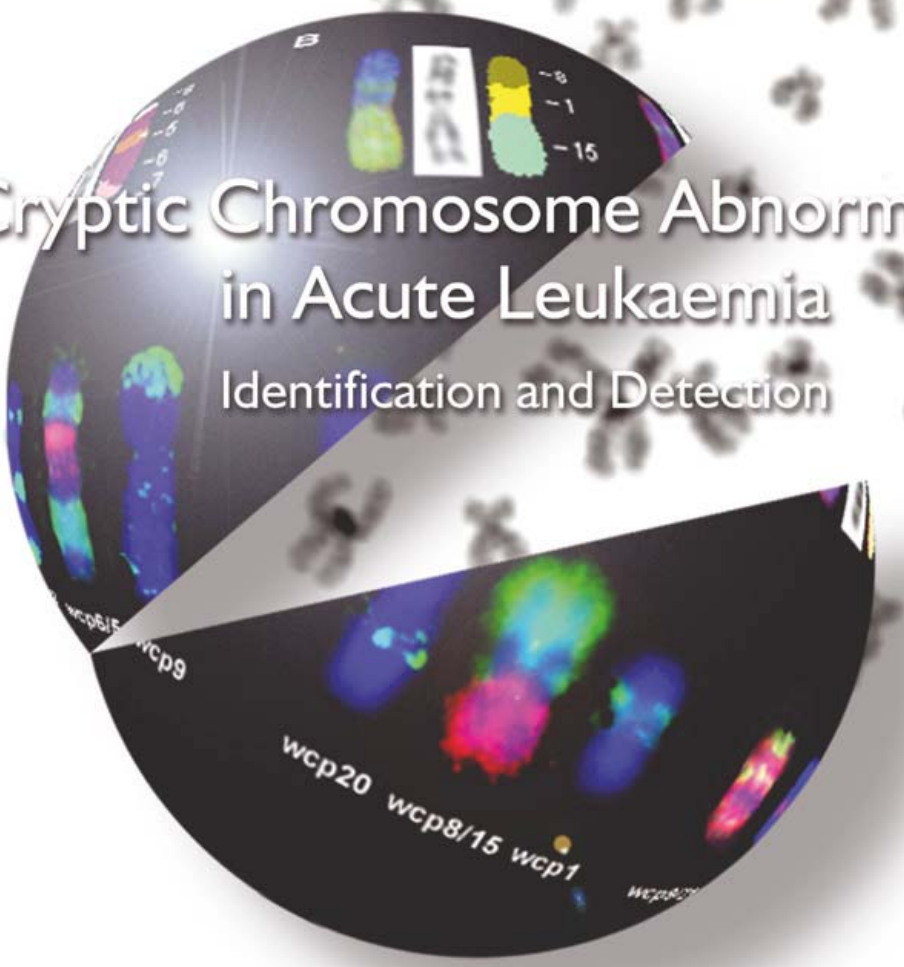

Cryptic Chromosome Abnormalities in Acute Leukaemia

Identification and Detection

Laura J.C.M. van Zutven

Cryptic Chromosome Abnormalities in Acute Leukaemia
Identification and Detection

Cryptische chromosoomafwijkingen in acute leukemie
Identificatie en detectie

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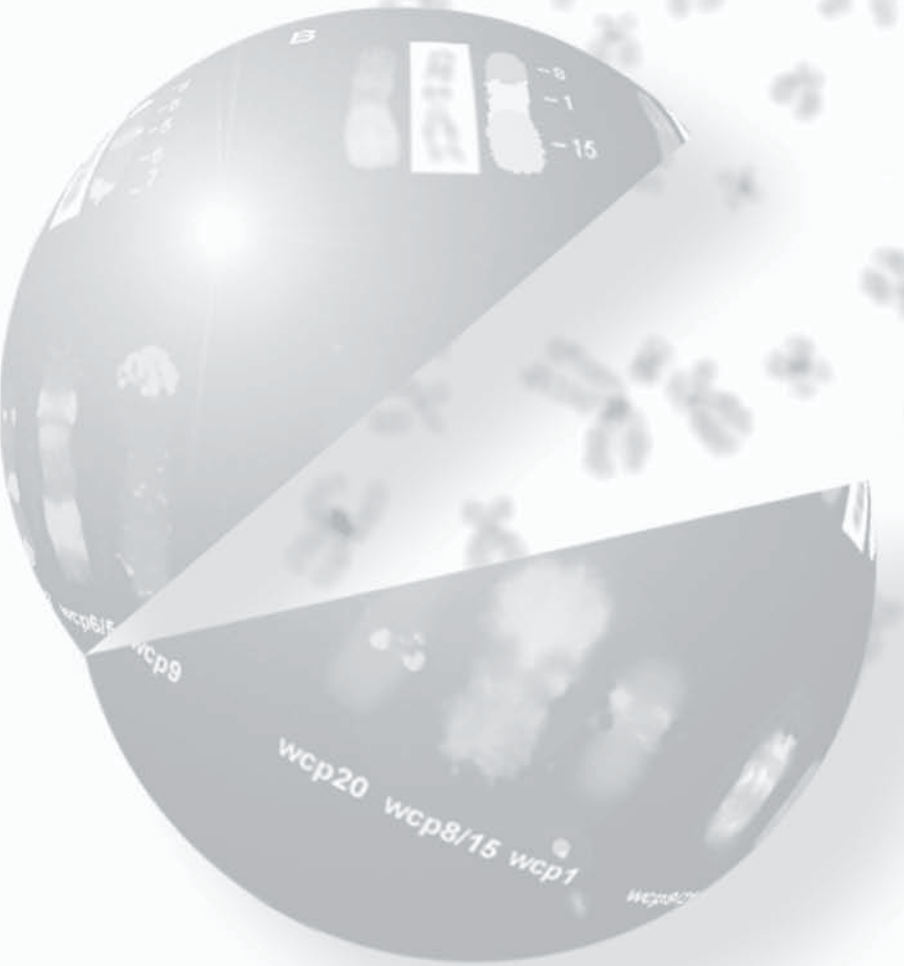
Chapter 1
General Introduction



Chapter 1
General Introduction

Normal haematopoiesis and leukaemia

Normal haematopoiesis

Blood contains many different cell types with different functions, such as lymphocytes, erythrocytes, granulocytes, monocytes and platelets. The two types of lymphocytes, B- and T-lymphocytes, are involved in the antigen-specific immune responses. Erythrocytes transport O_2 from the lungs through the blood vessels to the organs and CO_2 from the organs to the lungs. Granulocytes and monocytes are important for inflammatory response following infections, and the platelets are essential for blood clotting. Interestingly, all these different cells are generated from a common pluripotent stem cell in the bone marrow with a life-long self-renewal capacity (Figure 1). This cell gives rise to committed progenitor cells, which can self-renew a limited number of times. However, in the end they are irreversibly determined to differentiate into one or a few blood cell types. Two main lineages of blood cell differentiation exist, the lymphoid and the myeloid differentiation. The lymphoid progenitors differentiate into B-lymphocytes via precursor-B-cell development in the bone marrow, or into T-lymphocytes via precursor-T-cell development in the thymus. The other blood cell types develop through the myeloid lineage (Figure 1). As soon as the lymphoid or myeloid differentiation process is completed, the mature cells are released from the bone marrow or the thymus into the blood circulation [1]. Abnormalities in the normal program of blood cell differentiation or proliferation result in haematological diseases, including leukaemia.

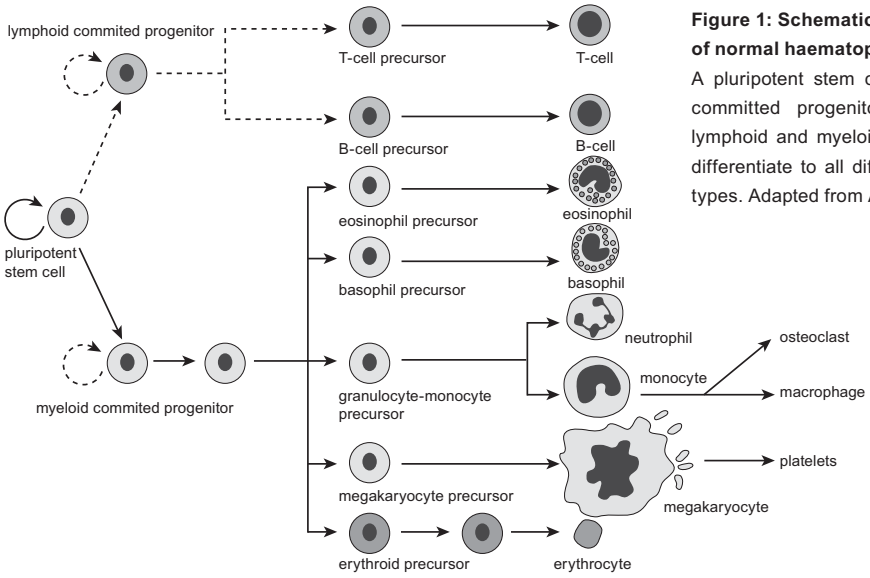


Figure 1: Schematic representation of normal haematopoiesis.
A pluripotent stem cell gives rise to committed progenitor cells of the lymphoid and myeloid lineage, which differentiate to all different blood cell types. Adapted from Alberts *et al.* [2]

Leukaemia

Leukaemia is usually defined as the uncontrolled proliferation or expansion of haematopoietic cells that do not retain the capacity to differentiate normally to mature blood cells. According to this definition, some haematological disorders are not strictly leukaemias because they display only part of the full leukaemic phenotype, either growth expansion (myeloproliferative syndromes, MPS) or differentiation block (myelodysplastic syndromes, MDS). However, both can progress to acute leukaemia, and are sometimes called pre-leukaemic disorders [3].

Usually, leukaemia is divided into two main classes: acute and chronic leukaemia. Acute leukaemia shows a fast clinical pattern, and can result in death in a relatively short period of time, whereas chronic leukaemia is generally less aggressive and patients might live with this disease for several years if not treated [4]. Both acute and chronic leukaemia are subdivided into different categories, depending on the lineage and cell type involved. In acute myeloid leukaemia (AML), a clonal expansion of immature myeloid blasts occurs in the bone marrow, whereas in acute lymphoblastic leukaemia (ALL) a neoplasm of lymphoblasts of either the B-cell lineage (pro-B, common (c) ALL or pre-B-ALL) or the T-cell lineage (T-ALL) occurs in the bone marrow [5]. MDS is characterized by dysplasia (abnormal development and growth) and ineffective haematopoiesis in one or more of the major myeloid cell lineages [5] (see Figure 1). Chronic myeloid leukaemia (CML) is a myeloproliferative disease, originating from an abnormal pluripotent bone marrow stem cell. The initial phase is chronic, followed by the more aggressive stages, acceleration and/or blast crisis [5].

The leukaemic cells overgrow the normal cells in the bone marrow because of the uncontrolled proliferation of the haematopoietic cells with a differentiation block. This can result in strongly reduced numbers of normal mature blood cells, such as anaemia (low red blood cell count), thrombocytopenia and neutropenia, leading to fatigue, problems with blood clotting (e.g. bruising and prolonged bleedings) and infections respectively [5].The worldwide incidence of acute leukaemia is approximately 5 per 100,000 inhabitants per year [6], comprising just a very small fraction (~1%) of all cancers. Around 70% of these cases (worldwide) display AML, of which the vast majority of cases occur in adults, with a median age of 60 years. ALL is predominantly a disease of children: worldwide, 75% of ALL cases occur in children under 6 years of age [5]. MDS occurs predominantly in older adults, with a median age of 70 years. The incidence, not corrected for age, is 3 per 100,000 inhabitants worldwide, but this rises to 20 per 100,000 in the group older than 70 [5]. CML occurs in 15-20% of all leukaemia cases, with a worldwide incidence of 1-1.5 cases per 100,000 inhabitants per year. It can occur at any age, but the median age at diagnosis is around 50-60 years [5].

