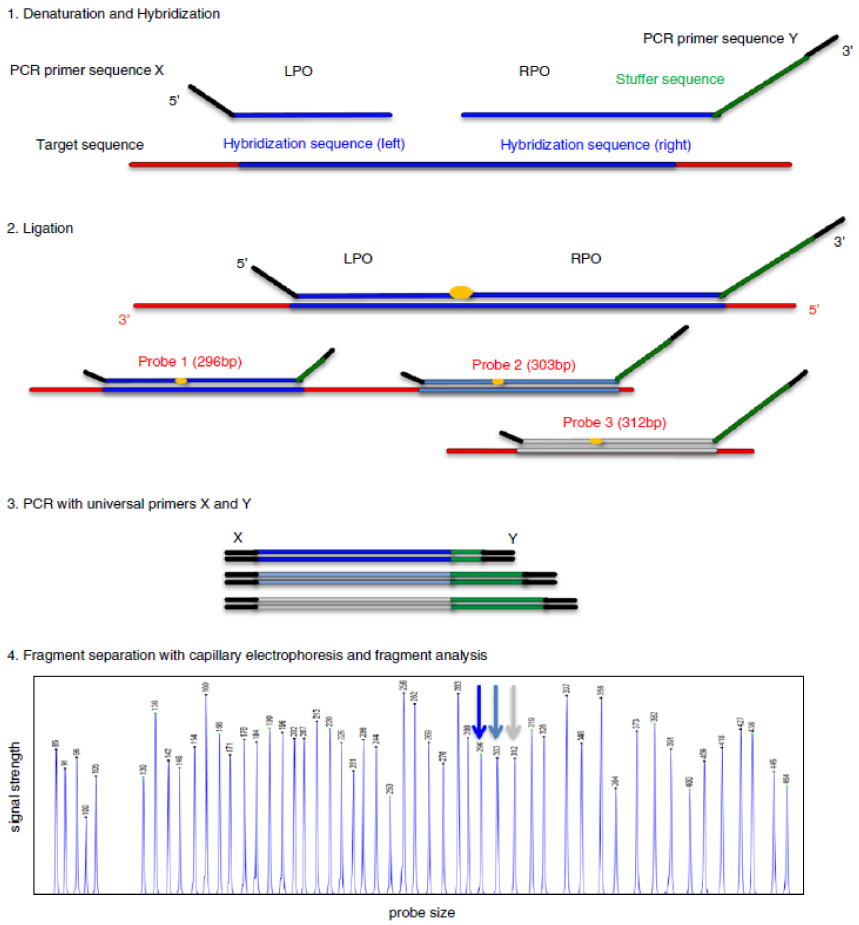

Figure 1.3. Principle of MLPA reaction including: 1) DNA dena-turation and hyb-ridization of probes; 2) ligation; 3) PCR; 4) separation of amplified products by electrophoresis and data analysis (adapted from Hömig-Hölzel and Savola 2012).

1.1.3.2. New high throughput approaches

A major breakthrough has been accomplished in DNA sequencing field since the first attempt in the late 1970s (Sanger et al. 1977). DNA sequencing is able to determine the precise order of nucleotides within a DNA molecule, so that it is considered as a gold standard technique for the detection of point mutations associated with inherited and acquired genetic disease. 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 hours or days by using different approaches, either by creating micro-reactors and/or attaching DNA molecules to solid surfaces or beads. The application of this approach is limited in routine cancer diagnostics due to the high cost, presence of specific artifacts for each type of NGS and partially high complexity of aberrations present in tumors. Still NGS should be taken into consideration for research and establishing new findings (Ilyas et al. 2015, Koboldt et al.2013).

1.2. The biology of leukemia

The emergence of blood cells is a complex and precise process, which is controlled by a number of humeral and cellular factors. It takes place primarily in the bone marrow, where all cellular blood components are derived from a pool of self-renewing haematopoietic stem cell (HSC) resides, which have the ability to differentiate into two main lineages: myeloid and lymphoid stem cells (Longo 2013).

Subsequently lymphoid progenitors can differentiate into B- , T-, or natural killer (NK) cell lines. The differentiation of B-lymphocytes (or B-cells) occurs in the bone marrow, whereas T-lymphocytes (or T-cells) progenitors migrate to the thymus. In Thymus they undergo several steps of proliferation and differentiation, and after that mature B- and T-cells migrate to peripheral lymphoid organs through the bloodstream (Hardy and Hayakawa 2001, Rothenberg et al. 2008).

The development of myeloid progenitors leads to the production of granulocytes, monocytes, erythrocytes, or platelets, which have different roles in the innate immunity, the adaptive immunity, and blood clotting (Kondo et al.2010).

The mechanisms which are involved in normal blood cells proliferation and differentiation are regulated very strictly; many factors have been identified in this process such as growth factors, interleukins, and transforming growth factor beta (TGF-β). The alterations in these regulation mechanisms can lead to uncontrolled cell proliferation and/ or failure in differentiation of progenitor cells to mature cells (Hardy and Hayakawa 2001).

Leukemia is a neoplastic proliferation of hematopoietic stem cells. It is classified according to the length of survival of the patients and the predominant cell lineage which is represented by the leukemic clone into four broad subtypes: acute lymphoblastic, acute myelogenous, chronic lymphocytic, and chronic myelogenous (Davis et al. 2014)

Whereas acute lymphoblastic leukemia appears more frequently in children, the other subtypes occur more often in adults. In addition to that acute leukemia is characterized by a rapid increase in the number of immature lymphoid or myeloid precursors in the bone marrow and the peripheral blood, i.e. immediate treatment is required (Mullighan et al. 2013, Dighiero et al. 2008). The development of chronic leukemia tends to be slower than acute leukemia, as the malignant cells in chronic forms are capable of relatively maturation despite the uncontrolled proliferating (Rodríguez-Vicente et al. 2013).

1.3. Chronic lymphoblastic leukemia (CLL)

Chronic lymphocytic leukemia (CLL) is the most frequent hematological malignancy in adults in Western countries, and it appears mainly in individuals >50 years of age (Chiorazzi et al. 2005). CLL is characterized by the accumulation of small B lymphocytes with a mature appearance in blood, bone marrow or other lymphoid tissues. Also it is considered as a heterogeneous disease, as the CLL patients show different and distinct clinical course and response to treatment according to the detected cell morphology, immunophenotype, as well as cytogenetic molecular genetic characteristics (Dighiero et al. 2008, Rodríguez-Vicente et al. 2013).

Whereas approximately one-third of CLL patients survive for long time without requirement of treatment and have no or minimal signs and symptoms during their entire disease course, others can develop an aggressive clinical outcome of the disease including enlarged lymph nodes, enlarged spleen, and severe immunoglobulin deficiencies. The diagnosis of CLL is, according to the National Cancer Institute (NCI) guidelines, based on a clonal expansion of at least 5,000 B lymphocytes per μl in the peripheral blood for the duration of at least 3 months, and a characteristic immunophenotype combining the presence of CD19, the T-cell antigen CD5, and CD23 (Dighiero et al 2000, Matutes et al. 1994, Cheson BD et al. 1996).

For the classification of CLL two staging systems are used: The Rai system which is applied more often in the United States, beside the Binet staging system which is the more prevailed in Europe. Both systems are suited for the assessment of disease progression and treatment planning, but they are not very effective for predicting early disease progression (Döhner et al. 2000, Zwiebel et al. 1998).

Along with the stage, additional prognostic markers are available to predict a patient's chances, in particular at early stages. The adverse prognostic factors such as advanced age, male gender, not-mutated *IGHV* (immunoglobulin heavy chain variable) gene, and high proportion of CLL cells containing ZAP-70 (more than 20%) or CD38 (more than 30%) are associated with shorter survival time. Favorable prognostic factors are low proportion of CLL cells containing ZAP-70 (20% or less) or CD38 (30% or less) and CLL cells with a mutated *IGHV* (Cramer et al. 2011, Rodríguez-Vicente et al. 2013).

1.3.1. Cytogenetic abnormalities in CLL

CLL is characterized by a high diversity in chromosomal aberrations. Several studies showed that detection and exact characterization of these abnormalities is essential in CLL prognosis and treatment. The International Workshop on Chronic Lymphocytic Leukemia guidelines consider the assessment of chromosomal abnormalities by FISH mandatory in clinical trials and desirable in general practice as a pre-treatment evaluation (Hallek et al. 2008). In the following the most important good, intermediate and adverse prognostic (cyto)genetic markers in CLL are presented.

1.3.1.1. 13q14 Deletion

Deletion within the 13q14 region is the most frequent aberration in CLL, with a prevalence of 40-60%, and it is associated with good prognosis; such CLL patients are also denominated being of "13q-"type. But during the last years, several studies indicated that the situation may be more complex, suggesting that the percentage of cells with deletion, as well as the size of the deletion itself could influence the prognosis. Deletion in this region can vary substantially in size, ranging from only 300 kbp up to >70 Mbp. Thereby, other (tumor suppressor) genes located in 13q14.2 (*RB1* gene) or 13q14.3 as microRNAs (miR-15a and miR16-1) and *DLEU7* gene can be deleted as well (Döhner et al. 2000, Dal Bo et al. 2011).

Thus, two types of 13q14 deletions are proposed: del(13q) type I (short), which includes only 13q14.3; and del(13q) type II (larger), which includes the *RB1* locus with significantly shorter time to first treatment (TTFT) and overall survival (OS). Also the CLL patients who are carrying a high percentage (≥70 %) of 13q- cells have a shorter overall survival (OS) than patients with <70% 13q- cells, as well as a shorter TTFT (Dal Bo et al. 2011).

In contrast to other recurrent abnormalities in CLL, 13q14 deletions could be heterozygous (monoallelic) or homozygous (biallelic). Biallelic losses in 13q14 are characteristically small and do not involve *RB1*. Also biallelic loss has been described in nearly 24% of 13q-type CLL patients (Garg et al. 2012).

Several studies suggested that 13q14 heterozygous deletion is an early event in CLL, whereas deletion of the second copy of this region occurs at a later stage. Nevertheless the clinical impact of the presence of biallelic losses in 13q has been discussed controversially. Some authors hypothesized that the biallelic status is associated with a more aggressive clinical course as it

results from a karyotypic evolution, while others suggested that homozygosity does not affect TTFT or OS (Chena et al. 2008, Van Dyke, et al. 2009, Garg et al. 2011, Puiggros et al 2013). Still it is noteworthy that CLL patients with a monoallelic del(13q) show lower lymphocyte growth kinetics than patients with biallelic deletions (Rodriguez-Vicente et al. 2013).

1.3.1.2. Trisomy 12

Trisomy 12 is the third most common chromosomal abnormality in CLL, occurring in 10-20% of cases. It can be associated with other chromosomal rearrangements such as trisomy of chromosomes 18 and 19, as well as IGH rearrangement (Rodriguez-Vicente et al. 2013).

Trisomy 12 is associated with an intermediate prognosis and an atypical morphology or immunophenotype. Nevertheless, this category still is controversial, as recent analysis of prospective trials suggested that although progression-free survival (PFS) may be shorter in CLL patients with trisomy 12, the overall survival is favorable. In concordance with this, trisomy 12 in CLL is only rarely accompanied by *TP53* mutations, but it is highly associated with mutated *NOTCH1*, as well as CD38 expression. The latter which could explain to some extent the bad prognosis of these subgroups of patients, and thus the different survival rates for this trisomy 12 patients (Hallek et al. 2010, Matutes et al. 1996).

Although trisomy 12 appears early in CLL evolution and thus could be a trigger for secondary chromosomal aberrations or mutations such as *NOTCH1* and *FBXW7*, until now the critical genes which are involved in formation of this aberration remain unknown (Puiggros et al. 2014).

1.3.1.3. 11q23 deletion

Deletions in 11q23 are detected in 5 to 20% of CLL patients, and they appear generally in younger patients. Also they are associated with a more rapid progression of the disease and a shorter overall survival (Marasca et al 2013, Puiggros et al. 2014).

11q23 deletions are highly variable in size, therefore these CLL-cases can be classified according to the size into “classical or large deletion” (more common and the deletion is normally >20 Mbp) and “atypical or small deletion” (uncommon and more frequently associated with *ATM* mutations) (Gunn et al. 2009).

The minimal affected region includes the chromosomal bands 11q22.3-q23.1, which harbor the *ATM* gene in almost all cases, as well as other genes such as *BIRC3* (Puiggros et al. 2014).

ATM gene activates cell cycle checkpoints, and it has a central role in the DNA damage pathway as it can induce apoptosis in response to DNA breaks. *ATM* mutations have been reported in only 8–30% of patients with del (11q), which indicates that other genes could contribute in the pathobiology of 11q deletions in CLL. One of these genes is *BIRC3*, which is located ~6Mb centromeric to the *ATM* gene locus, at 11q22 and it is considered to be a negative regulator of the MAP3K14 serin-treonine kinase, which is the central activator of non-canonical NF-κB signaling pathway (Rossi et al 2014, Rodriguez-Vicente et al. 2013).

BIRC3 disruption, mutations and/or deletions are rarely detected in CLL at diagnosis (4% of patients). However *BIRC3* involvement has been reported in 24% of fludarabine-refractory CLL patients. Thus *BIRC3* disruption has been suggested to be specifically associated with a chemo-refractory CLL subtype (Rossi et al. 2014).

1.3.1.4. 17p13 deletion

While deletions in 17p have been reported in 3-8% of CLL patients at diagnosis, the detection rate of this chromosomal abnormality increases up to 30% in CLL patients with advanced and/or relapsed disease. Thus, it is one of the most frequently acquired aberrations triggered after treatment, and most cases with del (17p) show loss of one copy and mutation of the remaining copy of *TP53* gene (Delgado et al. 2012, Döhner et al 1995).

17p-deletion is usually associated with a very aggressive clinical course and the shortest overall survival besides lack of response to therapy. Nonetheless, the percentage of aberrant cells has a clinical relevance, as the cut-off value for the percentage of 17p-deleted nuclei that predicted adverse outcome has been identified to be 20% (Puiggros et al. 2014, Greipp et al. 2013).

The critical tumor suppressor gene in this region is *TP53*. This gene plays an essential role in inducing apoptosis or cell cycle arrest after DNA damage so that the patients harboring 17p deletion and/or *TP53* mutations do not respond to standard initial therapy (fludarabine and alkylating agents), because the mechanism of these drugs is *TP53*-dependent. However, fludarabine refractoriness is caused by *TP53* disruption in approximately 40% of CLL patients who did not respond to treatment, i.e. here other treatment approaches should be considered (Zenz et al. 2009, Rodriguez-Vicente et al. 2013). 17p-deletion often encompasses most of chromosome 17 short arm, and also it can be associated with the formation of an isochromosome i(17q) (Scheurlen et al. 1999).

Generally the formation of isochromosomes can occur during mitosis and meiosis, either by a misdivision of the centromere or by chromatid exchange involving two homologous chromosomes. Recently i(17q) in CLL has been reported to be associated with more complex karyotype, which could be a hint for more aggressive course of the disease than deletion of *TP53* alone (Mertens et al. 1994, Thompson et al. 2015, Alhourani et al. in press).

1.3.1.5. 14q32.33 rearrangements

CLL-associated chromosomal rearrangements in 14q32.33 could be either deletions (in 12-15% and includes IGH locus) being associated with a good prognosis, or translocations which were initially associated with a poor prognosis. Overall, recurrent balanced translocations involving 14q32.33 are rare in CLL.

However, the chromosomal partners involved have influence on the prognosis. While translocation t(14;19)(q32;q13) (*IGH/BCL3*) involving, trisomy 12, complex cytogenetics and unmutated *IGHV* has an inferior prognosis, the translocation t(14;18)(q32;q21) (*IGH/BCL2*) is not accompanied with complex karyotype or aggressive course of disease.

On the contrary, translocations involving *IGH* and *MYC* gene in 8q24.2, identify a subgroup of CLL with higher incidence of poor prognostic features (Quintero-Rivera et al. 2009, Cavazzini et al.2008, Mayr et al 2006, Huh et al. 2008).

1.3.1.6. Other abnormalities

Several other recurrent chromosomal abnormalities have been described in CLL, such as deletion of the long arm of chromosome 6 which appears in approximately 6% of CLL patients and is associated with intermediate prognosis, atypical morphology, splenomegaly, and higher detectable rates of CD38. Also Schwaenen et al. (2004) described 2p gains, including *MYCN* gene in a low proportion of CLL cases. Also abnormalities like 8p losses and 8q gains, total or partial trisomy 3, trisomy 8 and /or trisomy 18 have been reported in CLL (Cuneo et al. 2004, Fabris et al. 2013, Puiggros et al. 2014, Rodriguez-Vicente et al. 2013, Stilgenbauer et al. 1999, Schwaenen et al. 2004).

1.3.1.7. Complex karyotype

The presence of three or more chromosomal aberrations per patient is considered as a complex karyotype. The incidence of complex karyotypes in CLL is 20% (Puiggros et al. 2014).

The genomic complexity is associated with progressive and aggressive disease, short survival, and decreased therapeutic efficacy. Also there is highly significant association between complex karyotypes and 11q or 17p deletions. Ouillette et al. (2010) demonstrated, that genomic complexity in CLL was a consequence of an impaired DNA double-strand break response due to multiple gene defects, including not only *TP53*, but also *ATM* and other genes located in 11q or *RB1* gene located in 13q14 (Kujawski et al.2008, Ouillette et al. 2010).

1.3.1.8. Somatic mutations in CLL.

The mutational status of immunoglobulin heavy chain variable (IGHV) gene in CLL has a strong and independent prognostic value. Accordingly CLL patients with unmutated *IGHV* genes have higher risks of relapse after stem cell transplantation and they reveal more frequently poor prognosis aberrations (11q-, 17p-); in addition to that they have shorter OS. Favorable aberrations (13q as a single abnormality) are more frequent in such CLL patients with mutated *IGHV* gene who have better OS (Oscier et al. 2002, Hamblin et al. 1999, Ritgen et al. 2003).

The *IGHV* mutation status is strongly associated with ZAP-70 expression levels: CLL patients with mutated *IGHV* genes are ZAP-70-positive, whereas ZAP-70-negative ones present more frequently the unmutated *IGHV* status. Thus, the ZAP-70 expression levels have been suggested as a surrogate marker for the investigation of *IGHV* mutation status (Wiestner et al. 2003, Orchard et al. 2004).

Also, next-generation sequencing (NGS) techniques provided better insights into genomic complexity and heterogeneity of CLL. Novel gene mutations have been identified in CLL, such as in *NOTCH1* and *SF3B1*. The detection rate of such abnormalities is 5% to 20%, and they are associated with advanced disease and poor prognosis (Quesada et al. 2012, Rodriguez-Vicente et al. 2013).

NOTCH1 encodes a transmembrane protein that acts as a ligand-activated transcription factor. *NOTCH1* signaling plays a critical role in CLL cell survival and apoptosis resistance. The mutation of *NOTCH1* leads to oncogenic pathway activation and it is more frequently associated with trisomy 12, unmutated *IGHV*, and poor prognosis. In addition to that the presence of

NOTCH1 mutation is considered as an independent predictor of shorter overall survival in CLL (Balatti et al. 2012, Campregher et al. 2014).

Finally, *SF3B1* gene is to mention as a core component of the spliceosome, which is involved in the splicing of precursor messenger RNA and in the formation of mature mRNA. Mutations in this gene appear in ~10% of CLL patients at diagnosis, and are associated with poor prognosis markers such as del (11q) and unmutated *IGHV* status. Interestingly, recurrent mutations of *SF3B1* have been reported in 17% of CLL patients who showed refractory to fludarabine treatment (Rossi et al. 2011, Quesada et al. 2012).

1.3.2. Treatment of CLL

Many factors play a role in the determination of the treatment of CLL such as the prognosis based on afore mentioned markers, patients' age, and the ability of the patient to tolerate side effects of treatments (Smolewski et al. 2013). The main used treatments are:

1.3.2.1. Standard chemotherapy and immunochemotherapy

Chemotherapy is recommended only for the CLL patients with advanced or progressive disease, as some CLL patients show a stable clinical course of the disease and they could survive for many years without any treatment (Dighiero et al. 2000).

Previously, chlorambucil was the drug of choice for the treatment of CLL patients with progressive or advanced disease. Currently, purine-nucleoside-analogue- (PNA-) based regimens such as fludarabine are considered the first line treatment for CLL. They could be used as a monotherapy or in a combination with cyclophosphamide, as the combination therapy appeared to be more effective than monotherapy with respect to overall response, and complete remission (Dighiero et al. 1998, Rai et al. 2000, Eichhorst et al. 2006). Addition of monoclonal antibodies such as rituximab to the combination of fludarabine and cyclophosphamide can increase the overall survival and complete remission of the CLL patients, and it is proved to be especially effective in refractory/relapsed CLL patients to the dual treatment (Hallek et al. 2010, Robak et al. 2010).

Rituximab is considered as anti-CD20, which is primarily found on the surface of B cells, therefore rituximab is used to treat diseases which are characterized by over-proliferation of B cells. Whereas this combination of drugs is acceptable for younger, physically fit patients, it has

limitations in the less fit group, mainly due to the risk of myelosuppression (Smolewski et al. 2013).