Chromosome aberrations in cancer

Cancer mostly arises due to somatically acquired genetic changes, such as (partial) gains or losses of chromosomes, deletions, translocations and inversions as well as microscopically invisible changes at the nucleotide level, such as point mutations and small deletions [7]. Numerical aberrations or unbalanced rearrangements are frequently observed in solid tumours, but are less common in haematological malignancies, in which translocations are

found more often. Point mutations or amplifications of oncogenes, rendering these genes constitutively or over expressed, thus altering the proportion of the encoded proteins, can cause uncontrolled proliferation of cells, which can lead to cancer. Deletions frequently result in the loss of tumour suppressor genes, thus deregulating the cell cycle or the normal process of apoptosis to eliminate cells with genetic damage. Knudson's "two-hit-hypothesis", originally proposed for retinoblastoma, states that two "hits" or mutagenic events are necessary for development of cancer [8]. The first hit can be present in the germ line, and thus in all somatic cells. However, before a tumour arises, a second somatic mutation is essential. Alternatively, both crucial mutations need to arise somatically within the same cell. This hypothesis is valid for numerous tumour suppressor genes [9], but for many cancers a multi-hit model holds true [10, 11]. It is now thought at least 6 essential alterations in cell physiology are needed to cause most cancers [12]. These alterations are acquired capabilities, i.e. self-sufficiency in growth signals, insensitivity to antigrowth signals, evading of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. Genome instability enables these alterations to occur in premalignant cell populations [12].

Chromosomal translocations and inversions can have two major consequences: firstly, regulatory sequences of a gene come to lie near a proto-oncogene, which thus becomes activated [7]. This often involves the regulatory sequences of genes coding for T-cell receptors (TCRs) or immunoglobulin (Ig) proteins. In lymphoid cells, these loci recombine to produce functional *Ig* or *TCR* genes (VDJ recombination). This process can accidentally lead to translocations and inversions. Because VDJ recombination naturally occurs in the immune system, high-level transcriptional activation of a proto-oncogene by juxtaposition to an *Ig* or *TCR* gene occurs only in lymphoid malignancies (reviewed in [7, 13]), e.g. t(8;14)(q24;q32) in Burkitt's lymphoma, where *C-MYC* (8q24) is activated through enhancers constitutively active in the *IGH* region (14q32) [14]. The other possible outcome of chromosomal translocations and inversions is that the breaks occur in two genes, which upon wrongly joining, create an in-frame fusion gene encoding a chimaeric protein [7], e.g. t(9;22)(q34;q11) in CML, fusing *BCR* (22q11) and *ABL* (9q34) in-frame [15, 16] (see below). The genes involved often encode signalling molecules or transcription factors, which indicates that altered signalling or transcription plays a major role in tumourigenesis (reviewed in [7, 13]). Translocations and inversions can be specific for certain types of tumours or idiopathic (non-specific), meaning that they are observed in different tumour types or only in one particular case.

Chromosome aberrations in leukaemia

Chromosome rearrangements frequently occur in haematological malignancies, including leukaemia. To date many specific translocations and inversions have been described for specific different subtypes of leukaemia, and more are still being identified (see "Translocations, fusion genes and leukaemogenesis"). Additionally, regions frequently lost or gained have been described, and the search for genes within these regions, thought to be involved in leukaemogenesis, is going on. Translocations can occur as easily visible abnormalities in conventional karyotyping when large or distinct parts of chromosomes have been exchanged. However, during the last 10 years, several frequently occurring translocations have been

identified which are cryptic by nature (i.e. not visible by classical cytogenetic staining techniques) because of the exchange of small fragments or fragments with very similar banding, e.g. t(12;21)(p13;q22) [17-19] and t(5;14)(q35;q32) [20, 21].

Relevance of cytogenetics in acute leukaemia

Cytogenetic analysis can provide information about the aberrations present in leukaemia. The presence of a specific cytogenetic aberration can support the diagnosis and classification of the involved leukaemia. Using cytogenetic approaches, one can investigate whether progression or regression of the disease has occurred and also discriminate relapses from secondary malignancies [13]. Furthermore, the presence of chromosome rearrangements constitutes a prognostic factor for the outcome of disease, as e.g. t(8;21)(q22;q22), t(15;17)(q22;q21) or inv(16)(p13q22), and t(9;22)(q34;q11) or t(4;11)(q21;q23) are predictive of good and bad outcomes in AML and ALL (Figure 2) [5]. Additionally, cytogenetics can help in the identification of cytogenetically different leukaemic subclones within one sample. Specific chromosome abnormalities can also be correlated to *in vitro* drug sensitivity, as measured using the MTT assay [22]. For example, these tests showed that *TEL-AML1* positive cases are more sensitive for L-asparaginase than *TEL-AML1* negative cases, and that *MLL* rearranged infant ALL cases are more resistant to L-asparaginase and prednisolone, but more sensitive to cytarabine and 2-chlorodeoxyadenosine than *MLL* non-rearranged cases [23, 24]. Thus, the presence of chromosome rearrangements might have therapeutic consequences as well.

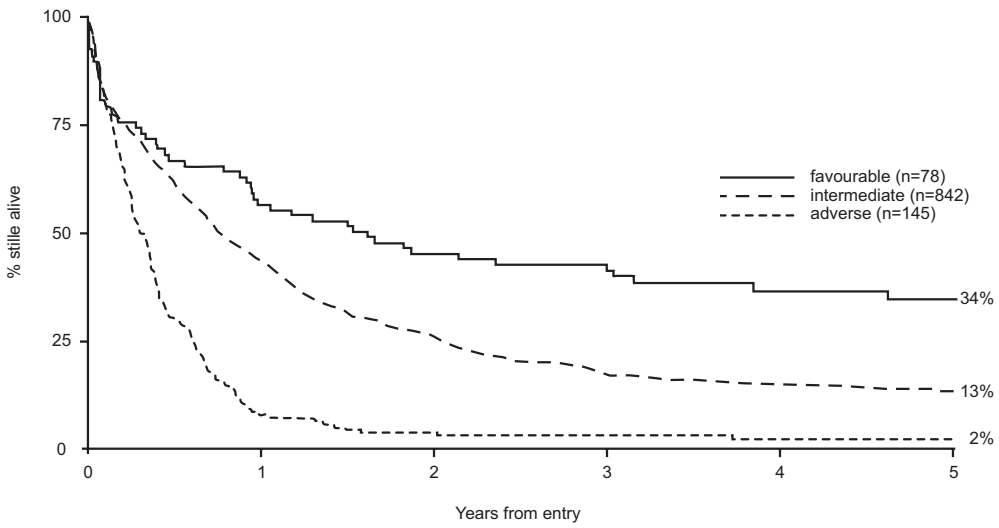


Figure 2: Prognostic value of chromosome abnormalities in AML. Kaplan-Meier overall survival curves of AML patients enrolled in MRC-11 trial, adapted from Grimwade *et al.* [25]. Risk groups were as follows: Favourable group: t(15;17), t(8;21), or inv(16), alone or in conjunction with other abnormalities. Intermediate group: normal karyotype and other non-complex abnormalities. The adverse group in this figure represents only patients with a.o. complex karyotype (5 or more unrelated abnormalities).

However, in approximately 50% of all new acute leukaemia patients, no or only non-specific clonal abnormalities are present. This absence of specific chromosomal abnormalities might be explained by the presence of small, cryptic rearrangements, which cannot be detected using conventional cytogenetic banding techniques, next to e.g. the presence of point mutations and small deletions. Therefore, specific aberrations may exist that have not been detected yet, as has been shown for childhood ALL where the cryptic t(12;21)(p13;q22) [17-19] and t(5;14)(q35;q32) [20, 21] are present in a large part of the cases (see below). Additionally, conventional cytogenetics showed the presence of marker chromosomes, but banding techniques do not allow elucidation of their composition. New molecular (cyto)genetic techniques are helpful tools in identifying these cryptic chromosome aberrations, as well as in elucidating the composition of marker chromosomes (discussed in the section “Molecular cytogenetic techniques”).

Translocations, fusion genes and leukaemogenesis

The combined use of conventional and molecular cytogenetics (see below) has led to the identification of many fusion genes resulting from numerous translocations. The most frequently observed chromosomal abnormalities, predominantly fusion genes, in haematological malignancies are listed in table 1.

Table 1: Chromosome abnormalities frequently observed in haematological malignancies

Chromosome abnormality	Phenotype	Genes involved
t(9;22)(q34;q11)	CML, ALL	*BCR - ABL*
11q23 rearrangements	AML, ALL, MDS	*MLL* - partner gene
t(12;21)(p13;q22)	Pre-B-ALL	*TEL - AML1*
t(15;17)(q22;q12)	AML	*PML - RARA*
inv(16)(p13q22)	AML	*CBFB - MYH11*
t(8;21)(q22;q22)	AML	*AML1 – ETO (also called CBFA2T1)*
t(1;19)(q23;p13.3)	Pre-B-ALL	*PBX1 - E2A*
-5/del(5q)	AML, MDS	
-7/del(7q)	AML, MDS	
+8	AML, ALL, MDS, CML	
1p32 aberrations	T-ALL	*TAL1 (also called SCL)*
11p15 aberrations	AML, MDS, CML, T-ALL	*NUP98* - partner gene
Hyperdiploidy (>50 chromosomes)	Pre-B-ALL	

Several genes (e.g. *MLL, TEL* or *NUP98*) are involved in more than one translocation, resulting in in-frame fusions with multiple different partner genes. Fusion gene networks can be constructed as some genes with more than one fusion partner are connected to other genes with different fusion partners.

Thus, it seems that a limited number of genes are involved to cause leukaemia, with a few major players in the centre of the network.

BCR-ABL

The first specific chromosome abnormality in cancer, in particular leukaemia, was identified in 1960 by the discovery of the Philadelphia chromosome [26], recurrent in CML and ALL and less frequently in AML [27]. In 1973, banding techniques showed that the Philadelphia chromosome was in fact a product of a translocation involving chromosomes 9 and 22, t(9;22)(q34;q11) [28]. As first demonstrated here in Rotterdam in 1983 with the molecular cloning of the breakpoint, the genes involved are *ABL1 (*aliases: *ABL, c-ABL, JTK7, p150, v-abl)* on chromosome 9q34 [15] and *BCR* on chromosome 22q11 [16], which are fused in-frame as a result of the translocation [15, 16]. The *BCR (*aliases: *BCR1, D22S11, D22S662)* breakpoints are mostly observed within 2 regions. In 95% of CML patients and approximately one third of ALL patients having t(9;22), the *BCR* gene breakpoint occurs within a 5.8 kb region, the major breakpoint cluster region (M-*bcr*) [16]. In the other two thirds of ALL patients, the *BCR* breakpoint occurs in the minor breakpoint cluster region (m-*bcr*) [29]. The *BCR-ABL1* fusion product was shown to have transforming potential, derived from deregulation of the tyrosine kinase activity of ABL [30]. The normally regulated tyrosine kinase activity of the ABL1 protein is constitutively activated by the juxtaposition of *BCR* sequences. BCR now promotes dimerisation of the oncoprotein: two adjacent BCR-ABL1 molecules phosphorylate their respective partners on the tyrosine residues in their kinase domains. The uncontrolled kinase activity of BCR-ABL1 then takes over the physiological functions of the normal ABL1 enzyme, resulting in upregulation of cellular proliferation, decreased adherence of leukaemia cells to the bone marrow stroma and reduced apoptotic response to mutagenic stimuli, all essential for oncogenic transformation [31].