Recent clinical trial suggested bendamustine, which is a bifunctional agent composed of an alkylating nitrogen mustard group and a purine-like benzimidazole ring to be included in CLL treatment regimens (Knauf et al. 2009). On the other hand, alemtuzumab, which is a recombinant, humanized anti-CD52 monoclonal antibody, is recommended as a first line treatment in the CLL patients with 17p deletion and as a second- or third line treatment alone or in combination with other antineoplastic drugs in the CLL patients without 17p deletion, especially for those with hyperleukocytosis and no bulky nodal disease (Badoux et al. 2011, Hillmen et al. 2007, Gritti et al. 2012)

1.3.2.2. Stem cell transplantation

Although allogeneic hematopoietic stem cell transplantation (allo HSCT) introduces the only potentially curative treatment option for CLL patients, this approach is suitable for only a minority of CLL patients, because fully ablative regimens are associated with significant morbidity and mortality. Thus it has been considered as the treatment of choice for physically fit CLL patients who carry poor-risk features, such as refractory to purine analogs, short response time (<24 months) to intensive treatments, and/or presence of 17p/*TP53* abnormalities (Jaglowski et al. 2012). Recently, reduced-intensity conditioning was introduced for allo HSCT (mini allo HSCT), which is better tolerated than the myeloablative one (Smolewski et al. 2013).

1.3.2.3. New anticancer agents in CLL

A number of novel therapies and antibodies are available now for the treatment of CLL, such as Obinutuzumab (GA101) which is the first humanized anti-CD20 monoclonal antibody. It has been reported in preclinical studies to be more effective than rituximab in depleting B cells, including CLL cells (Illidge et al. 2012).

Also Oblimersen, which is an antisense oligodeoxyribonucleotide blocking transcription of proapoptotic Bcl-2 protein can make cancer cells more sensitive to chemotherapy. Other agents, like flavopiridol is a synthetic flavone, which is considered as a potent inhibitor of cyclin dependent kinases (CDKs). Also it triggers tumor cell *TP53*-independent apoptosis, making it applicable for such CLL patients with deletion in 17p. It has to be considered also toxicity of

flavopiridol being significant, including tumor lysis, infections, or diarrhea (Smolewski et al. 2013).

Finally, the immunomodulatory agent lenalidomide, which is one of the novel drug agents used to treat multiple myeloma, shows also activity in CLL. Lenalidomide is an effective and well-tolerated treatment alternative for elderly, symptomatic patients with CLL (Chen et al. 2011).

1.6. Aim of study/Questions worked on

Presence of cytogenetic abnormalities is a hallmark in CLL, according to which different prognosis and treatment regimens should be considered. Thus the detection of theses aberrations is of extreme importance, and it can be performed by various techniques. GTG-banding has a detection rate of ~48%, even after using a suitable mitogen such as TPA. Alternative and/or complementary approaches are iFISH, MLPA and/or aCGH techniques.

The aims of the present work were to:

1. How many cryptic chromosomal aberrations in the 85 studied CLL cases could be detected by MLPA, in comparison with routine iFISH and GTG-banding?
2. Could the underlying chromosomal abnormalities in CLL be precisely identified, to avoid misinterpretation of the prognosis so subsequently incorrect treatment regimens?
3. What is the percentage of *BIRC3* disruption in the studied 117 CLL cases, and its correlation with *ATM* deletion?
4. Is *BIRC3* disruption specific only for CLL?
5. Is presence or absence of i(17q) in CLL able to identify a new subgroup with more aggressive clinical course of the disease, and what is the best way for its detection?

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. **Alhourani E,** Rincic M, Othman MA Pohle B, Schlie C, Glaser A, Liehr T. **Comprehensive chronic lymphocytic leukemia diagnostics by combined multiplex ligation dependent probe amplification (MLPA) and interphase fluorescence in situ hybridization (iFISH).** Mol Cytogenet, 2014; 7(1):79.

3. Capela de Matos RR, De Figueiredo AF, Liehr T, **Alhourani E,** De Souza MT, Binato R, Ribeiro RC, Silva ML. **A novel three-way variant t(8;13;21)(q22;q33;q22) in a child with acute myeloid leukemia with *RUNX1/RUNX1T1*: The contribution of molecular approaches for revealing t(8;21) variants.** Acta Haematol, 2015; 134(4):243-245.

4. **Alhourani E,** Othman MA, Melo JB, Carreira IM, Grygalewicz B, Vujić D, Zecević Z, Joksić G, Glaser A, Pohle B, Schlie C, Hauke S, Liehr T. **BIRC3 alterations in chronic and B cell acute lymphocytic leukemia patients.** Oncol Lett, in press.

5. **Alhourani E,** Rincic M, Melo JB, Carreira IM, Glaser A, Pohle B, Schlie C, Liehr T. **Isochromosome 17q in chronic lymphocytic leukemia.** Leuk Res Treatment 2015; 2015:489592.

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

EXPERT REVIEWS

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 dif-ferent kinds of probes available for fluores-cence in situ hybridization (FISH) and the current state of molecular cytogenetics in can-cer diagnostics is given. This includes espe-cially the commercially available probe sets applied in routine neoplasia diagnostics and those multicolor FISH (mFISH) tools used in research to identify new tumor-associated criti-cal genomic regions.

Cytogenetic & molecular cytogenetics

The history of human cytogenetics started not before the year 1879. At this time, micro-scopes 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 aberra-tions 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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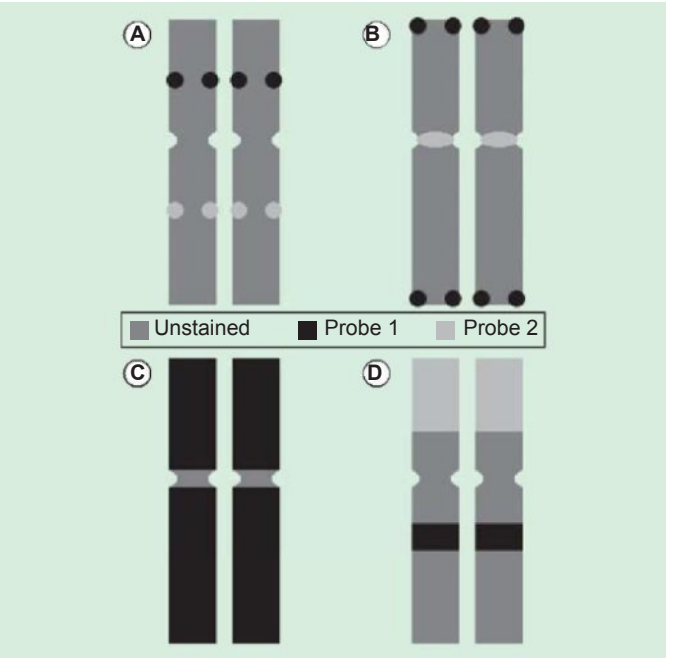

Figure 1. Schematic drawing depicting the four different kinds of fluorescence in situ hybridization-probes as differentiated in this review. (A) Locus-specific, single-copy probes, including subtelomeric probes. (B) Probes specific for repetitive sequences like telomeric (probe 1) and centromeric regions (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 over-come this kind of limitations, molecular cytogenetic approaches were and are necessary. In situ hybridization allows for examina-tion 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 can-cer 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 molecu-lar tumor-cytogenetic diagnostics, and they are even more con-siderable in research. mFISH is defined as the simultaneous use of at least three different ligands or fluorochromes for the spe-cific 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 col-leagues and E Schro□ck 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 dif-ferent 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 chro-mothripsis based on NGS [16]. However, it has to be empha-sized 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 diag-nostics, 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 onco-gene 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/PDGFRa
	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/RARa
	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 indepen-dently 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 informa-tion 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

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
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 applica-tions 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 leuke-mia [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 catego-rized 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 hyper-diploidy [5]) localized at different chromosomes.

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

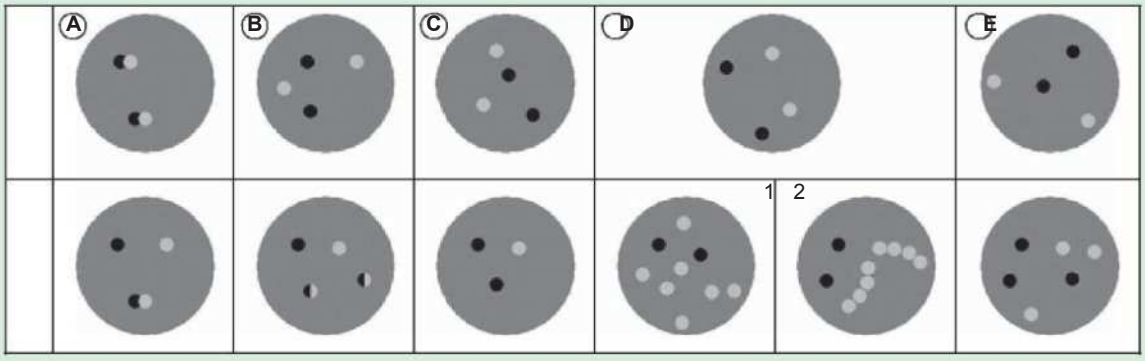

Figure 2. Schematic depiction of how locus-specific probes are normally combined in commercially available probe sets; the signal distribution as observed in an normal interphase cell is shown in the upper, the abnormal situation in the lower row.
(A) Dual-color break-apart probe; (B) dual-color dual 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, molecu-lar approaches have the advantage of being inexpensive and able to cover more targets at once. An approach that could the-oretically 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 cyto-genetic 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 per-form mFISH studies on a routine bases. Some countries in West-ern 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 can-cer needs to be done according to the results of tumor cytogenet-ics and/or the input of the referring clinician. Genetic counseling will not be necessary in most of neoplastic cases. However, excep-tions 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 super-numerary 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, pro-vides and will provide in future major input into the characteriza-tion 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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2.2. Article .2

Alhourani E, Rincic M, Othman MA Pohle B, Schlie C, Glaser A, Liehr T. **Comprehensive chronic lymphocytic leukemia diagnostics by combined multiplex ligation dependent probe amplification (MLPA) and interphase fluorescence in situ hybridization (iFISH).** Mol Cytogenet, 2014; 7(1):79.

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MOLECULAR CYTOGENETICS

RESEARCH Open Access

Comprehensive chronic lymphocytic leukemia diagnostics by combined multiplex ligation dependent probe amplification (MLPA) and interphase fluorescence in situ hybridization (iFISH)

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Abstract

Background: Banding-karyotyping and metaphase-directed-fluorescence-in-situhybridization (FISH) may be hampered by low mitotic index in leukemia. Interphase FISH (iFISH) is a way out here, however, testing many probes at the same time is protracted and expensive. Here multiplex-ligation-dependent-probe-amplification (MLPA) was used retrospectively in chronic lymphocytic leukemia (CLL) samples initially studied by banding cytogenetics and iFISH. Detection rates of iFISH and MLPA were compared and thus a cost-efficient scheme for routine diagnostics is proposed.

Results: Banding cytogenetics was done successfully in 67/85 samples. DNA was extracted from all 85 CLL samples. A commercially available MLPA probe set directed against 37 loci prone to be affected in hematological malignancies was applied. Besides, routine iFISH was done by commercially available probes for following regions: 11q22.3, 12p11.2-q11.1, 13q14.3, 13q34, 14q32.33 and 17p13.1. MLPA results were substantiated by iFISH using corresponding locus-specific probes.
Aberrations were detected in 67 of 85 samples (~79%) applying banding cytogenetics, iFISH and MLPA. A maximum of 8 aberrations was detected per sample; however, one aberration per sample was found most frequently. Overall 163 aberrations were identified. 15 of those (~9%) were exclusively detected by banding cytogenetics, 95 were found by MLPA (~58%) and 100 (~61%) by routine iFISH. MLPA was not able to distinguish reliably between mono- and biallelic del(13)(q14.3q14.3), which could be easily identified as well as quantified by routine iFISH. Also iFISH was superior to MLPA in samples with low tumor cell load. On the other hand MLPA detected additional aberrations in 22 samples, two of them being without any findings after routine iFISH.

Conclusions: Both MLPA and routine iFISH have comparable detection rates for aberrations being typically present in CLL. As MLPA can detect also rare chromosomal aberrations it should be used as an initial test if routine cytogenetics is not possible or non-informative. Still iFISH should be used additionally to distinguish mono- from biallelic deletions and also to determine rate of mosaicism for 13q14.2 to 13q14.3. In case MLPA is negative the corresponding CLL samples should be tested at least by iFISH using the standard probe set to.

Keywords: Chronic lymphocytic leukemia (CLL), Chromosomal aberrations, Multiplex ligation-dependent probe amplification (MLPA), Fluorescence in situ hybridization (FISH)

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Background

Chronic lymphocytic leukemia (CLL) is considered as the most common adult leukemia in Western countries with an estimated incidence of 5.8 in men and of 3.0 in women per 100,000 individuals and per year. It predominantly affects persons with more than 50 years of age [1,2]. A hall-mark of CLL is the presence of cytogenetic abnormalities; the latter help to estimate a patient's prognosis more ac-curately and also may provide insights into disease patho-genesis [3]. However, banding cytogenetics can only detect aberrations in ~30% of CLL samples [4]. Still, according to molecular (cyto)genetic data the major recurrent aberra-tions are:

(i) Deletions in 13q14 (50-60% of the samples) associated with a good prognosis, as are deletions in 14q32.33 (12-15% of the samples);
(ii) Trisomy 12 (15-25%) associated with intermediate prognosis; and
(iii) Deletions in 11q22 (ATM) (10-20%) or 17p13 (TP53) (5-10%) and/or recurrent balanced translocations go together with adverse prognosis [4- 9];
(iv) Less frequently observed aberrations in CLL are deletions in 6q associated with intermediate prognosis, 9p21 and 10q23, total or partial trisomies of chromosomes 3, 8, 18, or 19, and duplications in 2p24, the prognostic significance for these aberrations is unknown [1,10,11].

These aberrations were either detected applying cytogenetics and/or interphase fluorescence in situ hybridization (iFISH) [3] or more recently multiplex ligation-dependent probe amplification (MLPA) [7]. While iFISH provides information only for a limited number of genomic targets at the same time [1,5,7] MLPA can detect copy number alterations, methylation pattern changes and/or even point mutations simultaneously in multiple target regions [7,12]. Still iFISH can more reliably detect low level mosaics and mosaics of mono- and biallelic deletions [13].

In this study the efficiency of MLPA was compared with yet in our lab routinely performed cytogenetic and iFISH diagnostics of CLL. Based on the obtained results a new diagnostic scheme is proposed combining MLPA and iFISH leading to a more comprehensive characterization of each individual sample.

Results

85 samples of patients suffering from CLL (Additional file 1: Table S1 and Additional file 2: Table S2) were stud-ied here. Overall, including results from all here applied tests, chromosomal aberrations were detected in 70/85 (~85%) of the studied CLL-samples (Additional file 1: Table S1 and Additional file 2: Table S2). As summarized in Figure 1 between 0 and 8 aberrations were detectable per case. One chromosomal rearrangement per sample could be found most often (40%), followed by no aberration at all and three aberrations per sample. Four or more aberrations per sample were found in less then 10% of the cases.

Overall, 163 aberrations were detected in the 85 studied samples (Table 1, Additional file 2: Table S2). Cytogenetics revealed aberrant karyotypes in 15 (~22%) of the 67 samples where corresponding analyses was successful (Additional file 1: Table S1). In parts the cytogenetic findings could be substantiated by iFISH and or MLPA. As no corresponding probes were included neither in routine iFISH nor in MLPA, 15 (~9%) of the 163 detected ab-errations were found additionally by cytogenetics (Table 2). Interestingly, in sample 57 which presented with 5 chromosomal aberrations after banding cytogenetics no aberrations could be detected at all by iFISH or by MLPA. Other samples gave either no, a normal cytogenetic result or a result which also was confirmed by MLPA and/or iFISH (Additional file 1: Table S1).

Concerning the detection rates, the applied MLPA test found ~58% and routine iFISH ~61% of the 163 aberrations (Table 1, Additional file 2: Table S2). del(13)(q14.3q14.3) was most frequently found, i.e. in ~28% of the samples), followed by del(11)(q22.3q22.3) in ~9%, del(14)(q32q32) in ~8%, and del(13)(q14.2q14.2) and del(17)(p13.1p13.1) in ~6% of the samples, each.

Discordant results of MLPA and routine iFISH were in parts due to the different target regions covered by the tests; thus e.g. del(14)(q32q32) were only detectable by routine iFISH. On the other hand, MLPA detected additional aberrations in 22 samples, three of the patients being without any aberrant findings according to routine iFISH (Additional file 2: Table S2, cases 68–70).

In Table 3 thirteen samples are listed, which had low level mosaic aberrations based on routine iFISH and were not picked up by MLPA. In contrary in Table 4 twelve other samples with similar low level mosaics are listed, which were picked up by MLPA.

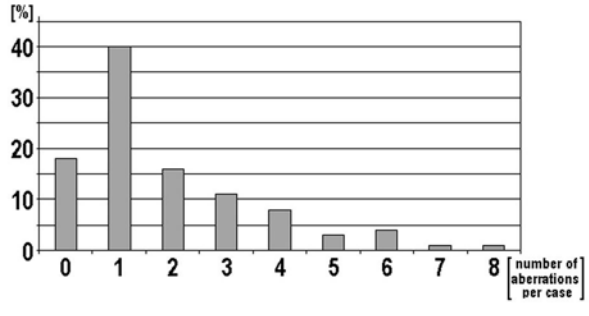

Figure 1 Number of aberrations present per sample as found in this study after application of all mentioned methods (banding cytogenetics, iFISH and MLPA) – values given in percent.

Table 1 Summary of 99 aberrations as detected by MLPA and 146 ones as detected or confirmed by iFISH; samples contributing to the discordant results of MLPA and iFISH are marked with asterisk *, ** or 'plus-sign' [+]

Affected regions	Genes	Detected in MLPA	Detected in iFISH
amp(2)(p24.3p24.3)	MYCN	3	3
amp(2)(p23.2 ~ 23.1p23.2 ~ 23.1)	ALK	3	3
del(6)(q21q21)	FYN	1	1
del(6)(q23.3q23.3)	MYB	2	2
del(6)(q25.1q25.1)	ESR1	1	1
del(6)(q27q27)	SMOC2	1	1
amp(6)(q27q27)	SMOC2	1[+]	0
amp(8)(q24.21q24.21)	MYC	1	1
t(9;22)(q34;q11)	BCR and ABL	n.a.	1
del(11)(q22.3q22.3)	ATM	12	14*
+12	ETV6, CCND2, MDM2	4	6*
del(13)(q14.2q14.2)	RB1	10	11**
del(13)(q14.2q14.2)x2	RB1	1	10**
del(13)(q14.3q14.3)	DLEU1, DLEU2, MIR15A	35	46*/**
del(13)(q14.3q14.3)x2	DLEU1, DLEU2, MIR15A	7	14**
del(14)(q32q32)	IGH	n.a.	13
rea(14)(q32.33) - > t(14;?)	IGH	n.a.	2
rea(14)(q32.33) - > ? + 14	IGH	n.a.	1
del(17)(p13.1p13.1)	TP53	9	10*
amp(17)(q25.1q25.2)	UNC13D	2	2
amp(18)(p11.21q11.21)	DCC	2[+]	1
amp(18)(q21.2q21.2)	RNMT	2[+]	1
amp(21)(q22.12q22.12)	RUNX1	2	2

Those with * are detailed in Table 2, those with ** in Table 4. Those with [+] could either not be tested in iFISH due to lack of corresponding probe or, in the two of the tested samples MLPA could not be confirmed by iFISH (routine and confirmatory together), most likely due to too large FISH-probe size.

Table 5 highlights 19 samples which were detected as carrying deletions in 13q14.2 and/or 13q14.3 according to MLPA and iFISH. Still iFISH revealed that there was a mix of monoallelic and biallelic deletion or only biallelic deletion, which could not always be detected by MLPA (Additional file 2: Table S2). Only such cases which had 100% biallelic deletions could be identified undoubtedly (e.g. sample 30); others showed biallelic deletions in MLPA but were indeed a mix of mono- and biallelic ones.

Finally, three copy number alterations found by MLPA could not be substantiated by additional iFISH studies (samples 65–67; Additional file 2: Table S2).

In Figure 2 a flow is suggested how a CLL-characterization could be performed most comprehensively and straight forward. Figure 3 shows how cases would have been grouped if only cytogenetics, only MLPA or only iFISH would have been done. Tables 6, 7 and 8 highlights how a step by step characterization and corresponding new results of would change the prognosis of the 95 studied cases.

Table 2 Aberrations only detected by banding cytogenetics in 9 samples of the present study

Sample number	Aberration only visible in GTG-banding [%]
1	del(5)(p1?3)[33]
32	-Y[44]
34	-Y[50]
38	t(3;?)(p21;?)[43]
41	-Y[80]
57	der(1)t(1;4)(q1?2;q?31)[90]
	der(4)t(4;?10)(q?31;q24)[90]
	?der(10)t(10;16)(q24;p?11.2)[90]
	der(15)t(1;15)(q1?2;q1?2)[90]
	der(16)t(15;16)(q1?2;p?11.2)[90]
58	der(2)t(2;13)(q?37;q?14)[21]
	?del(6)(p?23)[21]
61	t(3;?)(q2?9;?)[22]
	−7[22]
70	?add(1q)(q4)[50]

Discussion

When diagnostic screening for acquired genetic alteration in hematological malignancies is to be done, banding cytogenetics is still the gold standard, as it enables

Table 3 Detailed results in samples contributing to the discordant results of MLPA and iFISH marked with asterisk * in Table 1

Affected regions	Genes	Sample number	iFISH mosaic [%]
del(11)(q22.3q22.3)	ATM	1	30
del(11)(q22.3q22.3)	ATM	2	33
+12	ETV6, CCND2, MDM2	3	15
+12	ETV6, CCND2, MDM2	4	31
del(13)(q14.3q14.3)	DLEU1, DLEU2, MIR15A	5	18
del(13)(q14.3q14.3)	DLEU1, DLEU2, MIR15A	6	10
del(13)(q14.3q14.3)	DLEU1, DLEU2, MIR15A	7	10.5
del(13)(q14.3q14.3)	DLEU1, DLEU2, MIR15A	8	12
del(13)(q14.3q14.3)	DLEU1, DLEU2, MIR15A	9	18.5
del(13)(q14.3q14.3)	DLEU1, DLEU2, MIR15A	10	25
del(13)(q14.3q14.3)	DLEU1, DLEU2, MIR15A	11	34
del(13)(q14.3q14.3)	DLEU1, DLEU2, MIR15A	12	34
del(17)(p13.1p13.1)	TP53	13	11.5

Table 4 Detailed results in samples with concordance of MLPA and routine iFISH results but mosaic rates below 40% according to iFISH

Affected regions	Genes	Sample number	iFISH mosaic [%]
del(11)(q22.3q22.3)	ATM	14	23.5
del(11)(q22.3q22.3)	ATM	15	24
del(11)(q22.3q22.3)	ATM	16	11
del(13)(q14.3q14.3)	DLEU1, DLEU2, MIR15A	1	30
del(13)(q14.3q14.3)	DLEU1, DLEU2, MIR15A	2	18
del(13)(q14.3q14.3)	DLEU1, DLEU2, MIR15A	4	20
del(13)(q14.3q14.3)	DLEU1, DLEU2, MIR15A	14	34
del(13)(q14.3q14.3)	DLEU1, DLEU2, MIR15A	17	20
del(17)(p13.1p13.1)	TP53	1	16
del(17)(p13.1p13.1)	TP53	12	21
del(17)(p13.1p13.1)	TP53	18	19
del(17)(p13.1p13.1)	TP53	19	36

Table 5 Combination of biallelic and/or monoallelic deletion del(13)(q14.2q14.2) and del(13)(q14.3q14.3) – which is not clearly resolved by MLPA

Sample number	iFISH mosaic [%] del(13)(q14.2q14.2)		iFISH mosaic [%] del(13)(q14.3q14.3)	
	Monoallelic deletion	Biallelic deletion	Monoallelic deletion	Biallelic deletion
2	0	0	18	14
4	45	0	20	0
12	52	38	34	0
13	0	0	0	98.5
20	0	0	0	94
21	50	30	0	91
22	0	0	5	75
23	0	0	5	81
24	36	41	16	71
25	66	21	18	77
26	0	0	25	65
27	34	27	36.5	24
28	0	0	81	7
29	58	24	86	9
30	0	0	0	100
54	41	39	97	0
55	73	5	85	0
56	22	58	12	66
63	51	38	90	0

the untargeted search for gross chromosomal aberrations [14]. Malignant CLL cells derived from bone marrow are known to have a low mitotic index and in many cases only cytogenetically normal cells can be analyzed [4]. Thus, iFISH and MLPA are routinely applied additionally to or even as a replacement in tumorcytogenetics of CLL [7,15].

In this study, after directed diagnostics for 37 genetic loci (MLPA and routine iFISH together), still ~18% of the samples remained without an identified tumor marker. As highlighted by samples 32, 34, 41, 36 (see as well [16]) 38, 57, 58, 61 and 70 this can be due to unusual, not by tar-geted routine tests covered chromosomal aberrations; be-sides submicroscopic aberrations like point mutations [2] could be present in those 'normal' samples. Interestingly, in over 40% of the studied cases more than only one chromosomal aberration was identified (Figure 1). This may reflect in parts the slow progress of CLL. I.e. the ma-lignancy is detected after acquiring multiple aberrations and not as early as e.g. chronic myelogeneous leukemia (CML), which is already connected with severe clinical signs when only a t(9;22) is found, which is the only aber-ration in majority of the CML-cases [17].

As mentioned above, MLPA and routine iFISH are tar-geted tests, both. As they cover in parts different loci it was not unexpected that they have different detection rates. However, one would expect that iFISH technique underestimates the genomic complexity in CLL [1]. Still it is striking that the routine iFISH test found 61% of the 163 aberrations while MLPA only detected 58%, even though routine iFISH applied only 5 probes and MLPA had more than 7 times more, i.e. 37 target regions.

Concerning detection of low level mosaics (10% up to 36% of the cells being aberrant) this study showed that there are about alike amounts of cases being detectable and being missed by MLPA (Tables 3 and 4). There were cases detectable by MLPA with aberrant cell clone sizes down to ~10% according to iFISH (sample 16) and such being not detectable (samples 6, 7, and 8). To the best of our knowledge there are only few previous [18-20] and no systematic studies for the detection rates of low level mosaic in MLPA. Véronèse et al. [7] suggested that all false-negative cases occur in samples with only 12-21% of aberrant cells; thus they considered MLPA detection to be reliable when the fraction of aberrant cells is 25-30%, which is definitely less sensitive than iFISH detection. Overall, this problem has to be kept in mind when doing MLPA exclusively in routine diagnostics.

Still, the findings of this study are in concordance with Stevens-Kroef et al. [21] who claimed an almost perfect correlation between MLPA and iFISH, as long as identical genetic regions are tested in MLPA and iFISH. However, bi- and monoallelic deletions coming together in one sam-ple are not considered in this kind of comparison. Still, all apart from three MLPA findings not detectable in the

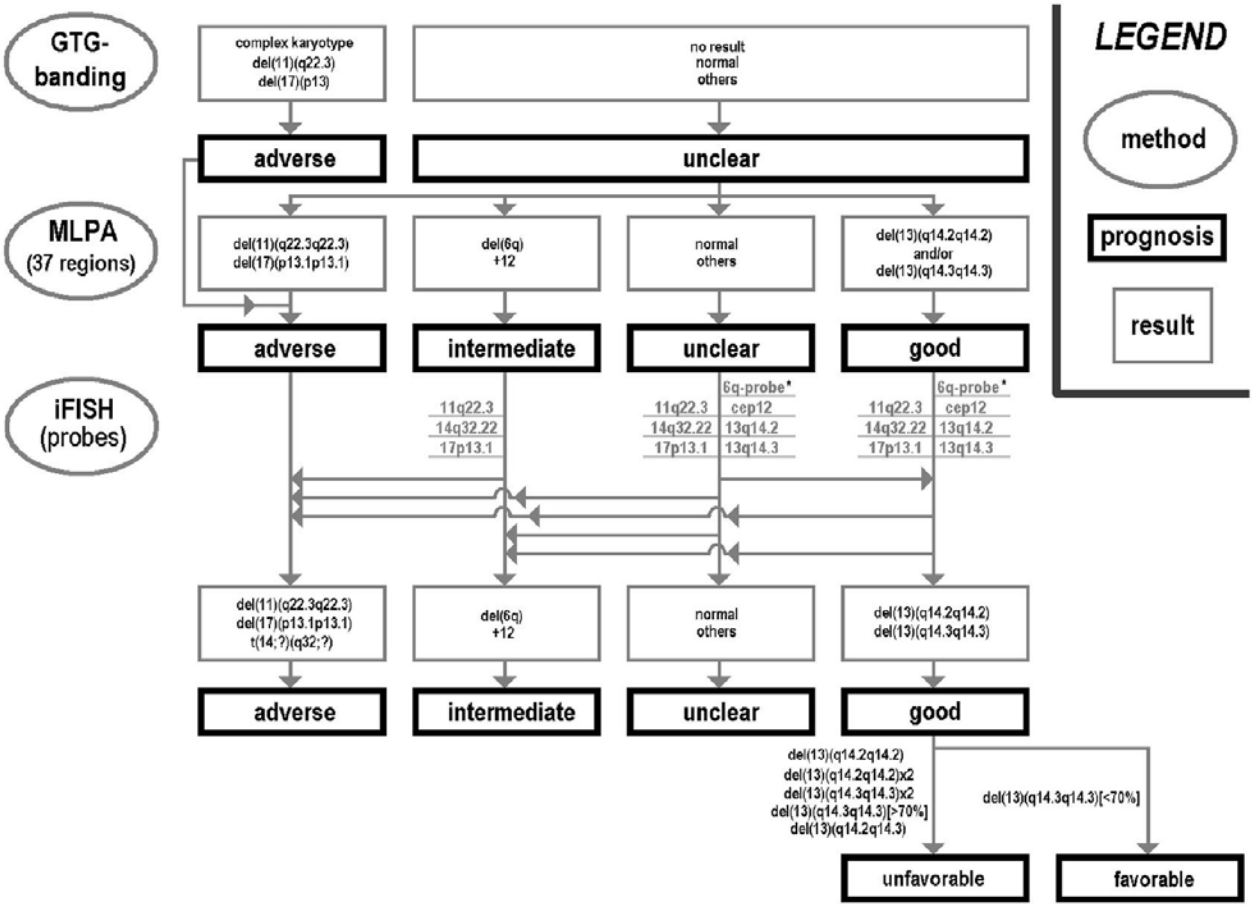

Figure 2 Suggestion how to proceed when doing MLPA as a primary test after GTG-banding: in case MLPA finds a tumor marker with adverse prognosis no further iFISH analyses is necessary. In case of an MLPA result suggesting intermediate, unclear or good iFISH for 3 to 6 target regions should be done. A probe for 6q may be also used; however, as case with a del(6q) are rare we would not recommend it at present as really indicated to be applied. According to the obtained results cases need to be regrouped. Finally, iFISH can be used to subclassify cases with good prognosis into such with favorable and unfavorable good prognosis.

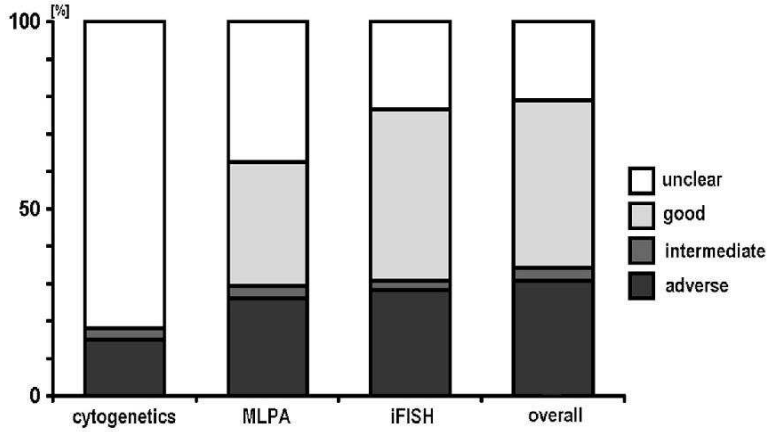

Figure 3 Detection rates of cytogenetics, MLPA and iFISH as standalone approaches are depicted and compared with overall result combining all three tests as suggested in Figure 2; the corresponding results obtained in the 85 cases were aligned with and are expressed as the resulting prognostic relevance of the identified chromosomal aberrations.

Table 6 Samples from Additional file 2: Table S2 are listed according to the groups suggested in Figure 2

Results according to MLPA	Adverse prognosis	Intermediate or unclear prognosis	Good prognosis including groups "favorable" and "unfavorable"	No aberrations
Samples	1, 10, 12, 14, 15, 16, 17, 18, 19, 24, 34, 35, 38, 39, 54, 58, 61, 63, 64, 65,	5, 37, 62, 68	2*, 4*, 13*, 20*, 21*, 22*, 23*, 25*, 26*, 27*, 28*, 29*, 30*, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 55*, 56*, 66, 67, 69	3, 6, 7, 8, 9, 11, 31, 32, 33, 36, 57, 59, 60, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85
Number of samples per group (absolute)	20	4	32	29
Number of samples per group (percent)	23.5	5	37.5	34

Samples marked with * have biallelic deletion in 13q14 as substantiated by iFISH or deletion of 13q14.2 and 13q14.3, thus going from favorable to unfavorable subgroup within good prognosis group after iFISH (see Table 7). Figures printed not bold and not in italics are case numbers; figures printed bold and in italics are absolute numbers of samples or same numbers in percent.

applied routine iFISH setting could be verified by subsequent targeted iFISH. In the not verified cases this can be due to too small size of the detected copy number alteration, not resolvable by iFISH.

It is well known that there are different clinical prognoses if a del(13)(q14.3q14.3) comes mono- or biallelic and alone or together with a del(13)(q14.2q14.2): larger deletions like del(13)(q14.2q14.3) and biallelic deletions have shorter time to first treatment [1,22,23]. To get reliable information for this question a combination of MLPA and FISH is necessary.

According to Campregher and Hamerschlak [2] the detected aberrations can be grouped in such with adverse, intermediate, good prognosis. Those cases with good prognoses are further subdivided in such cases with favorable and such with less favorable outcome. Especially cases with adverse prognosis have influence on the therapeutic decisions. Taken together with the results of this study we suggest a diagnostic flow as shown in Figure 2.

As both MLPA and routine iFISH have in principle comparable detection rates in CLL, MLPA is more cost ef-ficient than iFISH and it covers a more broad spectrum of target genes [12], we recommend MLPA to be the initial diagnostic test. The impact for the patient car-rying rare mutations can be evident: Fabris et al. [11] reported that 2p gain can be present already in early stages of the disease, particularly in those cases characterized by other poor prognostic markers (samples 5, 16 and 63); del(6q) is generally considered as an intermediate-risk factor [1,10] (samples 5 and 68); finally, López et al. [24] reported more rapid disease progression if trisomy 12 is accompanied by additional aberrations rather than if it is the only genetic abnormality (sample 62). Also new data may be acquired, as e.g. the impact of gain of MYC [1] (sample 16) or RUNX1 gene [25] (samples 5 and 69) are still unclear in CLL. If the diagnostic scheme suggested in Figure 2 would have been applied in the 85 patients pre-sented here in 20 of them (23.5%) no iFISH would have been necessary. In those 20 patients (Tables 6, 7 and 8) MLPA would have already identified one or more ad-verse chromosomal aberrations leading to a therapeutic consequence.

Four patients (Tables 6, 7 and 8) would have been grouped into 'intermediate prognosis' after MLPA, one of them just having a trisomy 12 (sample 37). So in this group of pa-tients, only three probes for the adverse prognosis regions should be applied in iFISH testing.

Normal MLPA result as found in 29 samples (= ~34%) all six (or seven, see legend of Figure 2) FISH probes as listed in Figure 2 should be applied to rule out low level mosaics of del(11)(q22.3q22.3), +12, del(13)(q14), del(17)(p13.1p13.1) or del(14)(q32q32). In the present

Table 7 Regrouping of samples from Table 6 after doing additional i-FISH as suggested in Figure 2

Results according to MLPA	Adverse prognosis	Intermediate prognosis	Good prognosis "unfavorable"	Good prognosis "favorable"	No aberrations
Samples	1+, 2, 3+, 10, 12, 13, 14, 15, 16, 17, 18, 19, 24, 34, 35, 38, 39, 54, 58, 60+, 61, 63, 64, 65,	4, 5, 37, 62, 68	20, 21, 22, 23, 25, 26, 27, 28, 29, 30, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 55, 56, 66, 67	6, 7, 8, 9, 11, 31, 32, 33, 40, 41, 42, 59, 69	36, 57, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85
Number of samples per group (absolute)	24	5	25	13	18
Number of samples per group (percent)	28	6	30	15	21

Samples marked with + have rea(14)(q32.33), thus they have to go to the adverse prognosis group. Samples with deletion of 13q14.2 and 13q14.3 detected by MLPA and/or deletion of 13q14.3 in ≥70% of the nuclei detected by iFISH go to unfavorable subgroup within good prognosis group. Figures printed not bold and not in italics are case numbers; figures printed bold and in italics are absolute numbers of samples or same numbers in percent.

Table 8 Final result after including result of GTG-banding based on from Tables 6 and 7

Results according to MLPA	Adverse prognosis	Intermediate or unclear prognosis	Good prognosis "unfavorable"	Good prognosis "favorable"	No aberrations
Samples	1, 2, 3, 10, 12, 13, 14, 15, 16, 17, 18, 19, 24, 34, 35, 38, 39, 54, 57*, 58, 60, 61, 63, 64, 65, 70*	4, 5, 36*, 37, 62, 68	20, 21, 22, 23, 25, 26, 27, 28, 29, 30, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 55, 56, 66, 67	6, 7, 8, 9, 11, 31, 32, 33, 40, 41, 42, 59, 69	71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85
Number of samples per group (absolute)	26	6	25	13	15
Number of samples per group (percent)	31	7	30	15	17

Samples marked with * have additional aberration not detectable by MLPA or routine iFISH. Figures printed not bold and not in italics are case numbers; figures printed bold and in italics are absolute numbers of samples or same numbers in percent.

cohort e.g. samples 2 and 13 go to "good prognosis", samples 3 and 60 to "adverse prognosis" and sample 4 to "intermediate prognosis" group (Tables 6, 7 and 8).

Finally, 32 patients (Tables 6, 7 and 8) have been classified as 'good prognosis' after MLPA. Here, the same FISH probes as for normal MLPA result should be used for further subclassification (Figure 2). Again patients then may have to be moved to other prognostic groups if additional or low level mosaics are identified. Also it is known that CLL cases with del(13)(q14.2q14.2) go into unfavorable subgroup, as do such cases with biallelic dele-tions in 13q14. Finally, del(13)(q14.3q14.3) detected in ≥70% of the cells are also an indication to group a patient in unfavorable subgroup of 'good prognosis' group [1,22]. Thus, further I-FISH studies are necessary also for patients with del(13)(q14.2q14.2) and/or del(13)(q14.3q14.3) in MLPA.

In case only MLPA and iFISH would have been done in the presently studied 85 patient still 3 samples would have been misclassified. Thus we suggest in Figure 2 still GTG-banding as the initial test for CLL diagnostics. Compared to a flow just applying banding cytogenetics and routine iFISH for diagnostics of CLL the introduction of the flow from Figure 2 would apply only 344 instead of 425 FISH-probes, i.e. 20% less.

Conclusion

The present study shows the importance of combining cytogenetics, molecular genetics and molecular cytogenetics to achieve a comprehensive characterization of acquired genetic alterations being present in CLL.

Methods

Patients and sample preparation

The present study included 85 samples of patients suffering from CLL (Additional file 1: Table S1 and Additional file 2: Table S2) diagnosed according to standard criteria [26]. The samples were obtained under informed consent of the corresponding patients and according to institutional ethical committee guidelines (Ethical commitee of the Friedrich Schiller University Jena).

DNA from lymphocytes was extracted by a commercial kit (Qiagen, Hilden, Germany) and was derived from different sources: 2 samples from heparinized bone marrow, 8 samples from heparinized blood, and 75 samples from cytogenetically prepared cells fixed in methanol/acetic acid (3:1) – 48 of them derived from bone marrow and 27 from blood (Additional file 1: Table S1).

GTG-banding and FISH analysis

The blood or bone marrow samples were stimulated with phorbol ester, i.e. 12-O-tetradecanoylphorbol-13-acetate (TPA) and cultivated for 96 hours, and a standard cytogenetic cell preparation following air drying method was done [27]. GTG-banding and iFISH analyses were routinely done in each sample following standard procedures [27,28]. In 67 samples chromosomes could be obtained from the material prepared.

For routine iFISH the following commercially available probe sets (Abbott/Vysis, Wiesbaden, Germany) were used: LSI p53/LSI ATM (in 17p13.1 and 11q22.3), LSI D13S319/LSI 13q34/CEP 12 (in 13q14.3, 13q34 and 12p11.1-q11.1), and LSI IGH dual color, break-apart probe (in 14q32.33).

Additionally, the following probes were used to validate and possibly confirm the results of MLPA:

- from Abbott/Vysis (Wiesbaden, Germany): LSI 13 (RB1 in 13q14.2), CEP 6 (D6Z1 in 6p11.1-q11,1), CEP 17 (D17Z1 in 17p11.1-q11.1) and CEP 18 (D18Z1 in 18p11.1-q11.1);
- from Zytovision (Bremerhaven, Germany): ZytoLight ®SPEC ALK Dual Color Break Apart (in 2p22.32 ~ 22.31), ZytoLight ®SPEC NMYC/2q11 Dual Color (in 2q24.3 and 2q11), ZytoLight ®SPEC MYB Dual Color Break Apart (in 6q23.3), ZytoLight ®SPEC ESR1/CEN 6 Dual Color (in 6q25.1 and 6p11.1-q11.1), ZytoLight ®SPEC CMYC/CEN 8 Dual Color (8q24.21 and 8p11.1-q11.1), ZytoLight ®SPEC ETV6/RUNX1 Dual Color Dual Fusion (in 12p13.2 and 21q22.12); and
- BACPAC Resources Center (Oakland, USA): RP1-142 L7 in 6q21 (gene FYN), RP11-318A15 in 17q25.1 (gene UNC13D), RP11-346H17 in 18q21.2 (gene DCC), RP11-37D8 in 6q27 (gene SMOC2) and RP11-411B in 18p11.22 (gene RNMT).