Small molecules capable of inhibiting the aberrant tyrosine kinase activity of *BCR-ABL* have been developed; most widely applied in clinical trials is imatinib mesylate [32]. This chemical (formerly known as STI571, available as Glivec (Europe) or Gleevec (USA) from Novartis Pharmaceutics) selectively inhibited growth of CML primary cells and *BCR-ABL* positive cell lines in vitro and in mice, whereas normal control cells were largely unaffected [33, 34]. The first clinical trials showed that Glivec had significant antileukaemic effects without major side effects in patients with CML, in whom interferon alpha treatment had failed, resulting in haematological and cytogenetic complete remissions (CR) in a considerable percentage of patients (53/54 patients showed haematological CR; 31% of patients showed a major cytogenetic response, of which 7 patients achieved cytogenetic CR) [35]. Administration of Glivec to newly diagnosed chronic phase CML patients resulted in significantly higher haematological and cytogenetic responses with better tolerance than combination of interferon alpha and cytarabine [36]. An emerging problem is the phenomenon of acquired resistance to Glivec in a subset of patients, which may be due to point mutations in the kinase domain of the *BCR-ABL* fusion gene [32, 37]. However, combination of Glivec with other chemotherapeutic agents might overcome (part) of this problem. In addition, new drugs are being developed which, hopefully, will not suffer from this problem.

The *BCR-ABL1* story nicely demonstrates how investigating chromosome aberrations can ultimately lead to new therapies, which might be more effective in treating patients than the current treatment regimens.

MLL

An important gene in the leukaemia fusion gene network is *MLL* or *ALL1* (11q23, aliases: *TRX1, HRX, HTRX1, CXXC7, MLL1A*), which has more than 60 translocation partner chromosomes of which at least 37 fusion partners have been cloned [38]. Abnormalities involving 11q23 are highly frequent in infant leukaemias. Additionally, *MLL* is frequently involved in therapy-related leukaemias that develop after exposure to topoisomerase II inhibitors [39]. The MLL protein is important for normal mammalian development and haematopoiesis by regulation of homeobox (*HOX*) genes. Fusion proteins appear to disrupt the ability of wild-type MLL to regulate *HOX* gene expression, resulting in leukaemogenesis. In most translocations, the 5' portion of *MLL* is fused to the 3' portion of a gene encoded on the reciprocal chromosome (reviewed in [39-42]). The partner genes appear to fall in two functional categories: signalling molecules that normally localize to the cytoplasm/cell junctions or nuclear factors implied in various aspects of transcriptional regulation [39]. Besides translocations, *MLL* is also involved in partial tandem duplications, e.g. of exons 2 through 6, suggesting that in these cases rearrangement of the *MLL* gene itself is sufficient for leukaemogenesis (reviewed in [39-41]).

TEL and *AML1*

TEL or *ETV6* (12p13) is involved in at least 33 different translocations [40] fusing this gene to at least 11 cloned partner genes [43]. Translocations involving *TEL* occur frequently in ALL, but also in AML and MDS. *TEL* is a member of the Ets-family of transcription factors and functions as a sequence-specific DNA-binding transcriptional regulator. Normal *TEL* function is required for the maintenance of the developing yolk sac vascular network, for the survival of selected cell populations and for haematopoiesis within the bone marrow [44]. Several types of fusion genes can be formed. Firstly, *TEL* provides the 5' fusion partner, which lead to in-frame fusions, resulting in modulation/stimulation of transcription factor activity or activation of kinases, dependent on the 3' partner gene. The *TEL* promoter can be fused to the complete partner gene, driving its expression, but also the other way around has been observed: the 5' promoter of partner genes drive expression of the chimaeric transcription factor. Additionally, translocations have been described without formation of fusion genes, where proto-oncogenes, brought into proximity of *TEL* can be ectopically expressed in leukaemic cells in contrast to normal cells [45]. Often, the non-rearranged *TEL* allele is lost, suggesting a role as tumour suppressor gene as well [46]. The t(12;21)(p13;q22), occurring in 25% of childhood precursor-B-ALL [18, 19], involves, besides *TEL*, another important gene in the fusion gene network, *AML1* or *RUNX1 (*alias: *CBFA2)*, located on 21q22 [43]. This gene codes for the DNA-binding component of core-binding factor (CBF) and binds DNA through the runt domain. The AML1 protein is essential for expression of genes important in haematopoietic cell development, function and differentiation [47-49]. *AML1* is translocated to at least 14 other partners in AML, MDS, CML and therapy-related leukaemias. In all translocations, except t(12;21)(p13;q22),

AML1 is the amino-terminal component of the fusion protein [40]. In most cases, the generation of a fusion gene inhibits *AML1* function, resulting in a granulocytic differentiation block, which is usually accompanied by enhanced cell proliferation [50].

Other chromosomal aberrations

Many other chromosome abnormalities are found in acute leukaemia [51]. This section introduces three specific chromosome aberrations, subject of research described later in this thesis (Chapters 4-6).

t(5;14)(q35;q32)

The recently described t(5;14)(q35;q32) is a cryptic translocation occurring mainly in childhood T-ALL [20, 21]. Fluorescence in situ hybridisation (FISH) demonstrated that the 5q breakpoint is heterogeneous. However, in the majority of patients the breakpoint is located within or downstream of *RANBP17*, which does not seem to be deregulated as a result of the t(5;14)(q35;q32). Instead, the downstream juxtaposed *HOX11L2* (also called *TLX3*) is ectopically expressed in these patients [52], and in the paediatric T-ALL cell line HPB-ALL, which carries the same translocation [53]. A second breakpoint on 5q35, located 2 Mb telomeric of *RANBP17*, was found in the T-ALL cell lines PEER and CCRF-CEM immediately upstream of the homeobox gene *NKX2-5* or *CSX* [54]. Thus far, this variant translocation has not been observed in T-ALL patients. The breakpoints on chromosome 14q32 seem to be heterogeneous as well. No single clone was found so far encompassing the breakpoints in all patients tested; however, the breakpoints in the patient samples were all shown to be downstream of *BCL11B,* which lies approximately 6.6 Mb centromeric of *IGH* [20].

NUP98 rearrangements (11p15)

Chromosomal aberrations involving *NUP98* are observed in different haematological malignancies. The NUP98 protein is part of a nuclear pore complex, and is involved in RNA export from the nucleus [55-57]. To date 16 translocations and one inversion have been described, and in all cloned cases fusion genes with different partner genes are formed [58-74]. Two major groups of partner genes can be discerned. The first are the *HOX* genes, which are a family of transcription factors playing a role in early embryonic development. *HOX* genes are organized in four clusters (A, B, C and D) located on four different chromosomes (chromosomes 7, 17, 12 and 2 respectively). Three of these clusters (A, B and C) have been shown to regulate normal and leukaemic haematopoiesis [75]. The other group of *NUP98* partner genes does not seem to have an obvious theme and include, amongst others, transcriptional coactivators and genes involved in RNA metabolic pathways [75]. In all translocations described so far, the fusion transcript contains the 5' portion of *NUP98*, which is fused in-frame to the 3' portion of the partner gene. Thus, the N-terminal part of the fusion protein might be important in leukaemogenesis. The Phenylalanine-Glycine (FG) repeats in this region have been shown to activate transcription. The fusion partners could provide binding specificity for targets that normally do not bind *NUP98* and can determine downstream targets of the fusion proteins, causing altered localisation and possibly deregulation of these targets [75].

CDKN2 deletions

The *CDKN2* locus on 9p21 encodes three genes involved in cell cycle regulation, $p16^{INK4A}$, $p15^{INK4B}$ and $p14^{ARF}$ (Figure 3). $p15^{INK4B}$ and $p16^{INK4A}$ together encode INK4, which specifically inhibits cyclin dependent kinases (CDK) CDK4 and CDK6. CDK4 and CDK6 normally trigger retinoblastoma (RB) phosphorylation; INK4 thus prevents RB phosphorylation. RB phosphorylation triggers the E2F transcriptional program, resulting in progression into S-phase. As a result of INK4 expression, unphosphorylated RB keeps E2F sequestered, leading to G1 arrest (as reviewed in [76-78]). $p14^{ARF}$ links the RB and p53 pathways of cell cycle regulation. It shares exons 2 and 3 with $p16^{INK4A}$, but has an alternative exon 1 (1β) and an alternative open reading frame. The $p14^{ARF}$ protein is unrelated to INK4. It sequesters MDM2, a p53 negative regulator, upon E2F stimulation, resulting in p53 stabilization and accumulation, leading to G1 arrest (as reviewed in [77, 79]). All three genes are thought to be tumour suppressor genes, and therefore, inactivation or loss of these genes would be an expected observation in tumour cells. Indeed, rearrangements, deletions and/or inactivation through promoter hypermethylation of the *CDKN2* locus are frequently found in acute leukaemia and different other tumour types [80-82]. Inactivation of this locus is thought to be important in leukaemogenesis and could have important clinical significance.

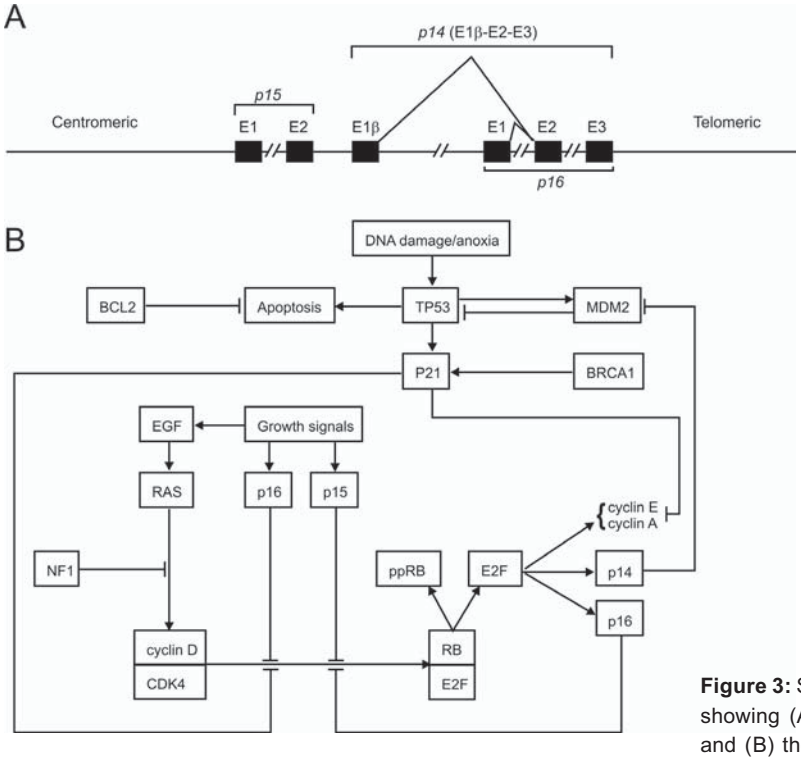


Figure 3: Schematic illustration showing (A) the *CDKN2* locus and (B) the pathways in which the genes in this locus are involved.