Table 9 Loci addressed in the commercially available MLPA kit used in this study

Targets	Loci	Number of probes included in kit
MYCN	2p24.3	2
ALK	2p23.2 ~ 23.1	1
MIR145	5q33.1	1
EBF1	5q33.3	2
MIR146A	5q33.3	1
FYN	6q21	1
MYB	6q23.3	1
ESR1	6q25.1	1
SMOC2	6q27	1
IKZF1	7p12.2	3
CDK6	7q21.2	1
RELN	7q22.1	1
MET	7q31.2	1
DPP6	7q36.2	1
MYC	8q24.21	2
MTAP	9p21.3	1
CDKN2A	9p21.3	1
CDKN2B	9p21.3	1
PAX5	9p13.2	2
PTEN	9p13.1	1
PTEN	10q23.31	1
ATM	11q22.3	4
ETV6	12p13.2	2
MDM2	12q15	1
CCND2	12p13.32	1
RB1	13q14.2	2
MIR15A	13q14.3	1
DLEU1	13q14.3	1
DLEU2	13q14.3	1
TP53	17p13.1	4
UNC13D	17q25.1	1
IKZF3	17q12	1
DCC	18q21.2	1
RNMT	18q21.2	1
CACNA1A	19p13.13	1
CHMP2A	19q13.43	1
RUNX1	21q22.12	2

For each iFISH analysis, at least 100–200 interphase nuclei were examined per sample and FISH-probe.

MLPA analysis

MLPA was performed using SALSA MLPA probemix P377-A1 for Hematological Malignancies Kit from (MRC-Holland, Amsterdam, The Netherlands). The P377-A1 probemix kit contains probes for 37 genes covered by overall 52 probes, which have diagnostic or prognostic significant role in hematologic malignancies (see Table 9).

MLPA was performed according to the manufacturer's protocol, which includes three reaction phases: hybridization, ligation, and PCR. Finally, a capillary electrophoresis was used to separate and analyze MLPA PCR products. Genemarker software was used to analyze the peak areas of the MLPA PCR products, and the ratio was normalized to a healthy control. Threshold of detection was set at 0.65-1.35, to minimize the false positive cases.

Additional files

Additional file 1: Table S1. Gender, age and cytogenetic results of the studied cases/samples.

Additional file 2: Table S2. Aberrations detected in 85 CLL samples and by which method the corresponding aberrations could be detected.

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

Authors' contributions
EA performed FISH experiments and drafted the paper; EA, MR and MAKO and did the MLPA experiments, BP, CS and AG perfomred the banding cytogenetic analyses, and TL planned the study and finalized the paper. All authors read and approved the final submission.

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2.2. Article .3

Capela de Matos RR, De Figueiredo AF, Liehr T, **Alhourani E,** De Souza MT, Binato R, Ribeiro RC, Silva ML. **A novel three-way variant t(8;13;21)(q22;q33;q22) in a child with acute myeloid leukemia with *RUNX1/RUNX1T1*: The contribution of molecular approaches for revealing t(8;21) variants.** Acta Haematol, 2015; 134(4):243-245.

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A Novel Three-Way Variant t(8;13;21)(q22;q33;q22) in a Child with Acute Myeloid Leukemia with *RUNX1/RUNX1T1*: The Contribution of Molecular Approaches for Revealing t(8;21) Variants

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The translocation t(8; 21)(q22;q22)/*RUNX1/RUNX1T1* is one of the most common translocations in pediatric acute myeloid leukemia (AML), accounting for 10–20% of all cases [1]. At the molecular genetic level, the rearrangement is defined by involvement of the *RUNX1 (AML1)* gene on chromosome 21q22 and the *RUNX1T1 (ETO)* gene on chromosome 8q22, resulting in the *RUNX1/RUNX1T1 (AML1/ETO)* fusion gene product [2]. The fusion protein disrupts the core binding factor transcription complex, leading to abnormalities in cell differentiation, proliferation and apoptosis. It is also thought that the fusion product is a driver of myeloid leukemogenesis in this AML subtype [3].

Approximately 3–4% of cases of AML with the t(8; 21)(q22;q22) occur in the context of complex rearrangements. Although t(8; 21) is associated with a good prognosis, the impact of the complex t(8; 21) variants is controversial. Some researchers have reported a favorable outcome for AML patients with complex t(8; 21) variants, while others have not confirmed these observations [4]; in these series, almost all of the patients were adults [2, 4, 5]. The clinical relevance and implications of t(8; 21) variants in pediatric patients are yet to be determined. These translocations are rare, so there is limited informa-tion on their prognostic impact. Thus, to make a contri-bution to the registry of t(8; 21) with complex variants, we present a case that belongs to this rare subgroup. We report on a child with AML harboring a novel three-way cryptic variant t(8; 13; 21), as revealed by detailed molecu-lar studies.

From May 2007 to March 2014, we analyzed samples from 114 children and adolescents (aged 0–18 years) with AML, 13 (11.4%) of whom harbored the t(8; 21) (q22;q22) and were examined by means of GTG band-ing, fluorescence in situ hybridization (FISH) and RT-PCR. Of these 13 RUNX1/RUNX1T1-positive patients, 3 (2.6%) did not have classic t(8; 21) by conventional karyo-typing. When FISH assay was performed, it was possible to observe that there was a third chromosome involved, with a *RUNX1/RUNX1T1* split signal, thus characterizing

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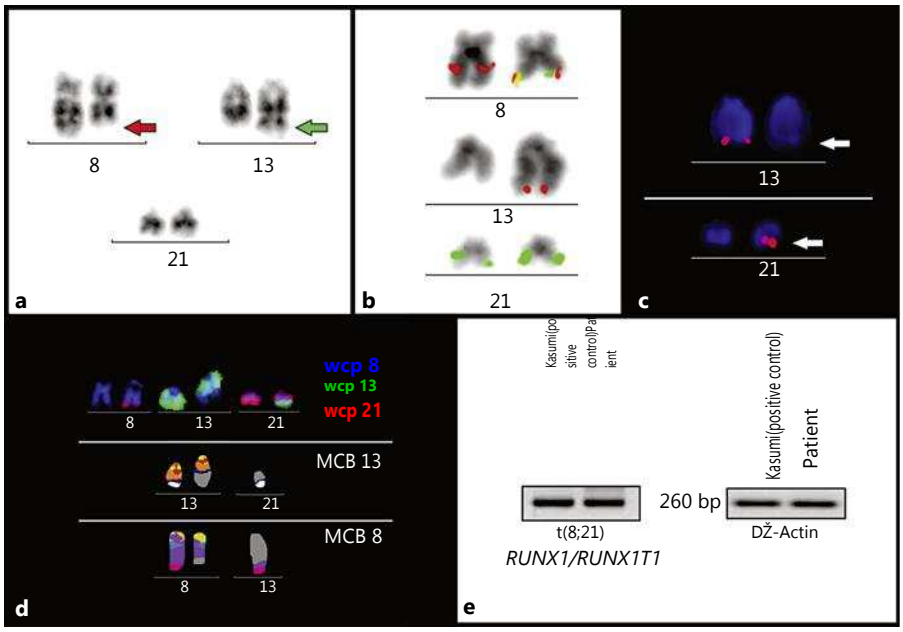

Fig. 1. **a** Partial G-banding karyotype. The red arrow shows a missing portion in chro-mosome 8, and the green arrow shows a gain of chromosomal material on chromo-some 13. **b** FISH with the *AML1/ETO* dual-color, dual-fusion probe, showing the *RUNX1/RUNX1T1* fusion on derivative chromosome 8 and a *RUNX1T1* split sig-nal to chromosome 13. **c** Complementary FISH, with a subtelomeric probe for the 13qter region, revealing that a portion of this region was translocated to chromo-some 21. **d** FISH with whole-chromosome painting (wcp) probes and MCB for chro-mosomes 8, 13 and 21, showing the origin and the breakpoints of each rearrange-ment. **e** RT-PCR confirmed the presence of the *RUNX1/RUNX1T1* fusion and revealed a PCR product of 260 base pairs (bp).

a masked variant of t(8; 21). These 3 cases were then se-lected to be studied by means of multicolor chromosome banding (MCB). We describe a novel t(8; 13; 21) variant in detail.

A 13-year-old girl was admitted with a 5-month his-tory of pallor and upper-airway symptoms associated with persistent fever, otalgia and dysacousia. At admis-sion, she had a white blood cell count of 22 × 10^9/l, a plate-let count of 96 × 10^9/l and hemoglobin of 6.2 g/dl. A chest X-ray showed consolidation in the lower/upper/left/right lobes, consistent with bacterial pneumonia. Physical ex-amination revealed lymphadenopathy in the cervical and inguinal regions, hepatomegaly (4 cm) and splenomegaly (7 cm). The bone marrow was hypercellular with 64% myeloid blast cells positive for CD45, CD34, CD117, MPO, CD33, CD13, HLA-DR, CD123, CD15 and CD19, i.e. compatible with AML.

GTG banding analysis defined the karyotype as 45,X,-X,del(8)(q22),der(13q3?) in 23 metaphases (fig. 1a). FISH analysis confirmed a cryptic fusion *RUNX1/ RUNX1T1* on derivative chromosome 8, with the pres-ence of a *RUNX1T1* split signal on derivative chromo-some 13 (fig. 1b). Complementary FISH analysis using a subtelomeric probe for the 13q region, showed a 13qter minor signal on chromosome 21 characterizing a cryptic translocation (fig. 1c). The application of whole-chro-mosome painting probes for chromosomes 8, 13 and 21 revealed a three-way translocation. To characterize the breakpoints of this complex rearrangement, MCB stud-ies were applied revealing the karyotype: 45,X,-X,t(8; 13; 21)(q22;q33;q22) (fig. 1d). RT-PCR for the *RUNX1/ RUNX1T1* fusion revealed a product of 260 base pairs (fig.1e).

The patient was stratified as being at standard risk, and was treated according to the AML-BFM-2004 protocol [6]. She achieved complete remission, but after receiving an intensification block, she developed febrile neutrope-nia and sepsis. She died of cardiac and respiratory failure 5 months after the initial diagnosis.

There are at least two steps for the formation mech-anism of the complex t(8; 21), following the formation of standard t(8; 21)(q22;q22) and the *RUNX1/RUNX1T1* fusion gene [7]. Material from the distal long arm of chromosome 21q22 translocates to the long arm of chromosome 8, but the end of chromosome 8 translo-cates to a third chromosome. The remainder of the third chromosome translocates to chromosome 21. The same behavior may have occurred in the transloca-tion in our patient and in 2 others previously reported [7, 8].

The involvement of chromosome 13 in a complex t(8; 21) variant has, so far, been reported in 3 patients [7, 8] including ours. In contrast to the other cases, the variant t(8; 13; 21)(q22;q33;q22) described here presented as a masked karyotype on GTG banding, and additional ma-terial was cryptically translocated on derivative chromo-

some 21, thus adding a novel t(8; 13; 21) variant to the literature.

Although the t(8; 21) complex variant accounts for only 0.05–1.1% of cases of childhood AML [2, 4, 5, 9], its frequency was higher (approx. 2.6%) in our cohort, suggesting that, in pediatric AML, a detailed characterization of *RUNX1T1* split signal via a combination of FISH, MCB and RT-PCR approaches may be necessary to uncover such complex variants. In our cohort as well as in the previously described cases [7, 8], the *RUNX1T1* gene (8q22) often splits to the third chromosome involved in the translocation. Thus, the observation that a similar formation mechanism of the complex t(8; 21) variant that preferentially involves the same chromosome regions, along with the higher frequency of complex t(8; 21) variants (that we observed in our cohort), reinforces the impor-tance of the clarification of such complex cases in order to investigate if the genes in these regions are involved in leukemogenesis.

Furthermore, it is important that cases with complex conventional karyotypes and a *RUNX1/RUNX1T1* split signal involving ≥3 chromosomes are analyzed by a combination of molecular assays. This approach can provide further knowledge about the heterogeneity of the *RUNX1/RUNX1T1* fusion gene and a possible association with prognosis. Finally, future studies involving bacterial artificial chromosome probes and next-generation sequencing are required to further pinpoint the breakpoint re-gions and describe the genes involved in all of the fusions that result from these complex rearrangements [10, 11].

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2.2. Article .4

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BIRC3 alterations in chronic and B-cell acute lymphocytic leukemia patients

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Abstract. Deletions within chromosome 11q22-23, are considered among the most common chromosomal aberrations in chronic lymphocytic leukemia (CLL), and are associated with a poor outcome. In addition to the ataxia telangiectasia mutated (*ATM*) gene, the baculoviral IAP repeat-containing 3 (*BIRC3*) gene is also located in the region. *BIRC3* encodes a negative regulator of the non-canonical nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) protein. Disruption of *BIRC3* is known to be restricted to CLL ludarabine-refractory patients. The aim of the present study was to determine the frequency of copy number changes of *BIRC3* and to assess its association with two known predictors of negative CLL outcome, *ATM* and tumor protein 53 (*TP53*) deletions. To evaluate the speciicity of *BIRC3* alterations to CLL, *BIRC3* copy numbers were assessed in 117 CLL patients in addition to 45 B-cell acute lymphocytic leukemia (B-ALL) patients. A commercially available multiplex ligation dependent probe ampliication kit, which includes four probes for the detection of *TP53* and four probes for *ATM* gene region, was applied. Interphase directed luorescence *in situ* hybridization was used to apply commercially available probes for *BIRC3*, *ATM* and *TP53*. High resolution array-comparative genomic hybridization was conducted in selected cases. Genetic abnormalities of *BIRC3* were detected in 23/117 (~20%) of CLL and 2/45 (~4%) of B-ALL cases. Overall, 20 patients with CLL and 1 with B-ALL possessed a *BIRC3* deletion, whilst 3 patients with CLL and 1 with B-ALL harbored a *BIRC3* duplication. All patients with an *ATM* deletion also possessed a *BIRC3* deletion. Only CLL cases possessed deletions in *BIRC3*, *ATM* and *TP53* simultaneously. Evidently, the deletion or duplication of *BIRC3* may be observed rarely in B-ALL patients. *BIRC3* duplication may occur in CLL patients, for which the prognosis requires additional studies in the future. The likelihood that *TP53* deletions occur simultaneously with *BIRC3* and/or *ATM* aberrations is low. However, as *ATM* deletions may, but not always, associate with *BIRC3* deletions, each region should be considered in the future diagnostics of CLL in order to aid treatment decisions, notably whether to treat with or without ludarabine.

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Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia observed in people aged >50 years in Western countries. CLL is characterized by a heterogeneous clinical course, with a time to progression ranging from months to decades (1). The presence of cytogenetic abnormalities is a hallmark of CLL. The most common recurrent aberrations in CLL affect chromosomes 11q, 13q, 14q, 17p and the whole of chromosome 12. Certain abnormalities, including deletions in 11q22.3, the ataxia telangiectasia mutated (*ATM*) gene (10-20%), and 17p13.1, the tumor protein 53 (*TP53*) gene (5-10%), are associated with a poor clinical outcome. Therefore, the detection of these aberrations is important for identifying high-risk patients, who suffer from rapid disease progression and a decreased overall survival time (2). Other frequent chromosomal aberrations in CLL are associated with a good (deletions in 13q14 or 14q32.33) or intermediate (trisomy 12) prognosis (1,3-5).

CLL is considered to be an insidious disease. Certain CLL patients, particularly patients with a good prognosis, survive for several years without requiring treatment; however,

another subgroup of patients experience an aggressive disease course and have a short life expectancy, despite aggressive treatment (6). The latter group tends to exhibit a particular lack of response to ludarabine□based regimens, which are generally considered to be the irst line of treatment for CLL (6). In a large fraction of these patients, the molecular basis of the aggressive clinical course remains unclear; however, in ~40% of patients, the molecular basis is hypothesized to be due to *TP53* disruption. In addition, the activation of the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) pathway is considered to be a mechanism of resistance to disease eradication (7).

From a clinical perspective, CLL cases may be divided into three major clinical phases: i) Newly diagnosed CLL; ii) progressive CLL; and iii) relapsed or ludarabine□refractory CLL. *TP53* abnormalities are observed in 40-50% of relapsed and fludarabine-refractory CLL cases and the deletion of 11q22-23 occurs in 25-30% of relapsed or ludarabine□refractory CLL patients (8). In a large previous study, 637 patients were classified into four risk groups according to a multivariate analysis of overall survival, which was based on genomic abnormalities and the mutational status of *TP53*, baculoviral IAP repeat-containing 3 (*BIRC3*), translocation-associated notch homolog 1 and splicing factor 3B subunit 1. Notably, the high-risk group was composed of patients that exhibited disruption to *TP53* and/or *BIRC3* (9).

The *BIRC3* gene is located on 11q22.2, is ~6 Mb centromeric to the *ATM* gene locus and is considered to be a negative regulator of the non-canonical NF-κB signaling pathway (10,11). *BIRC3* cooperates with tumor necrosis factor receptor-associated factors 2 and 3, in the same protein complex that negatively regulates the mitogen-activated protein kinase 14, a serine-threonine kinase and central activator of non-canonical NF-κB signaling (7). In addition, a frequent aberration associated with *BIRC3* is the recurrent t(11;18)(q21;q21) translocation, which involves the mucosa-associated lymphoid tissue lymphoma translocation gene 1 (*MALT1*), located on 18q21.32. This type of alteration appears in mucosa-associated lymphoid tissue (MALT) lymphoma (12).

In CLL, deletions within the long arm of chromosome 11 may be highly variable in size. The deletion may be distinguished as the more common ‘classical or large deletion’ or an ‘atypical or small deletion’, which are uncommon and more frequently associated with *ATM* mutations. This variation indicates that other genes may possibly contribute to the pathobiology of 11q deletions in CLL, and one of the genes that is hypothesized to be involved is *BIRC3* (13). *BIRC3* disruption, mutations or deletions are rarely detected in CLL at diagnosis (4% of patients), but are detected in 24% of ludarabine□refractory CLL patients. In a previous study, ludarabine□sensitive patients did not exhibit *BIRC3* mutations initially, which suggests that *BIRC3* disruption may be speciically associated with a chemo-refractory CLL subtype (7). Therefore, *BIRC3* disruption may be added to the panel of cytogenetic abnormalities, as the abnormality may be helpful in the early identiication of relapsed and ludarabine□refractory CLL patients. Affected patients should be considered for other treatment regimens, including cyclin-dependent kinase inhibitor, Bruton's tyrosine-kinase inhibitor, B-cell lymphoma 2 inhibitor or and alemtuzumab/corticosteroids (8,13). *BIRC3* abnormalities provide a molecular rationale for using NF-κB inhibitors, which remain under development (7).

Materials and methods

Patients and sample preparation. The present study included 117 CLL patients, and 45 B-cell acute lymphocytic leukemia (B-ALL) patients that were diagnosed according to standard criteria (14). The samples were obtained with the informed consent from the corresponding patients and according to the institutional Ethical Committee guidelines. For CLL cases, DNA was extracted from lymphocytes using a commercial kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. For B-ALL cases, DNA was derived from cytogenetically prepared cells, which were ixed in methanol/acetic acid (dilution, 3:1) (Table I).

Interphase luorescence in situ hybridization (iFISH) analysis. iFISH analyses were performed as previously described (2), using the following commercially available probes: LSI p53/LSI ATM (in 17p13.1 and 11q22.3), CEP 3 (D3Z1 in 3p11.1-q11.1), CEP 4 (D4Z1 in 4p11-q11), CEP 7 (D7Z1 in 7p11.1-q11.1), CEP 11 (D11Z1 in 11p11.11-q11), CEP 16 (D16Z2 in 16p11.1-q11.1), CEP 17 (D17Z1 in 17p11.1-q11.1) and CEP 18 (D18Z1 in 18p11.1-q11.1), all from Vysis (Abbott GmbH & Company, KG, Wiesbaden, Germany); and ZytoLight® SPEC *BIRC3*/*MALT1* DualColor Dual Fusion probe (in 11q22.2 and 18q21.32) from ZytoVision GmbH (Bremerhafen, Germany). For each iFISH analysis, 100-200 interphase nuclei were examined per patient and probe.

Multiplex ligation□dependent probe ampliication (MLPA) analysis. MLPA was performed using the SALSA MLPA probemix P377-A1 for Hematological Malignancies kit (MRC-Holland, Amsterdam, Netherlands). The P377-A1 probemix kit contains 52 probes for 37 genes. The *TP53* and *ATM* genes were assessed by four probes each; however, probes for the *BIRC3* gene were not included in the kit (2). MLPA was successfully performed on 85/117 CLL samples and 32/45 B-ALL samples. MLPA was not successful for the remaining samples due to fragmentation of DNA.

Array-comparative genomic hybridization (aCGH). aCGH was performed using the Agilent SurePrint G3 Human Genome Microarray 180 K (Agilent Technologies, Santa Clara, CA, USA), as previously described (15). aCGH was applied in 3 CLL patients that possessed a *BIRC3* duplication and in 1 B-ALL patient that possessed a *BIRC3* deletion.

Results

Gene copy numbers. BIRC3 gene copy number variations were detected in 23/117 (~20%) of CLL and 2/45 (~4%) of B-ALL cases, as summarized and detailed in Fig. 1. *BIRC3* deletions were identiied in 20 cases of CLL (cases C□1 to C-20) and in 1 case of B-ALL (case A-1). *ATM* deletion was detected in the identical 20 CLL and 1 B-ALL cases. Therefore, all patients with a *BIRC3* deletion also possessed an *ATM* deletion. However, in cases C-1, C-8, C-10, C-13 and

Table I. Gender, age and cytogenetic results of the B-ALL and CLL cases used in the present study.

Case no.	Gender	Age, years	DNA extracted from	Cytogenetic results
A-1	Male	84	BM	46,XY,-9,t(9;22)(q34;q11),del(11)(q),+mar[cp3]/46,XY[5]
A-2	Male	23	BM	Hyperdiploid/46,XY
A-3	Male	34	BM	46,XY
A-4	Male	19	BM	46,XY
A-5	Female	76	BM	45,X,-X[14]/46,XX[2]
C-1	Male	73	BM	46-47,XY,del(11)(q22q2?3),add(17)t(17;?)(p11.2;?)[cp5]/ 45-46,XY,del(11)(q22q2?3),del(17)(p11.2)[cp4]/ 43-46,XY,del(11)(q22q2?3)[cp2]/ 46,XY[7]
C-2	Female	50	B	n.a.
C-3	Female	39	BM	43-46,XY,del(11)(q2?2q2?4)[cp5]/ 45-46,XY,del(11)(q2?2q2?4),del(15)(q1?1q2?3)[cp11]/ 46,XY[1]
C-4	Male	64	BM	46,XY
C-5	Male	43	BM	46,XY
C-6	Male	67	BM	46,XY
C-7	Male	77	BM	46,XY,del(11)(q?21),add(20)(p13)[7]/ 45,X,-Y[10]/ 46,XY[3]
C-8	Male	53	BM	46,XY
C-9	Male	59	BM	n.a.
C-10	Male	73	BM	45,XY,der(2)t(2;13)(q?37;q?14),?del(6)(p?23), del(11)(q?21)der(12)t(12;13)(q?24;q?22),-13[cp4]/ 46,XY[19]
C-11	Male	72	B	n.a.
C-12	Female	73	BM	46,XX,add(11)(q?22)[3]/ 46,XX[12]
C-13	Male	54	B	46,XY
C-14	Male	68	BM	46,XY
C-15	Male	53	BM	46,XY
C-16	Male	75	BM	n.a.
C-17	Female	67	BM	46,XX[18] 45,X,-X[1]
C-18	Male	74	BM	n.a.
C-19	Male	65	BM	46,XY
C-20	Male	77	B	45-46,XY,del(11)(q?22q?23)[cp14] 46,XY[5]
C-21	Male	83	BM	47,XY,-11,+12,+mar[cp3]/ 47,XY,del(5)(p1?3),-11,+12,-17,+mar1,+mar2[cp6]/ 46,XY[9]
C-22	Male	72	BM	46,XY
C-23	Male	59	B	46,XY
C-24	Female	66	B	n.a.
C-25	Female	71	B	46,XX
C-26	Male	65	BM	46,XY,?t(3;?)(p21;?),add(17)(p?12)ort(17;?),-8,+mar[cp7] 46,XY[9]
C-27	Female	74	B	46,XX
C-28	Female	74	BM	46,XX,i(17)(q10)[1]/ 46,XX,+12,i(17)(q10),-21[9]/ 46,XX,t(3;?)(q2?9;?)[4],-7[4],+12[4],i(17)(q10)[4][cp4]/ 46,XX[4]
C-29	Female	90	B	n.a.
C-30	Male	56	BM	n.a.
C-31	Female	65	BM	46,XX

A-, B-ALL case; C-, CLL case; BM, cell pellet in Carnoys fixative from bone marrow; n.a., data not available.

Table II. Summary of MLPA and iFISH results of *TP53*, *ATM*, *BIRC3* and *MALT1* in all studied cases.

	TP53 (%)		*ATM* (%)		*BIRC3* (%)	*MALT1* (%)
Case no.	MLPA	iFISH	MLPA	iFISH	iFISH	iFISH
A-1	N	N	D	D (76.5)	D (75.0)	N
A-2	n.a.	A (100.0)	n.a.	A (100.0)	A (100.0)	A (100.0)
A-3	D	D (8.5)	N	N	N	N
A-4	D	D (10.0)	N	N	N	N
A-5	D	D (10.0)	N	N	N	N
A-6 to A-33	N	N	N	N	N	N
A-34 to A-45	n.a.	N	n.a.	N	N	N
C-1	D	D (86.0)	D	D (11.0)	D (80.0)	N
C-2	D	D (21.0)	N	D (23.0)	D (22.0)	N
C-3	N	N	D	D (98.0)	D (90.0)	N
C-4	N	N	D	D (23.5)	D (30.0)	N
C-5	N	N	D	D (24.0)	D (25.0)	N
C-6	N	N	D	D (88.0)	D (85.0)	N
C-7	N	N	D	D (90.0)	D (80.0)	N
C-8	N	N	D	D (77.0)	D (50.0)	N
C-9	N	N	D	D (98.0)	D (75.0)	N
C-10	N	N	D	D (87.0)	D (60.0)	N
C-11	N	N	D	D (95.0)	D (90.0)	N
C-12	N	N	D	D (83.0)	D (80.0)	N
C-13	N	N	D	D (93.0)	D (25.0)	N
C-14	N	N	N	D (33.0)	D (15.0)	N
C-15	N	N	N	D (12.0)	D (13.0)	N
C-16	n.a.	N	n.a.	D (80.0)	D (78.0)	N
C-17	n.a.	N	n.a.	D (10.0)	D (9.0)	N
C-18	n.a.	N	n.a.	D (73.0)	D (64.0)	N
C-19	n.a.	N	n.a.	D (9.0)	D (10.0)	N
C-20	n.a.	N	n.a.	D (96.0)	D (42.0)	N
C-21	D	D (16.0)	N	A (50.0)	A (50.0)	A (50.0)
C-22	D	D (40.0)	N	A (40.0)	A (40.0)	A (40.0)
C-23	N	N	N	N	A (36.0)	N
C-24	D	D (19.0)	N	N	N	N
C-25	D	D (36.0)	N	N	N	N
C-26	D	D (89.0)	N	N	N	N
C-27	D	D (77.0)	N	N	N	N
C-28	D	D (95.0)	N	N	N	N
C-29	N	D (11.5)	N	N	N	N
C-30	n.a.	D (86.5)	n.a.	N	N	N
C-31	N	N	N	N	N	A (75.0)
C-32 to C-91	N	N	N	N	N	N
C-92 to C-117	n.a.	N	n.a.	N	N	N

Data are expressed as type of change to copy number (% of cells with aberration). *TP53*, tumor protein 53; *ATM*, ataxia telangiectasia mutated; *BIRC3*, baculoviral IAP repeat-containing 3; *MALT1*, mucosa-associated lymphoid tissue lymphoma translocation gene 1; MLPA, multiplex ligation-dependent probe ampliication; iFISH, interphase luorescence *in situ* hybridization; A-, B-ALL case; C-, CLL case; N, no aberration; D, deletion; A, ampliication; n.a., data not available.

C-20 the detected clone sizes with deletions in *BIRC3* and *ATM* were extremely varied from one another. In case C-1, the clone with the *ATM* deletion was 8x smaller compared with that with the *BIRC3* deletion, whereas in cases C-8, C-10, C-13 and C-20, the clone with the *BIRC3* deletion was 2-3x smaller than that with the *ATM* deletion. A *BIRC3* duplication was identiied in 1 case of B-ALL (case A-2) and in 3 CLL patients (cases C21 to C23) (Table II).

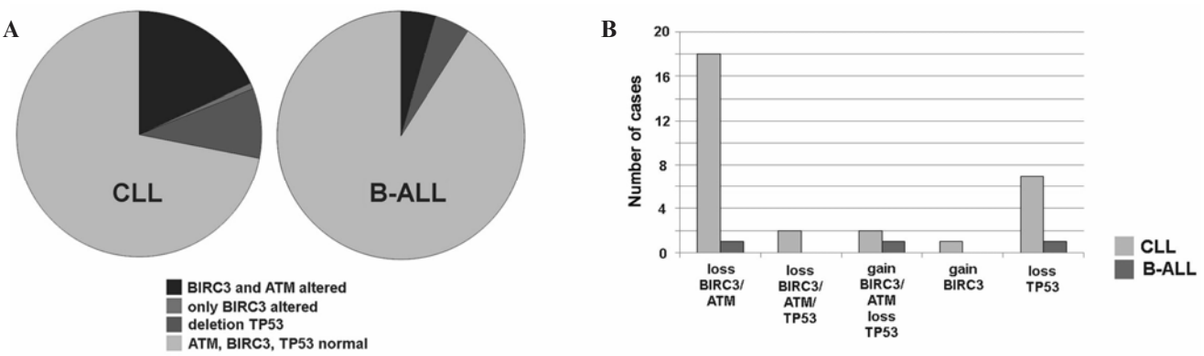

Figure 1. The distribution and of *BIRC3*, *ATM* and *TP53* aberrations in CLL and B-ALL patients are summarized. (A) In CLL and B-ALL, the majority of patients did not show alterations in the three genes. Distribution of the loss and gain of copy numbers in the two patient groups is depicted. (B) Combinations of loss and gain of the three genes were identiied in the patients with CLL and B□ALL, with alterations from part (A). *BIRC3*, baculoviral IAP repeat-containing 3; *ATM*, ataxia telangiectasia mutated; *TP53*, tumor protein 53; CLL, chronic lymphocytic leukemia; B-ALL, B-cell acute lymphocytic leukemia.

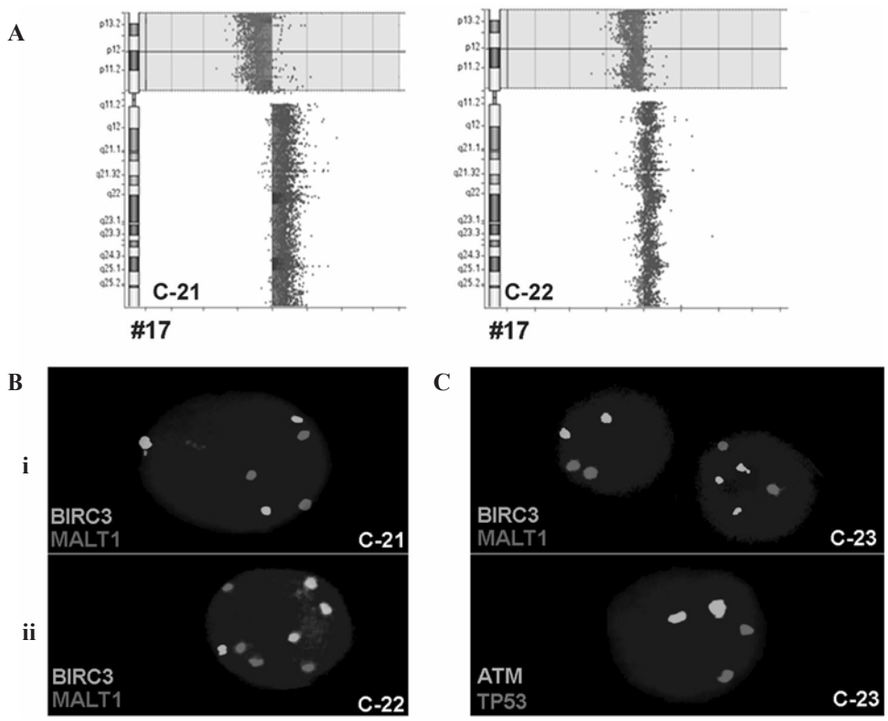

Figure 2. (A) Array comparative genomic hybridization conirmed the deletion in *TP53*, which was detected initially using iFISH and multiplex ligation dependent probe ampliication for CLL cases C-21 and C-22. The whole short arm was deleted and the long arm was possibly duplicated due to an isochromosome 17a formation, at least in case C-21. (B) Examples for gain of copy numbers for *BIRC3* and *MALT1* in the 2 cases by iFISH: i) C-21, an example of 3 copies and ii) C-22, an example of 4 copies. (C) iFISH results of the CLL case C-23. *BIRC3* had 3 copies in certain cells; however *ATM*, *MALT1* and *TP53* exhibited only 2 copies each, in all cells. *TP53*, tumor protein 53; CLL, chronic lymphocytic leukemia; iFISH, interphase fluorescence *in situ* hybridization; *BIRC3*, baculoviral IAP repeat-containing 3; *MALT1*, mucosa-associated lymphoid tissue lymphoma translocation gene 1; *ATM*, ataxia telangiectasia mutated.

With regard to *TP53* abnormalities, 3 patients with B-ALL possessed a *TP53* deletion in the absence of any aberrations in *BIRC3*. *TP53* deletions were present in 11 CLL patients, 7 of which possessed no associated *BIRC3* aberrations and, notably, 2 of which were accompanied by *BIRC3* and *ATM* ampliication.

BIRC3 duplication. In total, 3 CLL patients harbored a *BIRC3* duplication (cases C-21 to C-23), 2 of which (C-21 and C-22) were accompanied by *ATM* and *MALT1* duplications in addition to *TP53* deletion. To study these cases in greater depth, iFISH was performed using the centromeres of chromosomes (CEP) 3, 4, 7, 11, 16 and 18. For these chromosomes, 3 signals were detected in 11% (case C-21) and 25% (case C-22) of the cells, and 4 signals were detected in 29% (case C-21) and 25% (C-22) of the cells, respectively (Fig. 2).

The third CLL case (C-23) with *BIRC3* duplication was associated with normal copy numbers of *TP53* and *ATM*; the centromeric probes for chromosomes 11 and 17 only revealed 2 signals each (Fig. 2).

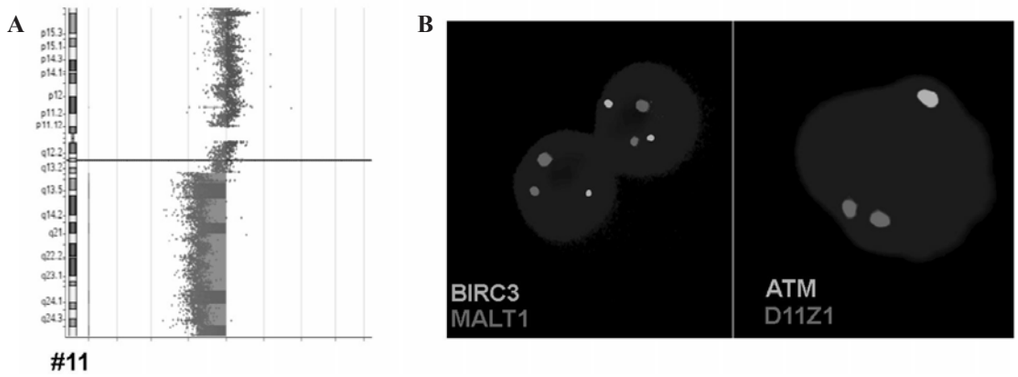

Figure 3. Results obtained for B-ALL case A-1 by array comparative genomic hybridization and iFISH are summarized. (A) Deletion in 11q22-q23, initially detected by iFISH, resulted in loss of the whole long arm of a chromosome 11. (B) Examples for heterozygote deletions of ATM and BIRC3 detected by iFISH are depicted. iFISH, interphase luorescence *in situ* hybridization; ATM, ataxia telangiectasia mutated; BIRC3, baculoviral IAP repeat-containing 3; MALT1, mucosa-associated lymphoid tissue lymphoma translocation gene 1; D11Z1, CEP 11 probe.

Based on the aCGH results for cases C-21, C-22 and C-23, the *TP53* deletion in C□21 and C□22 was conirmed; however, *BIRC3* was normal in all 3 patients (Fig. 2). Therefore, cases C-21 and C-22 had a mixture of a malignant triploid and tetraploid cell clones and a deletion in *TP53*. Case C-23 demonstrated the selective gain of copy numbers for *BIRC3*, without *ATM* involvement, in 36% of the cells; however, this inding was not detectable using aCGH.

MALT1 duplication. The patient with a *MALT1* duplication (case C-31) possessed a trisomy of chromosome 18, which was confirmed using MLPA and iFISH. The probes for the deleted in colorectal cancer gene on 18q21.2 and RNA (guanine-7-)methyltransferase gene on 18p11.22 revealed a duplication by MLPA, which was conirmed by iFISH in 75% of the cells.

B□ALL patients. Regarding B-ALL patients, 1 case revealed a deletion in *BIRC3* along with *ATM* (case A-1), and another case was identified as possessing a triploid/hyperdiploid karyotype in the iFISH analysis using CEP 11, 17 and 18 (case A-2, result not shown), as was observed in the cases C-21 and C-22.

The *BIRC3* deletion in B□ALL case A□1 was conirmed by aCGH, which reveled that the deletion in the long arm of chromosome 11 covered between chr11:67,773,863 and 134,945,165 (GRCh37/hg19) (Fig. 3). The *ATM* and *BIRC3* genes are located between positions 102,188,181 and 108,239,826.

Discussion

The present study regarding *BIRC3* copy number variations in 117 CLL and 45 B-ALL patients has revealed several major findings that, to the best of our knowledge, have not been previously reported. Firstly, *BIRC3* duplications were detected in 3 cases of CLL, and 2 of these were associated with *ATM* and *MALT1* duplications, in addition to *TP53* deletions; BIRC3 amplification was not more than a hint on a hyperdiploid cell clone as reported in CLL earlier, but not as a frequent event (16,17). In addition, 1 B-ALL patient possessed a duplication of *BIRC3* due to partial hyperdiploidy, which is more common in B-ALL compared with CLL, and is associated with good prognosis in pediatric patients (18). A CLL-case with a *BIRC3* duplication possessed normal *ATM* and *TP53* copy numbers; however, the duplication was not detected by aCGH, most likely due to the low sensitivity of aCGH for mosaic detection, despite being present in 36% of the cells. Previous studies on the interaction of *BIRC3* with the NF-κB pathway indicate that *BIRC3* duplication may lead to the inactivation of tumor suppressor activity (19-21).

As the predominant morphological feature of CLL is the accumulation of small B lymphocytes (1), B-ALL patients were chosen to be the second group to be tested for *BIRC3*-alterations in the present study. Therefore the second important inding of the present study is the detection of a *BIRC3* deletion in 1 of the 45 studied B-ALL cases. The aCGH for case A-1 revealed the deletion of almost all of the long arm of chromosome 11, and the most frequent aberrations associated with chromosome 11 in B-ALL patients are structural abnormalities in band 11q23, which harbors the myeloid/lymphoid leukemia gene (22).

Chromosomal deletions involving 11q have been reported in certain subtypes of hematological malignancies, including B-cell CLL, and are associated with a poor prognosis in mantle cell lymphomas or T-cell prolymphocytic leukemia (23). Therefore, the prognosis for the B-ALL patient in the present study may be poor or extremely poor. Additional studies are required to determine the role of *BIRC3* in the prognosis of B-ALL patients.

The disruption of *BIRC3* is specifically restricted to chemo-refractory cases in progressive CLL patients, and may selectively associate with fludarabine-refractory patients with normal *TP53* (7). Therefore, another notable inding of the present study is that *BIRC3* abnormalities were associated with *TP53* deletion in only 4/117 CLL cases. According to previous studies, the frequency of *BIRC3* disruption is low at diagnosis; however, *BIRC3* disruptions tend to accumulate among refractory CLL and emerge over time. Patients harboring a *BIRC3* disruption typically experience an aggressive disease course, even compared with other clinically aggressive groups (11,24). This aspect of the disease

was not the focus of the present study. However, examining *BIRC3* duplication cases for the presence of mutations may be interesting for future study.

In conclusion, the hypothesis by Rose-Zerilli *et al* (25) that *BIRC3* deletions are always associated with *ATM* deletions is questioned at least for a small percentage of cases. As screening of the *BIRC3* gene is not routinely undertaken for CLL patients (2,26), the results of the present study suggest that screening may be considered as necessary in the future, particularly to aid in making the correct treatment decisions; particularly, whether to treat with or without ludarabine.