Molecular cytogenetic techniques

As described above, chromosome aberrations can occur as easily visible abnormalities, with exchange of large or distinct chromosome parts, or as cryptic rearrangements. The latter result from the exchange of small fragments or fragments with similar banding patterns. These cryptic rearrangements are easily missed using conventional cytogenetics, and possibly explain the large proportion of diagnostic acute leukaemia cases with seemingly normal karyotypes or non-specific clonal chromosome abnormalities. Additionally, the composition of marker chromosomes cannot be elucidated using conventional banding techniques. Over the last 10-15 years, new technical developments have led to a revolution in cytogenetics. Using fluorescence in situ hybridisation techniques, and more recent variants thereof, new cryptic chromosome rearrangements have been identified and the composition of marker chromosomes has been elucidated. In this section, these molecular cytogenetic techniques are discussed.

Fluorescence in situ hybridisation (FISH)

FISH is a rapid technique, with high sensitivity and specificity, which can be used on single non-dividing interphase and metaphase cells. In FISH, nucleoside triphosphate analogues coupled to biotin, digoxigenin or directly to fluorochromes are incorporated into DNA probes [83, 84]. Probes have become available for specific chromosomal loci, specific genes, specific repeats, and also painting probes for whole chromosomes or chromosome arms. These probes can be hybridised to tumour cells, allowing detection of certain specific translocations, and visualization of gains and losses of specific genes or chromosomal loci [83, 84]. Because FISH can be used on both interphase and metaphase cells, it is possible to detect aberrations present in non-dividing leukaemic cells that are missed when analysing metaphases only, for instance when the aberrant cells do not divide under laboratory conditions. Probes directly labelled with fluorochromes can be analysed immediately after post-hybridisation washing. Biotin-labelled probes are detected using fluorochrome-conjugated-avidin. Signal strength can be increased using biotinylated-anti-avidin and another round of fluorochrome-conjugated-avidin. Digoxigenin-labelled probes are detected using specific fluorochrome-conjugated-anti-digoxigenin antibodies, eventually followed by a round of signal amplification using secondary fluorochrome-conjugated antibodies. After this, the chromosomes can be banded, to enable chromosome identification and probe localization [83, 84].

There are some limitations of FISH as well. For instance, data are provided only for those chromosomes/loci targeted by the probes, and for that reason only for those abnormalities for which probes are currently available. Not every part of DNA sequence can be used as a probe, because target size, the size of the unique DNA sequence to which the probe hybridises, should be large enough for detection. For example, hybridisation efficiency drops to 20-50% when probes containing 2 kb of target sequence are used [85]. In the future one can expect the number and specificity of DNA probes to increase dramatically on the basis of genome sequence information and development of protocols for their selective amplification. Additionally, using classical single or dual colour FISH only a few abnormalities can be

investigated simultaneously [85]. These problems, however, are largely overcome by the introduction of multicolour FISH-based techniques, such as 12-colour telomere FISH (M-TEL) [86], spectral karyotyping (SKY) and multicolour FISH (M-FISH), provided that dividing cells are present.

SKY and M-FISH

Several groups have shown that SKY [87, 88] and M-FISH [89] are valuable tools in cytogenetic analysis of haematological tumours [90-94]. Karyotypes based on banding techniques could be refined and marker chromosomes or incompletely identified abnormalities were further elucidated [90-92].

Both techniques are based on the simultaneous hybridisation of all 24 differentially labelled (human) chromosome-painting probes [87-89]. To prepare suitable probes, the chromosomes are first flow-sorted or microdissected, after which the DNA for each chromosome is amplified by, for instance, a degenerate oligo-primed polymerase chain reaction (DOP-PCR). Next, each chromosome is differentially labelled with one or a combination of fluorochromes. Using a combinatorial labelling scheme with five different fluorochromes, 31 different targets can be distinguished (2^n-1), which is sufficient for the differential labelling of all human chromosomes [87-89]. For SKY, hybridisation is followed by visualization using spectral imaging through a single optical filter and the spectracube that allow excitation and measurement of emission spectra of all fluorochromes simultaneously (Figure 4) [87, 88]. As a result, full spectral information is available for each pixel of the image. Subsequently a specific classification colour is assigned to each pixel with identical spectra based on the information available from the combinatorial labelling table (see appendix), resulting in 24-colour karyotyping of chromosomes. For M-FISH, each fluorochrome is excited separately and the separate emission channels are combined [89].

SKY and M-FISH, when using whole chromosome paints, are unable to detect chromosomal inversions and small deletions. With the currently available probes, the minimum alteration detected using SKY ranges from 1-2 Mb [95], but the sensitivity is also dependent on the degree of condensation of the obtained chromosomes. Small translocations or insertions can be detected depending on chromosome morphology and composition. However, detection might be troubled since at the border of 2 combinatorial-labelled painting probes, the fluorescence interferes, resulting in new, different spectra, which are classified according to the new spectral information. This could lead to classification problems. If the translocated or inserted fragment is very small, only the interfering segment can be visible, resulting in misclassification [96]. Interference, due to an unbalanced translocation e.g. involving chromosome 16 (labelled with B and D; B=Texas Red, D=FITC) and a small piece of chromosome 20 (labelled with A; A=Rhodamine), results in the combination ABD, which is the combination assigned to chromosome 18. Thus this translocation might be classified as t(16;18), instead of t(16;20). This problem of misclassification can be overcome by introducing more fluorescent labels for the combinatorial labelling, as was shown by Azofeifa *et al.* [97]. However, some fluorescence mixing will still be present, resulting in additional bands at the breakpoints, and some intrachromosomal rearrangements will still be missed. Careful analysis and verification by dual colour FISH are recommended.

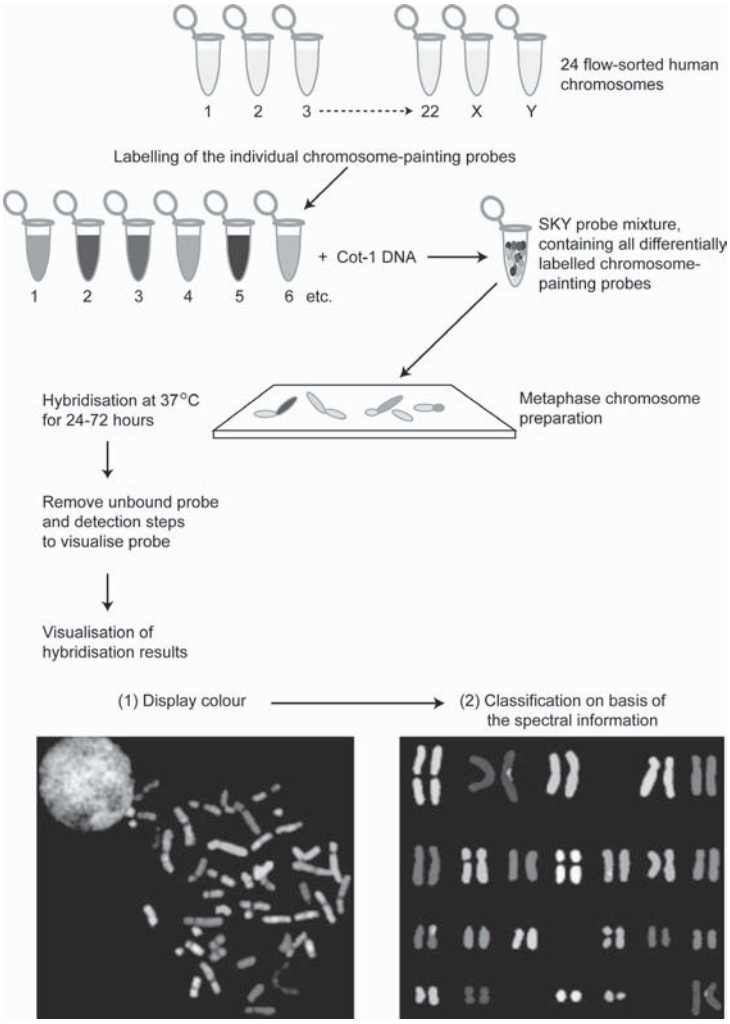


Figure 4: Principle of spectral karyotyping (SKY). A combinatorial-labelled painting probe mixture is hybridised to metaphase spreads. Hybridisation is followed by visualisation using spectral imaging through a single optical filter that allows excitation and measurement of emission spectra of all fluorochromes simultaneously. As a result, full spectral information is available for each pixel of the image, after which a specific classification colour is assigned to each pixel with identical spectra. This allows 24-colour karyotyping of chromosomes. Adapted from: http://www-ermm.cbcu.cam.ac.uk/0000199Xh.htm

Modifications of M-FISH with reduced colour complexity but increased banding resolution are multicolour bar coding [98] or cross species colour banding, Rx-FISH [99]. Both variations use a mixture of primate DNA probes instead of the set of 24 differentially labelled human DNA probes. As many structural rearrangements between human and nonhuman primate chromosomes have occurred during evolution, a banding pattern is obtained when this mixture

is hybridised to human chromosomes [98-100]. This could partly overcome the problem of detecting intrachromosomal rearrangements, but is complicated by the redundancy of some colour band combinations in different chromosomes. For this reason, the possibility of interchromosomal rearrangements cannot be excluded using multicolour bar coding or Rx-FISH. In fact these techniques have been used mainly for elucidating the composition of single aberrant chromosomes or exact breakpoint identification in such a single derivative chromosome [101, 102]. Spectral colour banding (SCAN), one chromosome at the time, identifies almost all bands of a chromosome in specific colours, almost identical to the corresponding G-bands [103]. Using SCAN intrachromosomal rearrangements can be detected, while excluding the possibility of interchromosomal rearrangements. Thus far, this technique has only been applied to chromosome 3, but it can be extended to other chromosomes. A similar technique, mBAND, uses region-specific partial chromosome paints to produce high-resolution multicolour banding [104], which has been shown to very useful in identification of breakpoints even in the case of highly complex intrachromosomal rearrangements [105]. As only one chromosome is investigated in one experiment, SCAN or mBAND will not be useful as a whole genome screening tool. Nevertheless, combination of SKY and SCAN/mBAND will probably be a powerful tool in tumour cytogenetics.