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2.2. Article .5

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Research Article

Isochromosome 17q in Chronic Lymphocytic Leukemia

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In chronic lymphocytic leukemia (CLL), presence of acquired cytogenetic abnormalities may help to estimate prognosis. However, deletion of *TP53* gene, which is associated with an aggressive course of the disease and poor prognosis along with a lack of response to treatment, is one of the alterations which may escape cytogenetic diagnoses in CLL. Thus, other techniques have emerged such as interphase fluorescence *in situ* hybridization (iFISH). Deletion of *TP53* may but must not go together with the formation of an isochromosome i(17q); surprisingly this subgroup of patients was not in the focus of CLL studies yet. This study was about if presence of i(17q) could be indicative for a new subgroup in CLL with more adverse prognosis. As a result, *TP53* deletion was detected in 18 out of 150 (12%) here studied CLL cases. Six of those cases (~33%) had the *TP53* deletion accompanied by an i(17q). Interestingly, the cases with i(17q) showed a tendency towards more associated chromosomal aberrations. These findings may be the bases for follow-up studies in CLL patients with *TP53* deletion with and without i(17q); it may be suggested that the i(17q) presents an even more adverse prognostic marker than *TP53* deletion alone.

1. Introduction

Chronic lymphocytic leukemia (CLL) is a relatively frequently observable acquired disease in men and women of >50 years of age [1]. Also CLL is a heterogeneous malignancy, as the survival of CLL patients can be in the range of months to decades according to the underlying genetic abnormalities [2]. The most frequent cytogenetic aberrations in CLL are involving chromosomal subbands 13q14 (50–60%), 14q32 (12–15%), 11q22 (10–20%), and 17p13 (5–10%) as well as trisomy 12 (15–25%); each group has different prognoses and survival rates [1, 3]. Deletion of *TP53* gene, which is located in the short arm of chromosome 17 towards the telomeric region in 17p13.1, is associated with poor prognosis and lack of response to fludarabine-based regimens.

TP53 deletion in CLL can be associated with isochromosome formation of the long arm of one chromosome 17 leading at the same time to partial monosomy 17p and partial trisomy 17q. In general, isochromosome i(17q) is the most frequently observed isochromosome in hematological malignancies and it can be present as primary or secondary aberration; that is, it may play roles during development as well as progression of the malignancy. Presence of i(17q) as a sole abnormality is associated with a high risk of progression and an aggressive clinical course, but i(17q) can also be found as part of a complex karyotype [4–6]. In solid tumors, i(17q) is reported predominantly in medulloblastoma [7], there often associated with c-myc amplification [8].

Overall, detection of acquired chromosomal abnormalities such as i(17q) just based on GTG-banding may be limited due to low mitotic potential of CLL bone marrow cells. Thus, nowadays other techniques are applied to overcome this problem, by name interphase fluorescence *in situ* hybridization (iFISH), multiplex ligation dependent probe amplification

(MLPA), and array-comparative genomic hybridization (aCGH) [2, 9, 10]. Here we studied 150 CLL samples and concentrated on the questions (i) if i(17q) can be detected reliably by MLPA and (ii) if i(17q) presence in patients with *TP53* deletion is associated with more complex cytogenetic aberrations. An association with the clinical outcome would have been favorable as well; unfortunately this was not possible due to lack of necessary clinical data.

2. Material and Methods

2.1. Patients and Sample Preparation. The present study included 150 CLL patients, which were diagnosed according to standard criteria [11]. The samples were obtained under informed consent of the corresponding patients and according to institutional ethical committee guidelines (Ethical Committee of the Friedrich Schiller University Jena).

DNA was extracted from lymphocytes of 85 CLL cases by a commercial kit (Qiagen) according to manufacturer's instructions. DNA was derived from different sources: 2 samples from heparinized bone marrow, 8 samples from heparinized blood, and 75 samples from cytogenetically prepared cells fixed in methanol/acetic acid (3 : 1)—48 of them derived from bone marrow and 27 from blood. Details on the studied patients can be found in the paper by Alhourani et al. (2014): the 10 here in more detail studied patients with *TP53* deletion (Table 1) were cases 61 (now 1), 1 (now 2), 17 (now 3), 19 (now 4), 12 (now 5), 38 (now 6), 18 (now 7), 16 (now 8), 39 (now 9), and 13 (now 10) from Alhourani et al. (2014) [1]. In the previous study, no special attention was given to the here treated i(17q) problem, and additional studies, esp. FISH experiments, and reinterpretation of MLPA and aCGH data were performed here.

For further investigation of i(17q) status, additional 65 CLL patients were included in this study with special focus on 8 cases (86 to 93) with *TP53* (Table 1).

2.2. GTG-Banding and Interphase-Directed Fluorescence In Situ Hybridization (iFISH) Analysis. GTG-banding and iFISH analyses were done as previously reported [1].

For iFISH, the following probes were used:

(i) Abbott/Vysis (Wiesbaden, Germany): LSI p53/LSI ATM (in 17p13.1 and 11q22.3), LSI D13S319/LSI 13q34/CEP 12 (in 13q14.3, 13q34, and 12p11.1-q11.1), LSI IGH dual color, break-apart probe (in 14q32.33), LSI SMS Region SpectrumOrange/LSI RARA SpectrumGreen (in 17p11.2 and 17q12-21), CEP 17 (D17Z1 in 17p11.1-q11.1), TelVysion 17p (282M16/SP6), and TelVysion 17q (D17S928).

(ii) From Zytovision (Bremerhaven, Germany): Zyto-Light SPEC CMYC/CEN 8 Dual Color (8q24.21 and 8p11.1-q11.1).

(iii) BACPAC Resources Center (Oakland, USA): RP11-318A15 in 17q25.1 (gene *UNC13D*) and RP11-94L15 in 17q12 (gene *IKZF3*).

For each iFISH analysis, 100–200 interphase nuclei were examined per case and probe.

2.3. Multiplex Ligation Dependent Probe Amplification (MLPA) Analysis. Multiplex ligation dependent probe amplification (MLPA) was performed on 85 CLL cases using SALSA MLPA probemix P377-A1 for Hematological Malignancies Kit from (MRC-Holland, Amsterdam, Netherlands).

The P377-A1 probemix kit contains 52 probes for overall 37 genes; *TP53* which is located on the short arm of chromosome 17 is covered by 4 probes; likewise *UNC13D* and *IKZF3* on q arm were covered by one probe for each of them [1].

2.4. High Resolution Array-Comparative Genomic Hybridization (aCGH). High resolution array-comparative genomic hybridization (aCGH) was performed using Agilent SurePrint G3 Human Genome microarray 180 K (Agilent Technologies, Santa Clara, CA, USA) as previously reported [12].

3. Results

Deletion of *TP53* has been detected in 9/85 cases by MLPA. Besides a screening for *TP53* deletion was done by iFISH in all of the studied 85 CLL cases to detect mosaic cases with low percentage of aberrant cells as well. Accordingly, *TP53* deletion was detected in one additional CLL case, being present there in only 11.5% of the studied cells (case 10).

The overall detected 10 cases with *TP53* deletion (Table 2) were further studied by iFISH using probes *IKZF3* in 17q12, *UNC13D* in 17q25.1, and subtelomeric probes (17pter and 17qter; Figure 1(a)); furthermore iFISH-probes for the most frequent aberrations in CLL and, in part, aCGH (case 3; Figure 1(b)) have been applied in those cases, as specified by Alhourani et al. (2014). So, overall 3/85 (~3.5%) of here studied CLL cases had the loss of *TP53* due to formation of an i(17q) which is equal to 30% of these patients.

Further 8 cases with *TP53* deletion were found in additional 65 studied CLL patients by iFISH-probe. Here, subtelomeric (17pter and 17qter) probes were applied to identify the three among them cases with i(17q). A probe for 17p11.2 and 17q12 confirmed the isochromosome status in those cases (Table 3).

In the here studied cases with i(17q), this alteration was accompanied by additional chromosomal aberrations (Table 4). For all of them, amplification of c-myc was excluded. While in the first 85 CLL patients, cases 1 and 2 were accompanied by five additional acquired chromosomal rearrangements and case 3 had only one additional change. In cases 1 and 2, at least one of these additional changes was correlated with an adverse prognosis; in case 3 the del(13) is considered to be a favorable prognostic factor. Cases 4–10, which showed just deletion of *TP53* without isochromosome formation, had either no further aberrations (cases 4 and 9) or just one additional chromosomal alteration associated with good prognosis (cases 5, 7, and 10). Only case 8 showed two additional chromosomal alterations with known adverse prognostic meaning.

Among the 8 cases with *TP53* deletion studied only by iFISH, three cases revealed i(17q). While case 88 showed 8 additional chromosomal rearrangements (two of them were associated with good prognosis), the other two cases, 86 and 87, had only one additional chromosomal alteration.

Table 1: Gender, age, and cytogenetic results of the 18 studied CLL cases which showed deletion of *TP53* gene.

Case number	Gender	Age [y]	DNA extracted from	Banding cytogenetics
1	F	74	bm	46,XX,i(17)(q10)[1]/ 46,XX,+12,i(17)(q10),-21[9]/ 46,XX,t(3;?)(q2?9;?)[4],-7[4],+12[4],i(17)(q10)[4][cp4]/ 46,XX[4]
2	M	83	bm	47,XY,-11,+12,+mar[cp3]/ 47,XY,del(5)(p1?3),-11,+12,-17,+mar1,+mar2[cp6]/ 46,XY[9]
3	M	72	bm	46,XY
4	F	71	b	46,XX
5	F	50	b	n.a.
6	M	65	bm	46,XY,?t(3;?)(p21;?),der(17)t(17;?),-18,+mar[cp7]/ 46,XY[9]
7	F	66	b	n.a.
8	M	73	bm	46~47,XY,del(11)(q22q2?3),der(17)t(17;?)(p11.2;?)[cp5]/ 45~46,XY,del(11)(q22q2?3),del(17)(p11.2)[cp4]/ 43~46,XY,del(11)(q22q2?3)[cp2]/ 46,XY[7]
9	F	74	B	46,XX
10	F	90	b	n.a.
86	M	74	n.a.	45~46,XY,i(17)(q10)[cp4]/ 45,X,-Y[2]/ 46,XY[14]
87	F	76	n.a.	46,XX,?t(6;19)(p22;p13),del(17)(p?11.2)[1] 46,XX[16]
88	M	65	n.a.	46,XY,t(10;13)(q2?2;q1?3)[10] 46,XY,i(18)(q10)[1] 45,XY,?del(6)(?q21),-17[1] 46,XY,-17,+mar[1] 44,XY,-11,-17[1] 46,XY,-4,-21,+2mar[1] 46,XY[5]
89	F	68	n.a.	n.a.
90	F	63	n.a.	n.a.
91	F	79	n.a.	n.a.
92	M	61	n.a.	n.a.
93	F	75	n.a.	n.a.

b = cell pellet in Carnoy's fixative from blood; bm = cell pellet in Carnoy's fixative from bone marrow; F = female; M = male; n.a. = not available; B = native peripheral blood.

The remaining 5 cases with *TP53* deletion and no i(17q) were associated with one additional chromosomal aberration with good prognosis (cases 89 and 90), or no additional chromosomal changes (cases 91, 92, and 93) (Table 1).

4. Discussion

Generally, isochromosome formation is characterized by the loss of the entire short arm with subsequent duplication of the entire long arm, resulting in two homologous arms attached to a single centromere as mirror images [4, 7, 13]. There are two hypotheses to explain the formation of isochromosome, either by transverse instead of longitudinal division of the centromere or by chromatid exchange involving two homologous chromosomes. The rate of the appearance of isochromosomes is different among the various types of tumors, with the highest occurrence in germ cell neoplasms (60%) and the lowest in chronic myeloproliferative disorders (2.3%) [14].

Surprisingly, although i(17q) appeared in 6/150 (4%) here studied CLL patients, that is, and 6/18 (~33%) of CLL patients with a deletion of *TP53*, this chromosomal aberration has not been studied in detail yet in this patient group. Still, there is one study including 2 CLL patients with i(17q) which showed that such isochromosome most likely forms due to clustered breakpoints in 17q11 and is not associated with *TP53* mutations of the intact chromosome 17 [4]. In 2006, i(17q) was found to be present in 4/16 (25%) CLL patients with *TP53*

Table 2: Summary of MLPA and iFISH results of *IKZF3*- and *UNC13D*-gene specific probes and subtelomeric probes for chromosome 17 in 10 CLL cases with *TP53* deletion in the first group.

Case number	*TP53* [%]		*UNC13D* [%]		*IKZF3* [%]		iFISH [%]	
	MLPA	iFISH	MLPA	iFISH	MLPA	iFISH	*Subtel. pter*	*Subtel. qter*
1	d	d [95]	a	a [90]	a	a [90]	d [90]	a [90]
2	d	d [40]	a	a [40]	a	a [40]	d [40]	a [40]
3	d	d [40]	n	a [25]	n	a [25]	d [25]	a [25]
4	d	d [36]	n	n	n	n	n	n
5	d	d [21]	n	n	n	n	n	n
6	d	d [89]	n	n	n	n	n	n
7	d	d [19]	n	n	n	n	n	n
8	d	d [86]	n	n	n	n	n	n
9	d	d [77]	n	n	n	n	n	n
10	n	d [11,5]	n	n	n	n	n	n
11 to 85	n	n	n	n.a.	n	n.a.	n.a.	n.a.

n = no aberration, d = deletion, a = amplification, n.a. = not tested, and [] = percentage of cells with aberration.

Table 3: Summary of iFISH results using SMS and RARA gene specific probes and subtelomeric probes for chromosome 17 in 8 CLL cases with *TP53* deletion in CLL cases only studied by iFISH and not by MLPA.

Case number	iFISH [%]	iFISH [%]		iFISH [%]	
	TP53	*Subtel. pter*	*Subtel. qter*	SMS	RARA
86	d [77]	d [77]	a [77]	d [77]	a [77]
87	d [77]	d [77]	a [77]	d [77]	a [77]
88	d [80]	d [80]	a [80]	d [80]	a [80]
89	d [69]	n	n	n.a	n.a
90	d [28]	n	n	n.a	n.a
91	d [75]	n	n	n.a	n.a
92	d [89]	n	n	n.a	n.a
93	d [95.5]	n	n	n.a	n.a
94 to 150	n	n.a	n.a	n.a	n.a

n = no aberration, d = deletion, a = amplification, n.a. = not tested, and [] = percentage of cells with aberration.

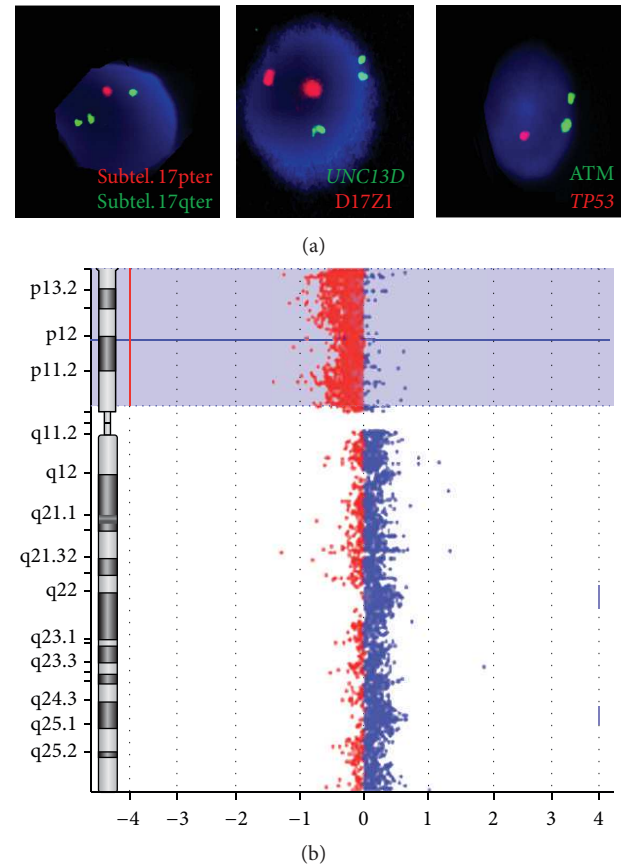

Figure 1: (a) Isochromosome 17q was detected initially by iFISH in this case; representative examples for heterozygote deletions of *TP53* and #17 subtelomeric region 17p (subtel. 17pter) besides three signals for *UNC13D* and subtel. 17qter. Only 2 signals for centromere of chromosome 17 (D17Z1) and ATM gene on chromosome 11 were detected. (b) aCGH showed deletion of short arm and gain of long arm of chromosome 17 in case 3.

gene loss [15]; that is, the here reported frequency is within the same range. However, the initial finding of an i(17q) in 2/21 (9.5%) CLL cases seems to be overestimated due to small sample size [16].

Even though here only 6 cases with i(17q) could be studied, the results summarized in Table 4 show a clear tendency: cases with i(17q) are associated with more aberrations compared to those which have just deletion of *TP53*. Cases 3 and 8 do not exactly fit into this suggestion. However, case 3 had only 25% of the cells with an i(17q) indicating an early phase of the disease; in case 8 cytogenetics provided a hint on an ongoing karyotypic evolution and already advanced stage of the disease.

Whereas both Baliakas et al. and Rigolin et al. reported that complex karyotype predicts a worse overall survival, also Baliakas et al. demonstrated that complex karyotype is identified as an independent prognostic factor for shorter time-to-first-treatment [17, 18].

Furthermore, Thompson et al. showed that relapsed/refractory CLL patients who reveal del(17p) and complex

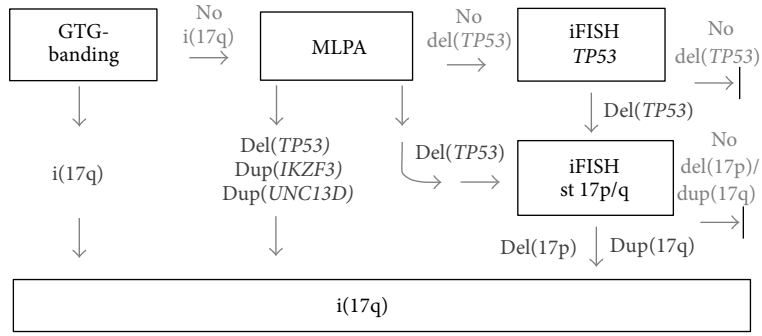

Figure 2: Here a scheme for the suggested procedures how to delineate an i(17q), if cytogenetics, MLPA, and iFISH are available.

Table 4: All 18 CLL cases which revealed *TP53* deletion are listed showing the additionally detected chromosomal aberrations and their clinical impact (1).

Case number	Additional aberrations not listing #17 aberrations [%]	Prognosis
1	t(3;?)(q2?9;?)[22]	n.a.
	-7[22]	Adverse
	+12[78]	Intermediate
	del(14)(q32q32)[94]	Good
	-21[50]	n.a.
2	del(5)(p1?3)[33]	n.a.
	del(11)(q22.3q22.3)[30]	Adverse
	+12[70]	Intermediate
	del(13)(q14.3q14.3)[30]	Good
	rea(14)(q32.33)[28] -> ?+14	Adverse
3	del(13)(q14.3q14.3)[20]	Good
4	None detected	Intermediate
5	del(13)(q14.2q14.2)[52] del(13)(q14.2q14.2)x2[38] del(13)(q14.3q14.3)[34]	Good
6	t(3;?)(p21;?)[44]	n.a.
	-18,+mar[44]	n.a.
7	del(13)(q14.3q14.3)[90.5]	Good
8	amp(8)(q24.21q24.21)[21]	Adverse
	del(11)(q22.3q22.3)[11]	Adverse
9	None detected	Intermediate
10	del(13)(q14.3q14.3)x2[98.5]	Good
86	-Y	n.a.
87	?t(6;19)(p22;p13),del(17)(p?11.2)	Adverse
88	t(10;13)(q2?2;q1?3)	Advesrse
	i(18)(q10)	n.a.
	?del(6)(?q21),-17	n.a.
	-17,+mar	n.a.
	-11,-17	n.a.
	-4,-21,+2mar	n.a.
	del(13)(q14.3q14.3)[57]	Good
	del(14)(q32q32)[75]	Good
89	del(13)(q14.2q14.2)[50] del(13)(q14.2q14.2)x2[7]	Good
90	del(14)(q32q32)[36]	Good
91	None detected	Intermediate
92	None detected	Intermediate
93	None detected	Intermediate

n.a. = not available.

karyotype have shorter overall survival than those with only del(17p) [19].

Due to lack of clinical data, the clinical impact of i(17q) could not be followed up, but in spite of that the present study gives first hints that i(17q) presence may be an indicator for more aggressive course of CLL disease than just *TP53* deletion without i(17q) formation. Similar findings were reported for other hematological neoplasia, like acute lymphocytic leukemia [20], acute promyelocytic leukemia [21], chronic myeloid leukemia [5], or other myeloid leukemia [22–24].

As previously outlined by us and others, MLPA is a quick and inexpensive screening tool for CLL diagnostics [1, 25]. However, its inability to detect low level mosaics needs to be considered and thus a diagnostic scheme combining cytogenetics, iFISH, and MLPA needs to be considered for reliable testing of CLL cases in diagnostics [1]. Thus, in Figure 2 we suggest a scheme of how to detect i(17q) reliably.

In conclusion, i(17q) presence in CLL cases with *TP53* deletion should be considered as a potentially adverse marker for more aggressive course of the disease than monosomy of 17p13.1 alone; it needs to be kept in mind that MLPA alone may be not sufficient to pick up all corresponding cases and a combination with iFISH may be considered additionally.

Conflict of Interests

The authors declare that they have no competing interests.

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3. Discussion.

Diagnosis and prognosis of CLL depend on presence or absence of chromosomal abnormalities, which can be detected by different molecular and cytogenetic techniques (Döhner et al. 2000, Rodriguez-Vicente et al. 2013). Thus, the first phase of the present work was dedicated to identify all possible chromosomal aberrations in 85 CLL cases by using GTG-banding, iFISH, MLPA and aCGH in selected cases as discussed in 3.1. As outlined in 3.1.1.-3.1.2., concordance and discordance between MPLA and iFISH and the potential clinical relevance for the additional detected cases by MLPA are discussed. In 3.1.3. a diagnostic cost efficient scheme combining the different techniques is suggested.

Furthermore, in this thesis the disruption of *BIRC3* gene was studied in 117 CLL-, and 45 B-ALL cases and the association between *BIRC3* disruption and deletion of *ATM* gene was analyzed (3.2.). Finally i(17q) status was examined in 150 CLL, and potential i(17q)-association with complex karyotypes.

3.1. Cytogenetic analysis for CLL diagnostics

Prognosis and hence treatment decisions for CLL patients vary according to the detected chromosomal abnormalities. The identification of such aberrations by conventional karyotyping and metaphase-directed FISH is obstructed by a low in vitro mitotic activity of malignant cells, which leads to the lack of the metaphase spreads in the analyzable sample (article 1, Döhner et al. 2000). Accordingly, iFISH has been introduced as a powerful tool for the detection of genomic aberrations in CLL, as it can be performed in both dividing and non-dividing cells, by that overcoming the limitation of low mitotic index. Still it is restricted to specific chromosomal regions according to the used probes panel (e.g. 13q14.3, 12p11.1-q11.1, 14q32.33, 17p13.1 and 11q22.3 in CLL (Rodriguez-Vicente et al. 2013, Haferlach et al. 2007). MLPA technique may be applied in CLL diagnostics to detect copy number variations in multiple chromosomal regions at the same time. Thus one of the objectives of this study was the evaluation of MLPA efficacy in identification of unbalanced genomic aberrations in CLL in comparison with GTG-banding and iFISH (article 2, Véronèse et al. 2013).

3.1.1. Concordances of MLPA and iFISH results

MLPA has been performed retrospectively on 85 CLL samples, which were initially studied by GTG-banding and routine iFISH. Overall MLPA and iFISH were in concordance in 70% of cases. However, 33 additional chromosomal aberrations were detected by MLPA, 30 of them confirmed later by iFISH (article 2).

The additional detected aberrations had an impact on the prognosis of individual CLL cases. 12 cases with known deletion in 13q14.3 revealed additional loss of 13q14.2; this implied a regrouping from favorable into unfavorable group (article 2, Dal Bo et al. 2011, Rodriguez-Vicente et al. 2013).

Also according to MLPA results previously unrecognizable deletions in the long arm of chromosome 6 were identified in 2 cases, being considered as an intermediate-risk factor, leading to short TTFT and OS (article 2, Cuneo et al. 2004).

In addition to that 3 cases had 2p amplification; they were associated with deletion in *ATM* in two cases and deletion in 6q in the third one. Fabris et al. (2013) reported that 2p gain which include *MYCN* gene can be present since the early stages of the disease, particularly in those cases characterized by other poor prognosis markers, i.e. del(11)(q23) and del(17)(p13). A more aggressive course of the disease was associated to those patients. Thus 2p amplification can be an indicator for poor prognosis with short OS (article 2, Fabris et al. 2013).

Only one case of this study showed trisomy 18 accompanied with trisomy 12; this changed the prognosis of this patients to be more adverse compared to such with only trisomy 12. Another case revealed duplication in 8q24 (*MYC*) accompanied with deletion in *TP53* and *ATM* genes. This aberration (Dup *MYC*) is considered as a rare one in CLL, and to be associated with shorter OS (article 2, López et al. 2012, Puiggros et al. 2014).

Duplication of *RUNX1* gene (21q22.12), also called *AML1*-gene, showed up in two cases of this study; in one of them as a sole abnormality, and in the second one it was accompanied by deletions in 6q ,13q14.3, and gains in 2p. Normally, amplification in *RUNX1* (*AML1*) gene appears in acute lymphoblastic leukemia and not in CLL, and is associated with a poor outcome. In addition to that translocation of this gene has been reported to be involved in a novel three-way variant t(8;13;21)(q22;q33;q22) in a child with acute myeloid leukemia (articles 2, 3, Robinson et al. 2003).

3.1.2. Discordances of MLPA and iFISH results

The results of array-CGH and iFISH showed no alterations in the chromosomal regions for the three aberrations (Dup in *RNMT*, *DCC*, *SMOC2*) being identified by MLPA, as well; thus, they have been considered as false positive results. This leads to the conclusion that genetic aberrations, which are detected by MLPA technique, especially those chromosomal regions which are covered by a single probe in the MLPA kit, should be confirmed or falsified by another molecular genetic technique such as iFISH (article 2, Hömig-Hölzel et al. 2012).

Although the used MLPA kit (SALSA MLPA probemix P377-A1 for Hematological Malignancies) has the ability to detect 37 target regions simultaneously, the overall by MLPA detected alteration was only 58% of the 163 aberrations, while routine iFISH test, which applied only 5 probes, revealed 61% of the overall present aberrations (article 2).

These unexpected results could be explained partly by the absence of probes targeted against chromosomal region 14q32 (*IGH* locus) in the MLPA kit, as this region is considered among the most frequent chromosomal regions being altered in CLL. *IGH* aberration were detected in this study by routine iFISH in 16 cases (article 2, Quintero-Rivera et al. 2009).

Also the cases with low percentage of aberrant cells (10% up to 34% of the cells being aberrant) could be missed by MLPA. Surprisingly in this study there cases were also detectable by MLPA with percentage of aberrant cells down to ~10% according to iFISH, and other cases with 34% of aberrant cells after iFISH could not be picked up by MLPA (article 2, Hömig-Hölzel et al. 2012).

Few previous studies reported the MLPA detection rates of low level mosaic CLL cases. Whereas, Coll-Mulet et al. (2008) and Abdool et al. (2010) demonstrated that false-negative MLPA results appeared in samples with less than 25% and 20% of aberrant cells, respectively, Véronèse et al. (2013) suggested that all false-negative cases occur in cases with 12-21% of aberrant cells. Thus it was estimated that 25-30% aberrant cells are sufficient for reliable detection by MLPA, which is definitely less sensitive than iFISH detection (Véronèse et al. 2013). On the other hand Al Zaabi et al. (2010) demonstrated that MLPA can reliably detect the 13q14 deletion in samples containing at least 36% of aberrant cells.

Still, the findings of this study are in concordance with Stevens-Kroef et al. (2009) who reported that the detection limit of MLPA could be down to 10% of abnormal cells, and they found an almost perfect correlation between MLPA and iFISH, as long as identical genetic regions were

tested. Finally, false negative MLPA results can be also due to the technical impossibility of MLPA in detection of balanced translocations (Hömig-Hölzel et al. 2012).

3.1.3. The combination of the different techniques in a CLL leads to a cost efficient diagnostic scheme.

In spite of the application of iFISH and other molecular techniques for the detection of chromosomal abnormalities in CLL, GTG-banding is still considered as the golden standard method, as it enables the untargeted search for gross chromosomal aberrations. In the present study this general statement (Keen-Kim et al. 2008, Wan et al. 2012) was confirmed, as 15 (~9%) of the 163 detected aberrations were identified exclusively by banding-cytogenetics (articles 1, 2).

For a better assessment of the prognosis and the diagnosis of CLL, a cost efficient diagnostic scheme is needed and has been suggested, which combines three techniques together in a systematic way based on the detected chromosomal aberrations (article 2). According to the revealed chromosomal abnormalities by GTG-banding, the next test which has been suggested to be done is MLPA. As shown MLPA and routine iFISH have in principle comparable detection rates in CLL, but MLPA covers a more broad spectrum of target genes and also it is more cost efficient than iFISH (article 2, Hömig-Hölzel et al. 2012).

The detection of an adverse diagnostic aberration by any of the applied methods in the recommended order is considered the end point of the tests to be done for an individual case. Thus for example it would be adequate if such an adverse acquired chromosomal aberration would be detected already by GTG-banding, no further procedures should be applied.

If the suggested diagnostic scheme would have been applied in the studied 85 CLL patients, iFISH would have been not necessary to be performed in 20 of them, as MLPA would have already identified one or more adverse chromosomal abnormalities.

Also four patients would have been classified in intermediate prognosis group after the application of MLPA, and then only three probes for the adverse prognosis aberrations would have been enough for iFISH test.

For the 29 cases which were normal according to MLPA results, all routine iFISH probes should be applied for the detection of the low level mosaics cases. So based on iFISH results 4, 1, and 6 cases went to adverse, intermediate, and good prognosis, respectively.

The CLL patients with good prognosis aberrations could be classified into favorable or unfavorable subgroups according to the size and /or percentage of the aberrant cells, also biallelic deletions in 13q14 which could be recognized by iFISH has been considered to be in the unfavorable subgroup. Thus, for 32 patients who revealed good prognosis aberrations according to MLPA, the same iFISH probes as for normal MLPA results should be applied (Garg et al. 2012, Puiggros et al. 2014).

If only MLPA and iFISH methods would have been applied for the studied 85 patient, in this situation still 3 cases would have been misclassified, as GTG-banding revealed poor prognostic aberrations in two of the cases, and intermediate-type aberration in the third one. Thus GTG-banding has been suggested to be the initial test for CLL diagnostics. Irrespective of that also two cases were normal by GTG-banding and iFISH but after the application of MLPA one of them showed del 6q and the other one revealed Dup *RUNX*, which showed the importance of additional MLPA test performance for a better prognosis of CLL disease.

The application of the suggested scheme would minimize the number of the applied iFISH probes, as 344 instead of 425 iFISH-probes, i.e. 20% less would be used (article 2).

3.2. *BIRC3* disruption in CLL and B-ALL

Attempts for a precise prognosis are undertaken to lead later to effective treatment regimens for CLL patients. Unfortunately the molecular basis for a subgroup of patients who experience an aggressive clinical course of the disease is still unclear, as they tend to have refractory and/or relapsed towards fludarabine-based regimens, which are generally considered as the first line treatment for CLL. However, ~40% of those CLL-patients are associated with *TP53* abnormalities and thus the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway could play a role in the mechanism of this therapy resistance. *BIRC3* gene is considered as a negative regulator of the non-canonical pathway of NF-κB. Disruption of *BIRC3* in CLL leads to the proliferation of cells and resistance to apoptosis due to activation of NF-κB which regulates anti-apoptotic genes especially the *TRAF1* and *TRAF2* (Rossi et al. 2013, Sun SC et al. 2011).

The previously reported aberrations which are related to *BIRC3* were either disruption in CLL or recurrent translocation t(11;18)(q21;q21) in mucosa associated lymphoid tissue (MALT)

lymphoma (Dierlamm et al., 1999, Rose-Zerilli et al., 2014, Morgan et al., 1999, Rosebeck et al., 2011).

Based on the results of a previous study, which included 637 CLL patients, the disruption of *TP53* and/or *BIRC3* has been considered as high risk prognostic factors. As *BIRC3* disruption did not appear in the fludarabine sensitive patients, an association with chemo-refractory CLL subtype was suggested (Rossi et al. 2013.)

As the *BIRC3* gene is located in 11q22.2 next to the *ATM* gene locus, the objective of this study was the evaluation if there is an association between *BIRC3* and *ATM* abnormalities in CLL (117 cases) – especially to answer the question if *BIRC3* and *ATM* deletion appear simultaneously. Furthermore, 45 B-ALL patients having the same original subtype of affected cells (B lymphocytes) were studied for presence of *BIRC3* alterations were (article 4, Puiggros et al. 2014).

BIRC3 duplication was detected in 3 CLL cases; two of them were due to a hyperdiploid status, which has been reported in CLL earlier, but not as a frequent event. Interestingly, the third CLL case had the *BIRC3* duplication as a sole abnormality, and the clinical impact of this is not known yet. Still, the duplication of *BIRC3* in that case was not detected by array-CGH, most likely due to the limitation of array-CGH in detection cases with low percentage of aberrant cells, despite being present in 36% of the cells, here (article 4, Shao et al. 2010, Specchia et al. 2002).

The unexpected result of *BIRC3* amplification could be explained by the tumor suppressive role of NF-κB via its non-canonical pathway which has been reported by Keller et al. (2010). Also several studies found that NF-κB mediates apoptosis in a variety of cell types, as the activation of NF-κB promotes the ability of *TP53* to induce apoptosis, and by this NF-kB plays an essential in p53-mediated apoptosis. It is notable that the studied case with sole *BIRC3* duplication is associated with normal *TP53* status. Taking together what is known about the interaction between *BIRC3* and NF-κB pathway, it has been proposed that *BIRC3* duplication could lead to inactivation of tumor suppressor activity (article 4, Ryan et al., 2000, Liu et al., 2012, Jing et al. 2014).

Also one of the B-ALL cases showed *BIRC3* duplication as a part of hyperdiploidy. A hyperdiploid status appears more frequently in B-ALL compared to CLL, and it has a good prognosis in pediatric patients (Kebriaei et al. 2002).

Previously it was reported that BIRC3 mutations are selectively restricted to CLL, while they are absent in other lymphoid tumors which are representative of the main categories of mature B-cell neoplasms (diffuse large B-cell lymphoma, Burkitt lymphoma, follicular lymphoma, extranodal marginal zone lymphoma, hairy-cell leukemia, and multiple myeloma). Therefore the second important finding of this study is the detection of a *BIRC3* deletion in one of the 45 studied B-ALL cases. Based on array-CGH result, the deletion included almost the whole long arm of chromosome 11. The most frequent aberration in B-ALL related to chromosome 11 is the structural abnormality of the 11q23 band harboring the *MLL* (myeloid/lymphoid leukemia) gene translocation in 3% to 7%, and being associated with an extremely poor prognosis (article 4, Rossi et al. 2012, Cox et al. 2004).

Chromosomal deletions involving 11q have been reported also in other subtypes of hematological malignancies, such as B-cell chronic lymphocytic leukemia (B-CLL), which is associated with a poor prognosis, and also in mantle cell lymphomas (MCL) and T-cell prolymphocytic leukemia (T-PLL) (Monni et al. 2001).

Based on that, the prognosis for the B-ALL case with *BIRC3* deletion could be poor or extremely poor. But it remains to be determined if in such cases *BIRC3* may also play a role for prognosis in B-ALL, as it does in CLL (Cox et al., 2004).

Although the frequency of *BIRC3* disruption is low at diagnosis, it tends to accumulate among refractory CLL patients, as it has been reported selectively in fludarabine-refractory patients with normal *TP53*. In this study *BIRC3* abnormalities were associated with TP53 deletion in only 4/117 CLL cases (article 4, Rossi et al. 2012).

Overall, based on the results of this study, *ATM* deletions may, but not always must be, associate with *BIRC3* abnormalities, as one of the CLL cases also showed *BIRC3* duplication with normal *ATM* gene status acc. to MLPA. Therefore, the screening of *BIRC3* in CLL patients is recommended particularly for correct treatment decisions and especially to decide whether to treat with or without fludarabine regime (article 4).

3.3. Isochromosome 17q in CLL.

As previously mentioned, among CLL patients with TP53 deletion there is a subgroup suffering from relapsed and/or refractory disease towards the used treatment regimens. It was previously suggested that deletion of *TP53* in CLL patients could be associated with the formation of

isochromosome 17q [i(17q)], which is described as a duplication of the whole long arm with a simultaneous deletion of the whole short arm. In addition to that, the appearance of a complex karyotype in CLL has been considered as a poor prognostic feature. Here overall 150 CLL patients were studied for possible correlation of i(17q) presence and complex karyotype (article 5, Puiggros et al. 2014, Scheurlen et al. 2004).

3.3.1. The detection rate of i(17q) by MLPA

In addition to that the assessment of MLPA in the detection of i(17q) in the first studied 85 CLL cases (article 2) 65 more cases were studied by iFISH experiments, and array-CGH in selected cases (article 5).

The used MLPA kit (SALSA MLPA probemix P377-A1) includes 4 probes for *TP53* gene, which is located on the short arm of chromosome 17, and one probe each for *UNC13D* and *IKZF3* genes, which are located on the long arm of chromosome 17. The presence of an i(17q) appears as deletion of *TP53* with concomitant duplication of *UNC13D* and *IKZF3* genes (article 5).

Based on the results, a diagnostic scheme combining GTG-banding, MLPA, and iFISH has been suggested for special detection of i(17q) cases (article 5). GTG- banding is proposed to be the first test to be done, later MLPA should be performed, and if an i(17q) has not identified by banding cytogenetics and MLPA further iFISH tests should be performed by using subtelomeric probes for chromosome 17, to detect the cases with low percentage of the aberrant cells (article 5).

Among 85 cases which have been studied by GTG-banding, MLPA, and iFISH. The presence of i(17q) has been identified in 2 out of 3 cases by MLPA, and the third case was detected by iFISH, only, and later confirmed by array-CGH. Also an i(17q) was identified already by GTG-banding in one case among them.

3.3.2. Association between i(17q) and complex karyotype

Overall among 150 CLL patients, deletion of *TP53* was detected in 18 (12%) cases; among those i(17q) has been identified in 6 (~33%) cases (article 5). Previously one study demonstrated that the formation of i(17q) is most likely due to clustered breakpoints in 17q11 and is not associated with *TP53* mutations of the intact chromosome 17 (Fioretos et al. 1999).

Whereas the detection rate of i(17q) in the present study is almost in concordance with the previously reported in 2006, which was 25%; the initial finding of an i(17q) in 2/21 (9.5%) CLL cases by Vahdati et al. (1989) seems to be overestimated due to small sample size (Fink et al. 2006).

According to the results presented here, it was obviously that complex karyotypes are more frequently in cases with i(17q) compared to the cases with just deletion of *TP53*. In addition to that, all here studied the cases with i(17q) showed associated aberrations, four of them were considered as poor prognosis, while among 12 cases with only *TP53* deletion, just one case showed concomitant poor prognosis aberrations, and 5 cases did not show any other chromosomal aberrations (article 5).

Several studies reported complex karyotypes as an indicator for a short OS and shorter TTFT (Rigolin et al. 2015, Baliakas et al. 2014).

Moreover, it was also demonstrated that the association between del(17p) and complex karyotype in the relapsed/ refractory CLL patients decreases the overall survival in comparison to those patients with only del(17p) (Thompson et al. 2015).

Due to lack of clinical data in the present study, the impact of i(17q) on the clinical course of the disease could not be followed up. Still this study suggested the association of i(17q) and complex karyotype, that presence of i(17q) could be an indicator for more aggressive course of CLL disease than just *TP53* deletion without the presence of i(17q).

Furthermore, the presence of i(17q) was reported as an adverse cytogenetic feature in other hematological neoplasia, like acute lymphocytic leukemia, acute promyelocytic leukemia, chronic myeloid leukemia, or other myeloid leukemia (Pui et al. 1988, Duan et al. 2013, McClure et al. 1998, Becher et al. 1990, Sousa et al. 2012).

4. Conclusions and outlook

The identification of, after GTG-banding cryptic chromosomal / genetic aberrations in CLL is necessary for a precise prognosis and thus correct determination of treatment protocols.

Although cytogenetics and molecular cytogenetics are and will continue to be indispensable tools in leukemia diagnostics, each technique has its own limitations and advantages. The present work highlights that only the combination between cytogenetics, molecular genetics and molecular cytogenetics can lead to most comprehensive insights into the (cyto)genetic abnormalities in CLL. Thus, all the three techniques should be used for accurate diagnosis and therapeutic decisions for CLL patients according to the cost efficient suggested scheme:

MLPA should be applied as an initial test if routine cytogenetics is not possible or non-informative as it has the ability to detect rare chromosomal aberrations in CLL, according to the obtained MLPA-results iFISH should be performed when it is needed to distinguish mono- from biallelic deletions and also to avoid missing of aberrations being present only at low percentages of the studied cells.

Furthermore, according to the results of the present study *BIRC3* abnormalities are not always going together with *ATM* deletions. Thus screening of *BIRC3* may be considered as necessary in future, particularly to help taking the accurate treatment decisions.

As deletion of *TP53* can be associated with the formation of an i(17q) and the latter is associated with more aggressive disease course this aberration should be specifically checked in *TP53*-deletion cases. However, MLPA alone is not sufficient to pick up all corresponding cases and a combination with iFISH should be considered additionally.

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

1. How many cryptic chromosomal aberrations in the 85 studied CLL cases could be detected by MLPA, in comparison with routine iFISH and GTG-banding?

Overall 163 aberrations were identified. 15 of those (~9%) were exclusively detected by banding cytogenetics, 95 were found by MLPA (~58%) and 100 (~61%) by routine iFISH.

2. Could the underlying chromosomal abnormalities in CLL be precisely identified, to avoid misinterpretation of the prognosis so subsequently incorrect treatment regimens?