CGH

CGH is a quantitative double colour FISH technique that identifies and maps DNA copy number changes in a single hybridisation experiment. Total genomic DNA is isolated from a tumour sample and from a reference individual with a normal karyotype. Both DNAs are labelled with either green or red fluorescent dyes, pooled and hybridised to normal human metaphase spreads in the presence of cot1 DNA to suppress repetitive sequences. Differences in fluorescence intensities, visualized using digital imaging devices, reflect the DNA copy number changes in the tumour (Figure 5, left part). If DNA of the chromosomes is present in equal amount in both the test and reference sample, the fluorescence will be an equal mixture of the green and red and visible as yellow. If a chromosome or chromosomal region is lost in the tumour sample, green fluorescence (derived from the tumour sample) of this region is relatively underrepresented in the mixture, thus shifting the resulting fluorescence towards red. If a chromosome or region is gained in the tumour sample, green fluorescence of this region will be relatively over-represented and the colour will shift towards green [88, 106, 107]. This technique has been shown to be a valuable tool for identification of genomic imbalances in a.o. haematological malignancies [108, 109]. It should be noted however, that balanced rearrangements, such as reciprocal translocations and inversions, cannot be detected, and the obtained CGH data represent the genomic status of the major proportion of cells in the sample. Heterogeneity within tumour samples, with gains or losses in a large percentage of cells, can be detected, but heterogeneity resulting in small cytogenetic subclones, each with different aberrations, may be undetectable [110]. The resolution for detecting low copy number gains and losses is approximately 10 Mb [106, 111]. Thus, losses and low copy number gains smaller than this will be missed. More recently, a high-resolution CGH-based technique has been developed: matrix or array-CGH [112, 113]. Here, metaphase chromosomes have been

substituted by large DNA fragments, cloned into vectors, which are spotted on slides, generating genomic microarrays (Figure 5, right part). An equal mixture of differentially labelled tumour and reference DNA is hybridised to the microarrays as for standard CGH, and fluorescence ratios are visualized with digital imaging devices. Low copy number changes of fragments ranging from 75-130 kb in size can be detected, whereas high copy number amplifications can be visualized for probes even smaller [112]. Thus, the resolution of CGH increases enormously using matrix/array-CGH. Tiled arrays are being made (33k chip), covering almost the entire genome. Furthermore, microarrays specific for a disease or chromosomal region of interest are being developed.

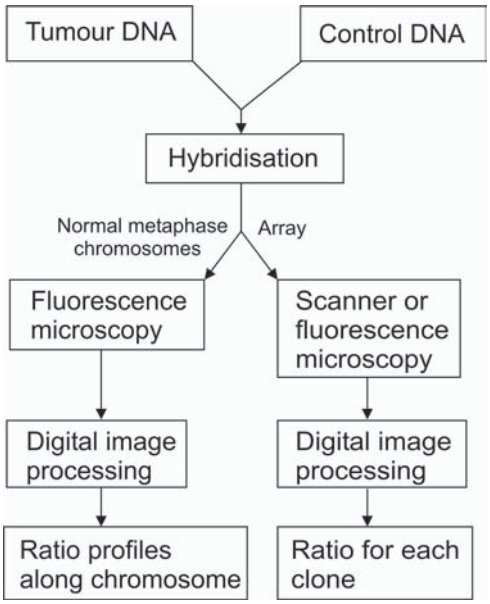


Figure 5: Principle of comparative genomic hybridisation (CGH) and array CGH. Tumour and normal reference DNA are differentially labelled and an equal mixture is hybridised to normal metaphases (left part). For array-CGH this mixture is hybridised to arrays containing genomic DNA clones (right part). Differences in fluorescence intensities, visualized using digital imaging devices, reflect the DNA copy number changes in the tumour.

Aim of thesis

Chromosome abnormalities are frequently observed in haematological malignancies. The presence of chromosome rearrangements constitutes a prognostic factor for the outcome of disease and has therapeutic consequences. However, in 50% of all acute leukaemia patients at diagnosis, no or only non-specific clonal abnormalities are present. This absence of specific chromosomal abnormalities might be explained, for example, by the presence of specific cryptic, rearrangements, which cannot be detected using conventional cytogenetic banding techniques, next to the presence of point mutations. Therefore, specific aberrations may exist that have not been detected yet. Additionally, marker chromosomes are observed with conventional cytogenetics, of which the chromosomal constitution remains unidentified.

The aim of this thesis was to identify new cryptic chromosome rearrangements and to investigate recently described aberrations in more detail using molecular cytogenetic techniques to study the probable importance for diagnosis, prognosis and treatment stratification. In general, research on chromosome abnormalities in leukaemia can be divided into different stages: the identification of new chromosomal abnormalities, the detailed characterisation of these newly identified aberrations and evaluation of the clinical relevance of chromosome aberrations. The research described in this thesis covers each of these 3 phases. The first part describes the identification of new abnormalities using SKY, FISH and CGH. Seventy AML, MDS and ALL patients with (complex) abnormal karyotypes were investigated with SKY to identify cryptic chromosome abnormalities, and to elucidate the composition of marker chromosomes present (Chapter 2). CGH was performed on those cases for which SKY showed chromosomes 2, 5, 7, 12, 17, 21 and 22 to be more frequently involved in structural aberrations than could be seen from conventional karyotyping only. The chromosomal regions lost or gained in the aberrations were identified and patients were compared to identify minimally gained and/or lost regions (Chapter 3). In the second part, we investigated (recently) reported recurrent abnormalities in more detail. The first translocation studied in this part was the t(5;14)(q35;q32) occurring in T-ALL. Since this translocation is cryptic, it is easily missed using conventional cytogenetics. Therefore, we developed split signal FISH probe sets for both known t(5;14)(q35;q32) variants involving *HOX11L2* or *CSX.* After validation, the probe sets were used to investigate 34 T-ALL cases (Chapter 4). The other recurrent aberration studied in this part of the thesis involved *NUP98* translocations, which are sometimes cryptic in nature. In Chapter 5, we investigated the occurrence of *NUP98* rearrangements in 34 patients with cytogenetically visible 11p abnormalities and 50 patients with normal karyotypes using *NUP98* specific FISH probes. Additionally, we investigated whether *NUP98* rearrangements exist that have not been identified yet. In the third part of this thesis, we evaluated the prognostic significance of *CDKN2* deletions in 109 childhood common/pre-B-ALL cases as determined by FISH (Chapter 6). Finally, Chapter 7 discusses the central theme of this thesis, cryptic chromosome abnormalities and links the different chapters.

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Chapter 2

Identification of new cryptic chromosome aberrations in acute leukaemia using molecular cytogenetic techniques

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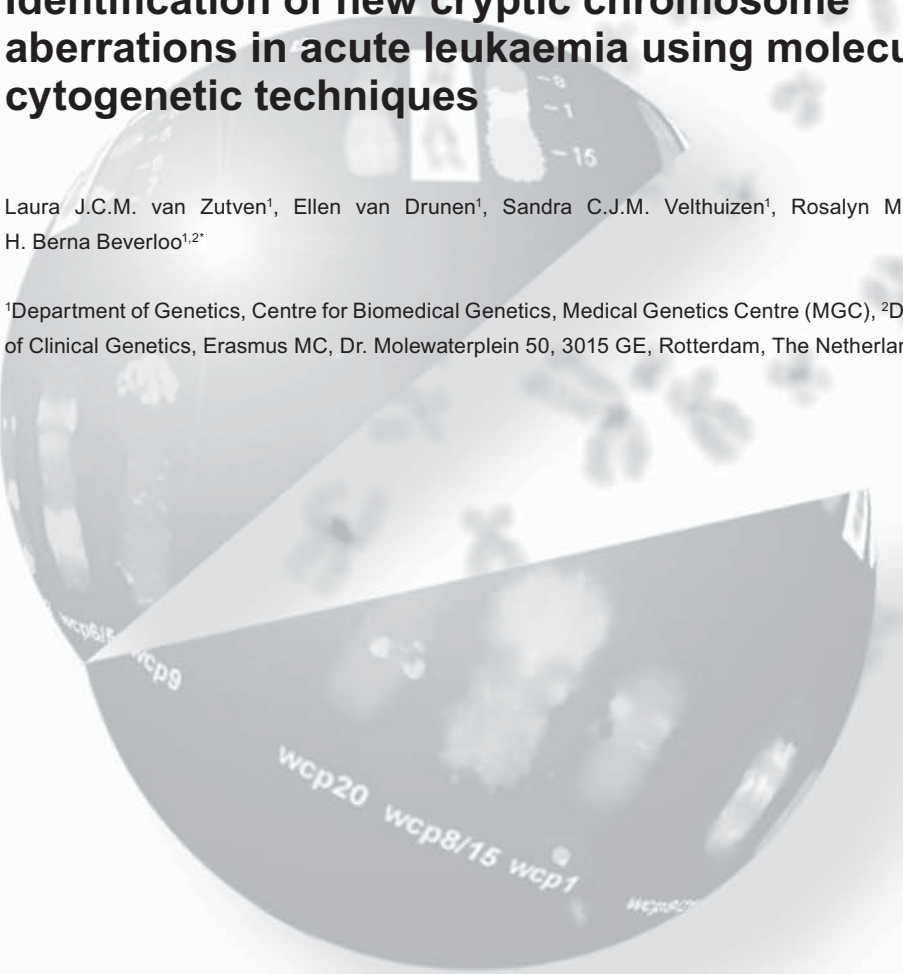

Chapter 2

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Abstract

Specific chromosome aberrations are observed in 50% of acute leukaemia patients. In the other 50% no or non-specific aberrations are found. In this latter group specific chromosome abnormalities might be present, which might be cryptic and not detectable using conventional banding techniques. We used spectral karyotyping (SKY) and fluorescence in situ hybridisation (FISH) aiming to identify new cryptic chromosome rearrangements in 48 AML, 19 MDS and 8 ALL patients; 50 patients with mainly complex karyotypes, and 25 AML/MDS patients with normal karyotypes.

In the cases with a normal karyotype, SKY did not identify any clonal cryptic abnormalities. In the AML cases with a complex karyotype, a frequent structural involvement of chromosomes 1, 3, 5, 11 and 12 was observed with karyotyping only. SKY confirmed this, but also showed chromosomes 5 and 17 to be even more often involved in the rearrangements. In 17 complex MDS cases many structural abnormalities of chromosomes 1, 2, 3, 5, 7, 12 and 15 were found. Using SKY new structural aberrations were found involving chromosomes 5, 17 and 22, and, to a lesser extent, chromosomes 2 and 12. In 8 ALL patients, chromosomes 7, 8, 9 and 22 were frequently involved based on karyotyping only. In addition, SKY showed frequent involvement of chromosomes 21, and, to a lesser extent, chromosomes 7 and 12 as well. In 5 cases we observed 6 rearrangements of apparently normal chromosomes: a der(2)t(2;3)(q3?;?) and a der(7)t(7;17)(q?;?p) in AML cases, a der(10)t(10;20)(p1?;) and a der(16)t(11;16)(?;?q) in MDS cases and a der(1)t(1;22)(p3?2;q1?) and a der(21)t(5;21)(?;p?) in an ALL case. Future investigations elucidating the exact breakpoints of these translocations are needed to determine whether these abnormalities are recurrent and eventually to identify the genes involved.

Introduction

Acute leukaemia (AL) is a worldwide disease, with an incidence of approximately 5 cases per 100,000 inhabitants per year. Acute lymphoblastic leukaemia (ALL) occurs most frequently in childhood AL, whereas acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS) occur predominantly among adult leukaemia patients. Chromosomal rearrangements are found in many AL cases. Some of these rearrangements are in particular associated with a

specific type of AL according to the FAB and WHO classifications [1]. The presence of certain chromosome aberrations might constitute an important prognostic factor for the outcome of disease and has therapeutic consequences. For example, t(8;21)(q22;q22), t(15;17)(q22;q21) or inv(16)(p13q22) in AML are associated with good prognosis, and are currently used for clinical stratification of therapy, whereas in contrast, t(9;22)(q34;q11) or t(4;11)(q21;q23) in ALL are associated with poor outcome [1]. However, in 50% of all *de novo* AL patients, no or only non-specific clonal abnormalities are detected. This absence of specific chromosomal abnormalities might be explained a.o. by the presence of small, cryptic, rearrangements, which cannot be detected using conventional cytogenetic banding techniques, next to the presence of e.g. point mutations. Therefore, specific aberrations may exist that have not been detected yet, as has been shown for childhood ALL where the cryptic t(12;21)(p13;q22) [2] and t(5;14)(q35;q32) [3] are present in a substantial proportion of the cases.

Molecular cytogenetic techniques that can help to identify these hidden chromosome abnormalities are fluorescence in situ hybridisation (FISH) and spectral karyotyping (SKY). Using FISH, fluorescently labelled probes are hybridised to tumour metaphases, allowing visualization of gains and losses of specific genes or chromosomal loci, and detection of certain specific translocations [4]. SKY [5, 6] or M-FISH [7] are based on the simultaneous hybridisation of all differentially labelled human chromosome-painting probes. For SKY, this is followed by visualization using spectral imaging through a single optical filter allowing excitation and measurement of emission spectra of all fluorochromes simultaneously. Full spectral information is available for each pixel of the image, after which a specific classification colour is assigned to every pixel with identical spectra, resulting in 24-colour chromosome karyotyping. SKY and M-FISH have been shown to be valuable tools in cytogenetic analysis of haematological tumours. Karyotypes based on banding techniques could be refined and marker chromosomes or incompletely identified abnormalities were further elucidated [8-10]. In addition, some hidden translocations have been identified as well [11, 12]. In this study we aimed to elucidate marker chromosome composition and to identify new specific chromosome rearrangements in samples of AML, MDS and ALL cases with mainly complex karyotypes on one hand, and AML/MDS cases with normal karyotypes on the other hand.

Materials and Methods

Conventional cytogenetic analysis and patients selection

Bone marrow or blood of 93 AL or MDS cases was obtained at diagnosis, cultured and harvested using standard cytogenetic protocols. For each case 20-32 metaphase cells were analysed using both QFQ- and RFA-banding. The chromosome aberrations observed were described according to ISCN 1995, An International System for Human Cytogenetic Nomenclature [13]. Remaining methanol/acetic acid (3:1) fixed cell suspensions were stored at -20°C and remaining blood or bone marrow was viably frozen in 0.1 volume dimethylsulfoxide (DMSO)/0.9 volume foetal calf serum (FCS) and stored in liquid nitrogen until use. At diagnosis, informed consent

of the patients and/or parents/guardians was obtained to use left-over material for research purposes.

Eight cases with known translocations e.g. t(9;22)(q34;q11), t(15;17)(q22;q21), t(9;11)(p21;q23), t(4;11)(q21;q23), t(8;21)(q22;q22) and the cryptic t(12;21)(p13;q22) were used for validation of the SKY probe set used (Table 1). For SKY analysis, a group consisting of 25 AML (age range 1-75 years), 17 MDS (age range 1-74 years) and 8 ALL (age range 11-76 years) cases, showing complex abnormal ($\geq$3 independent structural chromosome abnormalities) karyotypes in routine diagnostic cytogenetics and sufficient metaphase quality, was included (Tables 2, 3 and 4). In addition, 35 cases with simple chromosome aberrations (e.g. sole numerical aberrations) or a normal karyotype were chosen to investigate the possible presence of cryptic rearrangements (Table 1). The cases with normal cytogenetics did not harbour a cryptic 11q23/*MLL* translocation or 12p13/*TEL* rearrangement, investigated by FISH or Southern blot analysis, with the exception of cases 74, 81 and 92, which showed a partial tandem duplication of *MLL*.

Spectral karyotyping (SKY)

Metaphase spreads for SKY analysis were freshly prepared from stored methanol/acetic acid fixed cell suspensions. Hybridisation and detection of the SkyPaint™ kit probes (Applied Spectral Imaging (ASI), Migdal Ha'Emek, Israel) was performed according to the manufacturer's protocol with some minor changes. Briefly, slides were pre-treated with 200 μg/ml RNAse and 0.001% pepsin, fixed with 1% formaldehyde/PBS/50 mM $MgCl_2$ and dehydrated through an ethanol series (50%, 75%, 95%, 100%). Probes and chromosomes were denatured separately, followed by hybridisation in a moist chamber at 37°C for at least 36 hours. After this, the slides were washed with 50% formamide/2x SSC pH 7.0 at 39°C and immune detection was carried out using the solutions provided with the SkyPaint™ kit. Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted with anti-fade solution (Vectashield, Vector Laboratories, Peterborough, United Kingdom). For each case, 5-13 metaphases were captured using an epifluorescence microscope (Axioplan, Zeiss, Sliedrecht, The Netherlands) equipped with xenon light excitation and the Spectra Cube 200 system (ASI). The images were analysed using SKY View software 1.8 (ASI).

Table 1: Comparison of conventional cytogenetics and SKY in those cases with known specific chromosome aberrations or with relatively simple karyotypes

Case	Age (yr)	Sex	Diagnosis (FAB)	Conventional karyotype (ISCN, 1995)	SKY karyotype*
1	9	F	ALL	45,X,-X,t(2;18)(p11;p11)[7] (t(12;21)(p13;q22) positive by RT-PCR)	45,X,-X,der(2p)(5),**der(12)t(12;21)(p13;q22)**,der(18p)(2)[5]
2	3	M	ALL	Karyotype after FISH: 46,XY,t(12;21)(p13;q22)[12]/47, idem,+der(21)t(12;21)[2]/48,idem,+X,+der(21)t(12;21) [7]/46,XY[19]	46,XY,der(12)t(12;21)(p13;q22)[12]
3	56	F	ALL	47~49,XX,+X,t(9;22)(q34;q11),inc[cp3]/46,XX[58]	46,XX,der(9)t(9;22)(q34;q11)[1]/46,XX[3][a]
4	7	M	PreB-ALL	Karyotype after FISH: 46,XY,?t(12;21)(p13;q22)[31]	46,XY,der(12)t(12;21)(p13;q22)[8]/46,XY[3]
5	70	F	AML-M2	46,XX,t(8;21)(q22;q22)[45]	46,XX,t(8;21)(q22;q22)[6]
6	31	F	AML-M3	46,XX,t(15;17)(q22;q21)[19]/46,XX[1]	46,XX,t(15;17)(q22;q21),**der(19)(11)**[3][cp9]
7	29	M	AML-M5	50,XY,+5,+6,t(9;11)(p21;q23),+19,+22[4]/54,idem,+Y,+8, +13,+16,+19,-22[2]/55,idem,+Y,+8,+12,+13,+16,+19,-22 [4]/56,idem,+Y,+2,+8,+12,+13,+16,+19,-22[7]	50~55,XY,+2[2],+5[6],+6[5],+8[3],t(9;11)(p21;q23),+12[2],+13 [2],+16[2],+19[6],+19[2],+22[3][cp9]
8	34	M	t-AML-M4	46,XY,t(4;11)(q21;q23)[21]/46,XY[1]	46,XY,t(4;11)(q21;q23)[5]
9	55	M	AML	95,XXYY,add(5)(q1?5),-7,+13,+13,+18,+19[12]/46,XY[9]	95,XXYY,der(5q)(3),-7,+13,+13,+18,+19[cp6]
10	54	F	AML-M2	47,XX,+21[22]/46,XX[7]	47,XX,+21[7]
11	43	F	AML-M3	46,XX,add(12)(p1?3)[29]	46,XX,der(12p12)(20),der(20)(12)[9]
12	43	M	AML-M4	47,XY,+11[14]/46,XY[13]	47,XY,+11[7]
13	48	F	AML-M5	47,XX,+11[27]/46,XX[5]	47,XX,+11[9]
14	21	M	AML-M5 relapse	47,XY,+10[20]/46,XY[9]	47,XY,+10[4]/46,XY[5]
15	31	M	AML-M6	46,XY,del(9)(q22q34)[26]	46,XY,del(9)(q)[12]
16	69	M	MDS	46,XY,add(18)(q2?2)[22]	46,XY,der(18q)(9)[7]
17	74	F	RA	46,XX,del(5)(q1?3q3?2)[16]/46,XX[4]	46,XX,del(5)(q)[8]/46,XX[2]
18	7	M	cALL	46,XY[33]	46,XY[6]
71	67	M	AML-M0	46,XY[33]	46,XY[9]
72	58	F	AML-M1	46,XX[32]	46,XX[7]
73	40	M	AML-M1	46,XY[67]	46,XY[9]
74	52	M	AML-M1	46,XY[21]/45,X,-Y[3]	46,XY[12]
75	64	M	AML-M1	46,XY[32]	46,XY[11]
76	64	M	AML-M1	46,XY[32]	46,XY[12]
77	54	M	AML-M1	46,XY[69]	46,XY[11]
78	62	F	AML-M1/M4	46,XX[42]	46,XX[8]
79	36	F	AML-M2	46,XX[32]	46,XX[10]
80	68	M	AML-M2	46,XY[32]	46,XY[11]

81	11	F	AML-M2	46,XX[32]	46,XX[8]
82	74	F	AML-M2	46,XX[34]	46,XX[9]
83	61	M	AML-M2	46,XY[35]	46,XY[12]
84	76	M	AML-M2	46,XY[33]	46,XY
85	69	F	AML-M4	46,XX[35]	46,XX[cp11][b]
86	47	M	AML-M4	46,XY[32]	46,XY[10]
87	41	F	AML-M4	46,XX[66]	46,XX[13]
88	43	F	AML-M4	46,XX[31]	46,XX[12]
89	69	M	AML-M4	46,XY[21]	46,XY[12]
90	77	M	AML-M4/M5	46,XY[32]	46,XY[cp7][b]
91	61	M	AML-M5	46,XY[53]	46,XY[9]
92	34	F	AML-M5	46,XX[44]	46,XX[10]
93	71	F	AML-M5b	46,XX[32]	46,XX[11]
94	64	M	CMMol	46,XY[40]	46,XY[9]
95	72	M	CMMol	46,XY[33]	46,XY[7]

***New** and **refined SKY** observations are shown in bold.

[a] too few aberrant metaphases

[b] SKY showed a non clonal structural aberration, which could also not be confirmed with FISH