Based on the obtained results a cost efficient diagnostic scheme is proposed combining GTG-banding, MLPA, and iFISH for a better diagnosis and thus treatment for CLL patients.

3. What is the percentage of *BIRC3* disruption in the studied 117 CLL cases, and its correlation with *ATM* deletion?

Genetic abnormalities of BIRC3 were detected in 23/117 (~20%) of CLL, and one of the CLL cases showed duplication of *BIRC3* without any alteration in *ATM*.

4. Is *BIRC3* disruption specific only for CLL?

No, as *BIRC3* abnormalities were detected in 2/45 (~4%) studied B-ALL cases.

5. Is presence or absence of i(17q) in CLL able to identify a new subgroup with more aggressive clinical course of the disease, and what is the best way for its detection?

A scheme has been proposed for the detection of i(17q) combining GTG-banding, MLPA, and iFISH, and it was demonstrated that i(17q) is more frequently associated with complex karyotypes. Thus i(17q) presence is considered as a hint for identification of a subgroup with more aggressive clinical course of the disease.

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6. Appendix

6.1. List of tables

Supplementary Table 1 (Article 2)

Gender, age and cytogenetic results of the studied cases/ samples.

Abbreviations: b = cell pellet in Carnoys fixative from blood; B = native blood; bm = cell pellet in Carnoys fixative from bone marrow; BM = native bone marrow; F = female; M = male;

case / sample number	gender	Age [y]	DNA extracted from	Cytogenetics
1	M	83	bm	47,XY,-11,+12,+mar[cp3]/ 47,XY,del(5)(p1?3),-11,+12,-17,+mar1,+mar2[cp6]/ 46,XY[9]
2	M	68	bm	46,XY
3	F	62	B	n.a.
4	M	72	b	47,XY,?t(2;14),+12[3]/ 45,X,-Y[4]/46,XY[11]
5	M	65	b	46,XY
6	M	71	bm	46,XY
7	M	50	bm	46,XY
8	F	64	bm	46,XX
9	F	55	bm	46,XX
10	F	39	bm	43~46,XY,del(11)(q2?2q2?4)[cp5]/ 45~46,XY,del(11)(q2?2q2?4),del(15)(q1?1q2?3)[cp11]/ 46,XY[1]
11	F	66	B	46,XX
12	F	50	b	n.a.
13	F	90	b	n.a.
14	M	64	bm	46,XY
15	M	43	bm	46,XY
16	M	73	bm	46~47,XY,del(11)(q22q2?3),add(17)t(17;?)(p11.2;?)[cp5]/ 45~46,XY,del(11)(q22q2?3),del(17)(p11.2)[cp4]/ 43~46,XY,del(11)(q22q2?3)[cp2]/ 46,XY[7]
17	M	72	bm	46,XY
18	F	66	b	n.a.
19	F	71	b	46,XX
20	F	74	b	46,XX
21	M	71	bm	46,XY
22	F	76	b	46,XX
23	M	62	b	46,XY
24	M	67	bm	46,XY
25	M	83	b	n.a.
26	M	79	B	n.a.
27	F	73	bm	46,XX
28	F	49	B	46,XX
29	F	69	b	n.a.
30	M	78	b	46,XY
31	M	74	bm	46,XY
32	M	71	b	45,X,-Y[cp8]/ 46,XY[10]
33	F	63	bm	46,XX

Supplementary Table 1 (Article 2 - continued)

case / sample number	gender	age [y]	DNA extracted from	Cytogenetics
34	M	77	bm	46,XY,del(11)(q?21),add(20)(p13)[7]/ 45,X,-Y[10]/ 46,XY[3]
35	M	53	bm	46,XY
36	M	73	bm	45,X,-Y,t(9;22)(q34;q11)
37	M	74	bm	45,X,-Y[2]/ 47,XY,+12[1]/ 48,XY,-6,-8,+12,+mar,+mar,+mar[1]/ 46,XY[14]
38	M	65	bm	46,XY,?t(3;?)(p21;?),add(17)(p?12) or t(17;?),-18,+mar[cp7]/ 46,XY[9]
39	F	74	B	46,XX
40	F	72	b	46,XX
41	M	72	bm	45,X,-Y[4]/ 46,XY[16]
42	M	51	bm	46,XY
43	F	48	b	46,XX
44	F	47	b	46,XX
45	F	79	bm	46,XX
46	M	67	bm	n.a.
47	M	67	bm	n.a.
48	M	68	bm	46,XY
49	M	61	b	46,XY
50	M	73	bm	46,XY
51	M	75	bm	46,XY
52	M	77	b	n.a.
53	M	54	b	46,XY
54	M	59	bm	n.a.
55	M	59	b	46,XY
56	F	47	B	46,XX
57	M	71	bm	46,XY,der(1)(t(1;4)(q1?2;q?31),der(4)t(4;?10)(q?31;q24), ?der(10)t(10;16)(q24;p?11.2),der(15)t(1;15)(q1?2;q1?2), der(16)t(15;16)(q1?2;p?11.2)[17]/ 46,XY[2]
58	M	73	bm	45,XY,der(2)t(2;13)(q?37;q?14),?del(6)(p?23),del(11)(q?21), der(12)t(12;13)(q?24;q?22),-13[cp4]/46,XY[19]
59	M	72	bm	46,XY
60	M	54	B	n.a.
61	F	74	bm	46,XX,i(17)(q10)[1]/ 46,XX,+12,i(17)(q10),-21[9]/ 46,XX,t(3;?)(q2?9;?)[4],-7[4],+12[4],i(17)(q10)[4][cp4]/ 46,XX[4]
62	F	65	bm	46,XX
63	M	72	b	n.a.
64	F	73	bm	46,XX,add(11)(q?22)[3]/ 46,XX[12]
65	M	54	b	46,XY

66	F	69	bm	46,XX
67	M	53	bm	46,XY

Supplementary Table 1 (Article 2 - continued)

case / sample number	gender	age [y]	DNA extracted from	Cytogenetics
68	M	53	b	46,XY
69	M	75	bm	46,XY
70	M	56	b	46,XY,?add(1q)(q4)[3]/ 46,XY[3]
71	F	58	BM	n.a.
72	F	73	B	n.a.
73	M	66	bm	46,XY
74	M	74	bm	46,XY
75	F	51	bm	46,XX
76	M	52	bm	46,XY
77	M	63	bm	n.a.
78	M	60	b	46,XY
79	M	72	b	n.a.
80	F	49	bm	46,XX
81	F	64	bm	46,XX
82	F	72	bm	46,XX
83	M	82	b	46,XY
84	M	74	BM	46,XY
85	F	72	bm	n.a.

Supplementary Table 2 (Article 2)

Aberrations detected in 85 CLL samples and by which method the corresponding aberrations could be detected. Abbreviations: + = detected, (+) = detected but not specific as bi- or monoallelic deletion, o = not tested, - not detected, n = no aberration.

sample number	aberrations [%]	iFISH routine	MLPA	iFISH confirmatory for MLPA
1	del(5)(p1?3)[33]	o	o	o
	del(11)(q22.3q22.3)[30]	+	-	o
	+12[70]	+	+	o
	del(13)(q14.3q14.3)[30]	+	+	o
	rea(14)(q32.33)[28] -> ?+14	+	o	o
	del(17)(p13.1p13.1)[16]	+	+	o
	amp(17)(q25.1q25.2)[40]	o	+	+
2	del(11)(q22.3q22.3)[33]	+	-	o
	del(13)(q14.3q14.3)[18]	+	+	o
	del(13)(q14.3q14.3)x2[14]	+	(+)	o
3	+12[15]	+	-	o
	rea(14)(q32.33)[52] -> t(14;18)(q32;q21)	+	o	o
4	+12[31]	+	-	o
	del(13)(q14.2q14.2)[45]	o	+	+
	del(13)(q14.3q14.3)[20]	+	+	o
5	amp(2)(p24.3p24.3)[60]	o	+	+
	amp(2)(p23.2~23.1p23.2~23.1)[63]	o	+	+
	del(6)(q23.3q23.3)[68]	o	+	+
	del(6)(q25.1q25.1)[65]	o	+	+
	del(6)(q27q27)[23]	o	+	+
	del(13)(q14.3q14.3)[18]	+	-	o
	del(14)(q32q32)[65]	+	o	o
	amp(21)(q22.12q22.12)[86]	o	+	+
6	del(13)(q14.3q14.3)[10]	+	-	o
7	del(13)(q14.3q14.3)[10.5]	+	-	o
8	del(13)(q14.3q14.3)[12]	+	-	o
9	del(13)(q14.3q14.3)[18.5]	+	-	o
10	del(11)(q22.3q22.3)[98]	+	+	o
	del(13)(q14.3q14.3)[25]	+	-	o
11	del(13)(q14.3q14.3)[34]	+	-	o

Supplementary Table 2 (Article 2 - continued)

sample number	aberrations [%]	iFISH routine	MLPA	iFISH confirmatory for MLPA
12	del(13)(q14.2q14.2)[52]	o	+	+
	del(13)(q14.2q14.2)x2[38]	o	(+)	+
	del(13)(q14.3q14.3)[34]	+	-	o
	del(17)(p13.1p13.1)[21]	+	+	o
13	del(13)(q14.3q14.3)x2[98.5]	+	+	o
	del(17)(p13.1p13.1)[11.5]	+	-	o
14	del(11)(q22.3q22.3)[23.5]	+	+	o
	del(13)(q14.3q14.3)[34]	+	+	o
15	del(11)(q22.3q22.3)[24]	+	+	o
16	amp(8)(q24.21q24.21)[21]	o	+	+
	del(11)(q22.3q22.3)[11]	+	+	o
	del(17)(p13.1p13.1)[86]	+	+	o
17	del(13)(q14.3q14.3)[20]	+	+	o
	del(17)(p13.1p13.1)[40]	+	+	o
18	del(13)(q14.3q14.3)[90.5]	+	+	o
	del(17)(p13.1p13.1)[19]	+	+	o
19	del(17)(p13.1p13.1)[36]	+	+	o
20	del(13)(q14.3q14.3)x2[94]	+	+	o
	del(14)(q32q32)[97]	+	o	o
21	del(13)(q14.2q14.2)[50]	o	+	+
	del(13)(q14.2q14.2)x2[30]	o	(+)	+
	del(13)(q14.3q14.3)x2[91]	+	+	o
22	del(13)(q14.3q14.3)[5]	+	(+)	o
	del(13)(q14.3q14.3)x2[75]	+	+	o
23	del(13)(q14.3q14.3)[5]	+	(+)	o
	del(13)(q14.3q14.3)x2[81]	+	+	o
24	del(11)(q22.3q22.3)[88]	+	+	o
	del(13)(q14.2q14.2)[36]	o	+	+
	del(13)(q14.2q14.2)x2[41]	o	(+)	+
	del(13)(q14.3q14.3)[16]	+	(+)	o
	del(13)(q14.3q14.3)x2[71]	+	+	o
25	del(13)(q14.2q14.2)[66]	o	+	+
	del(13)(q14.2q14.2)x2[21]	o	(+)	+
	del(13)(q14.3q14.3)[18]	+	(+)	o
	del(13)(q14.3q14.3)x2[77]	+	+	o
26	del(13)(q14.3q14.3)[25]	+	+	o
	del(13)(q14.3q14.3)x2[65]	+	(+)	o

Supplementary Table 2 (Article 2 - continued)

sample number	aberrations [%]	iFISH routine	MLPA	iFISH confirmatory for MLPA
27	del(13)(q14.2q14.2)[34]	o	+	+
	del(13)(q14.2q14.2)x2[27]	o	(+)	+
	del(13)(q14.3q14.3)[36.5]	+	+	o
	del(13)(q14.3q14.3)x2[24]	+	(+)	o
	del(14)(q32q32)[12]	+	o	o
28	del(13)(q14.3q14.3)[81]	+	+	o
	del(13)(q14.3q14.3)x2[7]	+	(+)	o
29	del(13)(q14.2q14.2)[58]	o	+	+
	del(13)(q14.2q14.2)x2[24]	o	(+)	+
	del(13)(q14.3q14.3)[86]	+	+	o
	del(13)(q14.3q14.3)x2[9]	+	(+)	o
30	del(13)(q14.3q14.3)x2[100]	+	+	o
31	del(14)(q32q32)[92]	+	o	o
32	del(14)(q32q32)[81]	+	o	o
	-Y[44]	o	o	o
33	del(14)(q32q32)[58]	+	o	o
34	del(11)(q22.3q22.3)[90]	+	+	o
	del(14)(q32q32)[90]	+	o	o
	-Y[50]	o	o	o
35	del(11)(q22.3q22.3)[77]	+	+	o
36	t(9;22)(q34;q11)[94]	+	o	o
37	+12[49.5]	+	+	o
38	t(3;?)(p21;?)[43]	o	o	o
	del(17)(p13.1p13.1)[89]	+	+	o
39	del(17)(p13.1p13.1)[77]	+	+	o
40	del(13)(q14.3q14.3)[52]	+	+	o
41	del(13)(q14.3q14.3)[60]	+	+	o
	-Y[80]	o	o	o
42	del(13)(q14.3q14.3)[68]	+	+	o
43	del(13)(q14.3q14.3)[70.5]	+	+	o
44	del(13)(q14.3q14.3)[73]	+	+	o
45	del(13)(q14.3q14.3)[73]	+	+	o
46	del(13)(q14.3q14.3)[80]	+	+	o
47	del(13)(q14.3q14.3)[80]	+	+	o
48	del(13)(q14.3q14.3)[81]	+	+	o

Supplementary Table 2 (Article 2 - continued)

sample number	aberrations [%]	iFISH routine	MLPA	iFISH confirmatory for MLPA
49	del(13)(q14.3q14.3)[83]	+	+	o
50	del(13)(q14.3q14.3)[85]	+	+	o
51	del(13)(q14.3q14.3)[91]	+	+	o
52	del(13)(q14.3q14.3)[94]	+	+	o
53	del(13)(q14.3q14.3)[94.5]	+	+	o
54	del(11)(q22.3q22.3)[98]	+	+	o
	del(13)(q14.2q14.2)[41]	o	+	+
	del(13)(q14.2q14.2)x2[39]	o	(+)	+
	del(13)(q14.3q14.3)[97]	+	+	o
55	del(13)(q14.2q14.2)[73]	o	+	+
	del(13)(q14.2q14.2)x2[5]	o	(+)	+
	del(13)(q14.3q14.3)[85]	+	+	o
56	del(13)(q14.2q14.2)[22]	o	+	+
	del(13)(q14.2q14.2)x2[58]	o	(+)	+
	del(13)(q14.3q14.3)[12]	+	+	o
	del(13)(q14.3q14.3)x2[66]	+	(+)	o
57	der(1)t(1;4)(q1?2;q?31)[90]	o	o	o
	der(4)t(4;?10)(q?31;q24)[90]	o	o	o
	?der(10)t(10;16)(q24;p?11.2)[90]	o	o	o
	der(15)t(1;15)(q1?2;q1?2)[90]	o	o	o
	der(16)t(15;16)(q1?2;p?11.2)[90]	o	o	o
58	der(2)t(2;13)(q?37;q?14)[21]	o	o	o
	?del(6)(p?23)[21]	o	o	o
	del(11)(q22.3q22.3)[87]	+	+	o
	del(13)(q14.3q14.3)[87]	+	+	o
	del(14)(q32q32)[85]	+	o	o
59	del(14)(q32q32)[85]	+	o	o
60	rea(14)(q32.33)[96] -> t(14;?)(q32;?)	+	o	o
61	t(3;?)(q2?9;?)[22]	o	o	o
	-7[22]	o	o	o
	+12[78]	+	+	o
	del(14)(q32q32)[94]	+	o	o
	del(17)(p13.1p13.1)[95]	+	+	o
	amp(17)(q25.1q25.2)[22]	o	+	+

Supplementary Table 2 (Article 2 - continued)

sample number	aberrations [%]	iFISH routine	MLPA	iFISH confirmatory for MLPA
62	+12[80]	+	+	o
	del(13)(q14.3q14.3)[62]	+	+	o
	amp(18)(p11.21q11.21)[75]	o	+	+
	amp(18)(q21.2q21.2)[75]	o	+	+
63	amp(2)(p24.3p24.3)[62.5]	o	+	+
	amp(2)(p23.2~23.1p23.2~23.1)[62.5]	o	+	+
	del(11)(q22.3q22.3)[95]	+	+	o
	del(13)(q14.2q14.2)[51]	o	+	+
	del(13)(q14.2q14.2)x2[38]	o	(+)	+
	del(13)(q14.3q14.3)[90]	+	+	o
	del(14)(q32q32)[91]	+	o	o
64	amp(2)(p24.3p24.3)[65]	o	+	+
	amp(2)(p23.2~23.1p23.2~23.1)[75]	o	+	+
	del(11)(q22.3q22.3)[83]	+	+	o
	del(13)(q14.3q14.3)[58.5]	+	+	o
65	amp(6)(q27q27)[?]	o	+	-
	del(11)(q22.3q22.3)[93]	+	+	o
	del(13)(q14.3q14.3)[96]	+	+	o
66	del(13)(q14.3q14.3)[90]	+	+	o
	del(14)(q32q32)[81]	+	o	o
	amp(18)(q21.2q21.2)[?]	o	+	-
67	del(13)(q14.3q14.3)[60]	+	+	o
	del(14)(q32q32)[80]	+	o	o
	amp(18)(p11.21q11.21)[?]	o	+	-
68	del(6)(q21q21)[33]	o	+	+
	del(6)(q23.3q23.3)[92]	o	+	+
69	amp(21)(q22.12q22.12)[50]	o	+	+
70	?add(1q)(q4)[50]	o	o	o
71	None	n	n	o
72	None	n	n	o
73	None	n	n	o
74	none	n	n	o
75	none	n	n	o
76	none	n	n	o
77	none	n	n	o

Supplementary Table 2 (Article 2 - continued)

sample number	aberrations [%]	iFISH routine	MLPA	iFISH confirmatory for MLPA
78	none	n	n	o
79	none	n	n	o
80	None	n	n	o
81	none	n	n	o
82	none	n	n	o
83	none	n	n	o
84	none	n	n	o
85	none	n	n	o

6.2. List of own publications

Alhourani E, Othman MA, Melo JB, Carreira IM, Grygalewicz B, Vujić D, Zecević Z, Joksić G, Glaser A, Pohle B, Schlie C, Hauke S, Liehr T. BIRC3 alterations in chronic and B cell acute lymphocytic leukemia patients. Oncol Lett, in press.

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Othman MA, Melo JB, Carreira IM, Rincic M, **Alhourani E,** Wilhelm K, Gruhn B, Glaser A, Liehr T. 2014 . 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, 33(2):625-30.

Othman MA, Rincic M, Melo JB, Carreira IM, **Alhourani E**, Hunstig F, Glaser A, Liehr T. 2014. 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, doi: 10.1155/2014/357123.

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Alhourani E, Abou KI, Attaya A. 2012. Role of Anti-MCV in Early Diagnosis of Rheumatoid Arthritis. Journal of Syrian clinical Laboratory Association, 4:7-12.

6.4. Curriculum Vitae

Personal information

- **Full Name:** Eyad Alhourani
- **Date of birth:** 24.08.1982
- **Place of birth:** Damscus, Syria
- **Nationality:** Syrian
- **Address:** Stauffenbergstr.2a, 07747 Jena
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Education

- **1988 – 1994** Teshreen Primary school, Damascus, Syria.
- **1994 – 2000** Ibn-Khaldon Secondary school, Hama, Syria.
- **2001 – 2006 B.Sc.** in Pharmacy, Damascus University, Syria.
- **2006 – 2011 M.Sc.** in Laboratory diagnosis, Damascus University, Syria.

Award

KAAD fellowship for Ph.D. study in Friedrich Schiller University of Jena (2013-2016).

Research experience

Cell Culture and Chromosomal analysis with GTG Banding.
Molecular Cytogenetics; Fluorescent in-situ hybridization (FISH) techniques.
Multiplex ligation probe dependent amplification (MLPA).
DNA isolation (Peripheral blood, Bone marrow, fixed cells and celllines),
DNA Reamplification and Labelling techniques.

Employment record

2005 – 2006: Trainee at Almouna pharmacy.
2006 – 2007: Part-time worker at Almouna pharmacy.
2007 – 2009: Part-time worker at Barakat Company as a medical representative
2009 – 2011: Part-time worker at Cheise Company as a medical representative
04.2012 – 09.2012: Practicum in Tübingen University.
09.2012 – 02.2013: Practicum in Heidelberg University.

04.2013 – till now: Ph.D student in group of PD Dr. rer. nat./
med. habil., h.c. (YSU) Thomas Liehr,
Molecular Cytogenetics Department,
Institute for Human Genetics, Jena University Hospital,
Friedrich Schiller University of Jena.

Personal skills

Languages: Arabic; Mother tongue.
English; Independent user.
German; Basic user / B2.

Computer skills: Windows & MS Office.
Online databases like ENSEMBL, BLAST, DGV and UCSC genome browser.
Karyotyping softwares.

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6.5. Ehrenwörtliche Erklärung

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

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

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.

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, 23.02.2016

Eyad Alhourani