Table 2: Comparison of conventional cytogenetics and SKY in AML cases

Case	Age (yr)	Sex	Diagnosis (FAB)	Conventional karyotype (ISCN, 1995)	SKY karyotype*
21	45	M	AML	43,XY,add(5)(q1?2),-7,?der(12)(p),-17,-18,-20,add(21)(p11), +mar[2]/43,idem,dic(12;?)(p12;?)[14]/44,idem,add(8)(q23), add(11)(p12),dic(12;?)(p12;?),add(17)(p11),+18[3]/46,XY[1]	43,XY,add(5q)(17),-7,**der(17q)(20)**,-18,-20,add(21p)(20)[4]/ 43,XY,idem,dic(12)(18)[7]/44,XY,idem,der(8q?)(17),add(11p) (8),add(12p)(10),**add(17q)(20)**,+18[1]
22	68	M	AML	47,XY,+5,t(6;11)(q26~27;q23)[13]/45,X,-Y[13]/46,XY[6]	47,XY,+5,der(6q)(11),del(11)(q)[6]
23	72	M	AML	45,X,-Y,add(5)(q1?4),add(11)(q2?3),inv(16)(p1?3q2?2)[18]/ 45,idem,del(9)(q1?2q3?2)[3]	45,X,-Y,der(5q)(8),der(11q)(6)[6]/45,idem,del(9)(q)[6]
24	56	F	AML-M0	45,XX,add(1)(p36),ins(4;4)(q1;q3),-9[9]/85,idemx2[3]	45,XX,der(1),del(4)(q),-9[13]
25	55	F	AML-M0	46,XX,-2,?add(5)(q?21),t(11;12)(q13;p12),+ring[23]/45,idem,-7 [3]/46,XX[6]	46,XX,**r(2)**,der(5q)(2),der(11q)(12),der(12p12)(11)[10]
26	73	F	AML-M1	43,XX,add(4)(p16)[2],add(5)(p12),-7,add(12)(p10),add(13)(p10) [12],-17,add(17)(p13),-18[cp19]/46,XX[1]	40~43,XX,der(4p)(2)**[2]**,**der(5p)(7)**[6],-7,**der(7q)(17)**,der(12p) (7)[7],der(13p)(?)[6],-17,**der(17p)(7)**,-18[cp8] FISH: der(13p)=der(13)t(2;13)
27	69	F	AML-M1	44~46,X,-X,?der(2)(p)[3],der(3)t(3;15)(p?21;q1?5),?der(4)(p)[6], -5,add(7)(q2?2),-15[17],-16[17],-17[17],?der(18)(p)[4],-22, +mar1,+mar2,+mar3[14],+mar4[11],+mar5[7],+mar6[2],+mar7 [14],+1-2mar[10][cp18]	43~45,X,**der(Xq)(17)**[4],**i(Xp)**[3],**del(X)(p)[3]**,der(3p)(15), **der(5p)(22)**,der(7?q)(3),**del(16)**[7],**del(17)**[5],**der(?)(22)**[cp10]
28	59	F	AML-M1	43,XX,del(3)(p21p26),-4,-5,add(6)(?q10),-7,+8,add(16)(q21),-17, -18,+mar1[5]/45,XX,del(3)(p21p26),-5,-6,-7,add(16)(q21),-17, +idic(22)(p11),+psudic(?;6)t(?;6)(?;q11)t(?;5)(?;q13),+mar1[5]/ 45,XX,del(3)(p21p26),-5,-6,idic(11)(p11),add(16)(q21),-17, +psudic(?;6)t(?;6)(?;q11)t(?;5)(?;q13),+mar1[5]/43~49,XX, del(3)(p21p26),add(6)(?q10),+8[2],add(16)(q21),-17,+mar1, +mar2[cp3]/46,XX[1]	45~48,XX,del(3)(p),-4,**der(5q)(?)**,der(6q)(6::17::5)[2], der(6q)(17)[9],-7[5],+8[5],dic(11p)[4],der(16q)(21),-17,-18[5], +21[2],+22[4][cp11] FISH: der(5q)(?)= der(5)t(5;17)
29	62	F	AML-M1	44,XX,del(1)(p13p22~31),del(3)(?p10),del(3)(q2?2),del(5) (q21q34),-7,add(11)(p10),-17,add(22)(q1?3)[32]	44,XX,del(1)(p),der(3),der(3),del(5)(q),-7,add(11p)(3::17::11), -17,?ins(22q)(3)[13]
30	60	F	AML-M1/M2	46,XX,del(11)(q13q25),+add(11)(p10),-16,-20,+mar1[12]/46, idem,del(7)(q21q31)[11]/47~49,idem,+X[1],del(7)(q21q31),+8, +8[2],+mar[1][cp10]/46,XX[2]	46,XX,del(11)(q),der(16p)(11),**der(20p)(16)**[4]/46,idem,del(7) (q)[5]/46,idem,del(7)(q),+8,**del(17)(q)**,**+del(17)(q)**[2]
31	68	M	AML-M2	50,XY,+Y,add(1)(q21),add(2)(q35),-12,+?der(14),-15,+?der(21), +22,+22,+mar[27]/46,XY[12]	45~50,XY,+Y,der(1q)(12)[7],der(2q)(15)[7],**der(3p)(1)**[7], **der(5q23~31)(12)**[3],**+der(5)t(5;12)**[4],-12[7],+14[4],-15[7], **der(17p)(?)[7]**,+21[4],+22[3],+22[4][cp8]/46,XY[1] FISH: der(17p)(?)=der(17p)(5)
32	53	M	AML-M2	42~46,X,-Y,-2[13],add(3)(q1?3)[12],-5,add(7)(q21),der(8)t(8;11) (q24;q11)[12],der(8)t(8;12)(p22;q11)[15],-12,-13,ins(18;Y)(p11;	41~47,X,der**(Y)**[6],**del(2)**[6],der(3q)(2)[6],-5[3],**r(5)**[6],?der(6) [2],-7[3],**der(7q)(3)**[7],der(8q)(11)[6],der(8p)(12)[7],-12[7],-13
				q12q12),+mar1[14],+r1[14],+r1[3],+r2[14],+1-2r2[2],dmin[7] [cp16]/46,XY[2]	[2],**der(13)**[7],+der(13)[5],+der(13)[1],der(18p)(Y)[8],+mar[2] [cp11]
33	60	F	AML-M2	45,X,add(X)(q13),add(4)(q28)[27],del(5)(q1?4q3?2),-17[27],-19 [4],add(19)(q13)[7],del(19)(p13)[12][cp30]/66~85,idemx2[2]	45,X,der(Xq)(9),der(4),del(5)(q),-17,der(19q)(17),**der(20p)(17)** [5]

34	75	F	AML-M2	46,XX,del(5)(q12~13q3?2)[14]/40~46,idem,-3,add(5)(p14),add(12)(p1?2),der(15)t(15;20)(q21;p1?2),add(16)(q2?2),-17,-20,+22[4],+mar1,+mar2[cp16]	46,XX,del(5)(q)[2]/44~45,XX,-3[9],del(5)(q),der(5p)(15),der(12p)(7),-15[3],**del(15)(q)**[5],der(15q)(20)[2],der(16q)(17),-17,**der(20p)(15)**,+22[5][cp12] FISH: der(12p)=der(12)t(12;17)
35	56	M	AML-M4	46,XY,t(1;3)(p36;q22),der(5)add(5)(p1?4)add(5)(q2?2q3?3),t(13;13)(q13;q14)[17]/46,XY[1]	46,XY,der(1p)(3),del(3)(q),der(5),del(13)(q)[9] FISH: der(5)=inv(5)
36	62	F	AML-M4	47,XX,+1,add(5)(q1?2),+add(11)(p1?1),-16,add(17)(p12),add(18)(p11),-20,+mar[4]/48~52,XX,+1,add(5)(q1?2),+9[2],add(11)(p1?1),+add(11)(p1?1),-16,add(17)(p12),add(18)(p11),-20,-22[30],+idic(22)(q1?2),+idic(22)(q1?2)[22],+idic(22)(q1?2)[5],+idic(22)(q1?2)[3],+idic(22)(q13),+mar[cp31]	48~50,XX,+1,der(5q11)(11),ins(11p)(18),+der(11p)(18),**del(16)**[6],**der(16?q)(5)**[3],der(17p)(17::5::20),der(18p)(20),-20,der(22),+i(22),+der(22)[7],+der(22)[1][cp11]
37	69	M	AML-M5	41~52,XY,-3,-5,-7,idic(8)(p1?2),+idic(8)(p1?2)[10],?der(10)(q),-12,-14,-17,-18,+mar1,+mar2,+mar3,+2-9mar[cp23]/72~130,idemx2-3,inc[3]/46,XY[2]	44~46,XY,-3,der(5)(12::5::12::17)[6],der(5)(5::12::17)[5],-7,idic(8q)[10],+idic(8q),der(8),+der(8)[6],+der(8)[4],der(12q)(12::5::17),del(14)(q)[10],-17,der(18p)(?::14::15::22::18)[6][11]
38	68	M	AML-M6	42~44,XY,der(3)t(3;6)(p13;p12),-5,-6,-7,-9,add(12)(p11),-18,+mar1,+mar2[8]/44,idem,+8,-add(12)(p11),+add(12)(p12)[6]/88,idemx2,+8,+8,-add(12)(p11),+add(12)(p12)x2[3]/43,idem,-12,-add(12)(p11),+mar2[3]	41-45,XY,der(3p)(6),-5,-7,+8[3],-9[8],der(12p)(18),-18,**der(?6)(9::6::5::6::7)**,**der(9)**[cp12] FISH: der(9::6::7::5::7::6::7)
39	67	M	AML-M6	43~45,XY,-3,-5[4],der(5)t(5;17)(q1?3;q2?1)[15],-7,i(8)(q10)[11],-11[13],-17,ider(19)(q10)add(19)(q13)[18],add(20)(q1?3)[13],+mar1[17],+mar2[15],+1-2mar[5][cp19]/46,XY[2]	43~46,XY,**del(3),**-5[2],der(5q)(17)[8],-7,i(8q)[4],-11[2],**del(11)**[7],**der(13)(6)**[2],-17,**der(17)**[2],**der(19)**,der(19)[6],der(20)(11)[8][cp11]
40	61	M	AML-M6	45~48,XY,der(1)del(1)(p13p31)inv(1)(p13q24)add(1)(q3?2)[18],add(4)(q?12)[17],-5[19],-7[18],del(7)(p1?2p2?2)[18],+8[18],+11[14],-17[18],-20,+mar1[18],+mar2[17],+mar3[14],+2-5mar[cp19]/46,XY[2]	47,XY,der(1q)(7),del(4)(q),**der(5q)(1)**,-7,del(7)(p),+8,+11,**der(?1)(17::1::17::1)**,**der(20q)(5)** or **ins(20q;5)**[cp12]
41	1	M	AML-M7	46,XY,der(1)t(1;13)(p3?6;q1?4),add(11)(p15),der(13)t(1;13)(p3?6;q1?2),add(21)(p13)[31]/46,XY[7]	45,XY,der(1p)(13)[3],-13,del(16)[3],del(19)[2],-21[2],+mar[cp4]
42	1	M	AML-M7 relapse	46,Y,t(X;1)(q24;q23)[3]/46,idem,t(3;12)(q22;p13)[22]/46,idem,t(3;12),t(9;16)(q34;q12)[7]/46,idem,der(3)t(3;12),der(7)t(7;12)(q22;p11)t(3;12),der(12)t(7;12)t(3;12)[3]/46,XY[11]	44~47,Y,t(X;1),t(3;12),?del(16)[a]
43	60	F	t-AML	43~45,XX,-3,-5,der(7)t(7;?)(q22;?)t(?;15)(?;q1),der(10)t(10;11)(q25;q12),-12,-15,add(16)(p1?),-17,der(19)t(19;?)(p1?3;?)t(?;12)(?;q11),+ring[3],+mar1,+fr[12],+mar4[12][cp14]/46,XX[1]	44,XX,**der(2q)(3)**,-3,**der(5q)(3)**,der(7q)(15),der(10q)(11),-12,**der(12)**,-15,der(16)(3),**der(17)(7)**[2],der(19?q)(12)[cp3] FISH: der(7)=der(7::17::15)
44	70	F	t-AML-M0	44,XX,del(3)(p11p21),?add(4)(q3?4),del(5)(q32q33),der(6)t(6;9)(q2?5;q34),-7,-9,add(10)(q22),add(10)(q23),-11,-12,del(15)(q2?2q2?4),add(18)(p11),der(19)t(12;19)(q1?3;p13.3),add(21)(p11),+mar1,+mar2[15]/46,XX[6]	44,XX,del(3)(p),der(4)dup(4),del(5)(q),der(6q)(9),**+der(6)(6::7::9)**,-7,-9,der(10q)(7),der(10q)(7),**der(11)**,-12,del(15)(q),der(18p)(10),der(19q)(12),der(21p)(15)[10]
45	62	F	t-AML-M6	39~42,XX,?der(2)(q3?6)[7],-5[10],-5[9],der(7)(p10)[10],-8[3],-13,-15[10],-16,-18,-19[10],-21,-22,-22[9],+mar1,+mar2[9],+mar3[9],+mar4[8],+mar5[8],+mar6[4][cp11]/46,XX[10]	39~45,XX,**der(3q)(22::X)**[2],-5[7],**der(5p)(15)**[5],-6[2],der(7p)(5)[9],-8[2],**der(10q)(17)**[1],**der(12)(12::14::7)**[1],**r(11)**[1],**der(11q)(?)**[1],**der(13p)(5)**[6],-15[4],-16[8],-18[5],**add(18p)(22)**[4],-19[8],-20[3],-21[8],-22[6],-22[2],+mar1[6],+mar4[2][cp10]/46,XX[2]

New and **refined SKY** observations are shown in bold.

[a] no conclusion possible about t(9;16)

Table 3: Comparison of conventional cytogenetics and SKY in MDS cases

Case	Age (yr)	Sex	Diagnosis (FAB)	Conventional karyotype (ISCN, 1995)	SKY karyotype*
46	54	F	CMMol	43~45,XX,add(2)(q36),der(5)t(5;12)(q13;q14),-7,-12,-15,-16,-17, ?der(19)t(7;19)(p13;q10),add(20)(q12~13),der(?)t(?;7)(?;q11)[16],+r,+mar1[15],+mar2[15][cp18]/46,XX[1]	42~46,XX,der(2q)(12)[4],der(5q13)(12)[5],**+der(7)**[3],der(7p)(7::19::17),-12[5],**der(12q)(2)**[3],**r(15)**[6],**del(16)**[3],-16[4],-17, der(19q)(7),**der(20q)(17)**[cp8]
47	1	M	JMML	46,XY,del(1)(p3?43?6),del(2)(q3?5q3?),add(5)(p1?2),add(5)(q3?3),add(6)(q2?5),add(7)(q2?1),add(11)(q23),add(15)(q2?5)[41]	46,XY,der(1p)(21),der(2q)(12),der(5p)(11),der(5q)(2),der(6q)(3),der(7q)(12),der(11q)(5),der(15q)(3)[12]
48	74	M	MDS	45,XY,add(1)(p32),add(5)(q2?1),del(5)(q?21q31),add(6)(q1?3),add(6)(q?21),-7,-17,add(19)(?p12),+ring[11]/44,XY,idem,der(1)add(1)(p32)t(1;12)(q4?3;q1?2),der(8;19)(q10;p10),add(12)(q13),add(18)(q12),+ring[9]	45,XY,del(1)(p),der(5q)(19),der(6q)(6),der(6q)(5),-7,**r(17)**,add(19q)(1)[cp6]/44,XY,idem,der(1q)(12),**der(8q)(2::5::6)**[1],add(12q)(22),add(18q)(19)[cp2]
49	56	M	MDS	43,X,-Y,r(3)(?),-4,-5,-7,der(12)t(12;15)(p1?;q1?),-15,add(17)(p1?2),-21,-22,+mar1x2,+mar2,+mar[45]/86~88,idemx2[4]/44,X,-Y,r(3)(?),-4,-5,-7,add(12)(p1?),-17,+mar1,+5mar[2]/46,XY[4]	43,X,-Y,der(3)r(3;22)**,der(4)(3)**,-5,-7,der(12p)(15),-15,der(17p)(5),**der(21p)(4)**,**der(22)(3)**,**+der(22)(3)**
50	54	F	MDS relapse	46,XX,t(6;10)(q21;p14),t(18;19)(p11;q13)[64]/46,XX[7]	46,XX,der(6q)(10),der(10p)(6),der(18p)(19),der(19q)(18)[9]
51	69	F	RA	46,XX,-4,add(5)(q2?1),add(6)(q1?5),der(11)t(4;11)(p1?3;q1?4),-12,add(13)(p11),add(16)(p1?2),-17,+mar1,+mar2,+mar3[11]/45,idem,-7,add(21)(p11)[9]/46,idem,t(2;3;15)(q2?3;p11;q21)[8]	44~46,XX,der(2q)(15)[3],der(3q)(2)[3],**der(4q)(12)**,der(5),der(6q)(16),der(7q)(21)[2],der(11q)(4),**der(12q)(11)**,der(13p)(12)**,**der(15q)(3)[3],der(16p)(6),**der(17)**[cp9] FISH: mar1=der(4)t(4;11)t(4;12) mar2=der(12)t(4;12)
52	61	M	RAEB	44,XY,-2,add(3)(p12),add(3)(q11),-5,-7,add(11)(p14),der(12)t(2;12)(q2?3;p12),add(17)(p11.2),+mar[26]/46,XY[6]	44,XY,**der(2q)(12)**[7],der(3p)(5),ins(3q)(5),-5,-7,der(11p)(?)[2],der(12p)(2)[7],der(17p)(2)[7][cp8] FISH: der(11)(?)= der(11)(12)
53	45	M	RAEB-T	43,XY,-2,add(3)(p1?4),-5,add(7)(q21),der(11)add(11)(p11)del(11)(q23),add(12)(p12),ins(12;?)(q11;?),add(13)(p11),-20[15]]/43,idem,2dmin[3]/46,XY[2]	43,XY,-2,der(3p)(2),der(5)(11)[8]**,**der(7q)(2)[8]**,**der(12p11~12)(20)[8],ins(12q)(2),der(13p)(20)[8],-20[7],1dmin[cp9] FISH: der(13)=ins(13;20) dmin = 11
54	68	F	RAEB-T	45,X,-X,-5,-16,-17,-21,+mar1,+mar2,+mar3,+mar4[27]/45,idem,+X,-15[3]/46,idem,+X[2]	45,XX,-X[10],**del(5)ins(5q)(16)**,**der(16p)(17)ins(16q;5)**,-17,**+21**[7],**+i(21q)**[3][cp12]
55	41	F	RAEB-T	44~46,XX,-3,-5[3],add(7)(q1?1),-9,-21,der(22)t(21;22)(?q11;p1?3),+der(?)t(?;9)(?;p1?2),+0-3mar[cp20]/46,XX,-3,-5,add(7),-9,-21,+22,+der(?),+2mar[2]/46,XX[2]	44~46,XX,-3,**der(7q)(5)**,der(9q)(3),del(21)(q),der(22p)(21),+1~2mar[3]
56	74	M	RAEB-T	41~45,XY,add(1)(q12)[19],der(7)t(1;7)(q31;q32),-8[19],add(15)(p11)[18],-16,-20,+mar1[18],+mar2[9],+r1[15],+r2[2][cp20]/87~91,idemx2,-8[2]/46,XY[1]	38~46,XY,der(1q)(2)[7],der(7q)(1)[9],der(8),+der(8)[4],**+der(?)(8::1::15)**[8],-15[8],**r(16)**[7],-20[9][cp9] FISH: der(8::1::15)= der(8::1::8::20::15)

57	54	M	RAEB-T	40~42,XY,-5,-7,der(8)dup(8)(?)t(8;17)(q24;q1?2),add(9)(p2?3), t(13;22)(q11;p13)[2],-15,add(15)(q2?),add(16)(p13),-17,-18,-21, psudic(18;7)(p11;p22)der(7)del(7)(p11q14)del(7)(q10q11),+ring [cp33]/77~82,idemx2[2]/46,XY[1]	41,XY,-5,**der(5),**+r(5),-7,der(8q)(17),der(9p)(5),-15,der(15), der(16q)(16::6::15),-17,-18,psudic(18?p)(7),-21, **psutri(16::21::15)**[12] FISH: r(5) not confirmed, but der(5)
58	55	F	RAEB-T	49~56,XX,add(5)(q1?2),+add(5)(q1?2)[2],+6,+8,-10,?der(10)(p), +11,del(12)(p11p13),+13,+14,add(17)(p11),add(20)(q1?1),+add (20)(q1?1),+21,+21[3],+22[2][cp21]	47~58,XX,der(5q12)(10),+der(5q12)(10),+6[4],+8,-10, **der(10p)(20)**,+11,del(12)(p),+13[4],+14,der(17p)(10), der(20q)(10)[4],+der(20q)(10)[2],+21,+21[2],+22[1][cp7]
59	63	M	RAEB-T	44~45,X,-Y,add(3)(p10),add(5)(q?15),+8,-10,-21,der(21)(qter- >cen::q21->q22::q22->qter),+mar[2][cp16]/51,X,-Y,+del(2) (q2?2q37),add(3)(p1?),add(5)(q?15),+6,+8,+8,add(10)(q21), +13,+19,add(21)(p11),add(21)(p11),+mar[2][cp13]/50~51,X,- Y,+del(2)(q2?2q37),add(3)(p10),add(5)(q?15),+add(6)(p1?3), +8,+8,-10,+13,+19,der(21)(qter->cen::q21->q22::q22->qter)x2, +2mar[2][cp3]	44,X,-Y,der(3)(10),der(5q23)(10),+8,-10[3],**der(10)(3)**[1], i(21)[2],-21[2]cp[4]/51,X,-Y,del(2)[4],del(3)[2],der(3)(10)[5], +der(3)(10)[1],der(5q23)(10),+6[4],+8,+8,**der(10)(3)**[5],+13, +19,i(21),+i(21)[3],-21[3][cp6]
60	66	M	RAEB-T	43~51,XY,+Y[2],-4[18],-5[18],+6[3],-11[17],add(13)(p11)[2], +14[2],-17[12],-20[6],der(20)t(1;20)(p2?1;q13)[11],-21[3],der(21) t(21;22)(q2?2;q1?1)[12],-22[19],+1~6mar[8],0~6dmin[4],inc [cp17][21]/90~94,idemx2[6]/46,XY[1]	43~46,XY,+Y[2],-4[10],-5,+6[2],**del(11)**,der(13)(19)[1], **der(16p)(2)**[3],**der(17?p)(11)**,**der(17)(19 or 20)**[4],**der(18p) (22)**[3],**der(19)(22)**[3],der(20)(1)[7],der(21)(22)[5],-22[9], **der(22)(18)**[2],dmin[5][cp11]
61	53	M	RAEB-T	40~47,XY,der(1)t(1;9)(p11;p11)[13],add(7)(q2?2)[13],der(8)t(Y;8) (q11;q24)[1],dic(9;15)(p12;p13)del(15)(q15q21)add(9)(p12),-13, der(14)t(1;14)(p3?;p11),-15,add(16)(p1?1)[14],der(17)t(13;17) (q21;p12),-20,-21,+1~5mar[cp17]	45,XY,del(1)(p),der(7q)(20),der(9p13)(15),-13,-15,ins(16) (16::18::16::18),der(17p)(13),-20,**ins(21;9)**,**+der(13)[**2]/ 44,idem,-Y,der(8q)(Y)[3]/45,idem,del(1)(p),der(14p)(1)[7] FISH: ins(21)=(9::21::9::21::20::21) ins(16)=(16::20::16::20) mar=fr13/fr16/18/fr20
62	73	M	t-RAEB-T	41~45,XY,add(8)(q23)[12],-11,add(11)(p11)[11],-15[11],add(15) (p11)[12],-17[10],?der(20)[5],add(22)(p11)[cp22]/46,XY[2]	39~45,XY,der(8q)(15)[2],-11[6],add(15p)(8)[2],**der(16?q)(11)** [2],add(17p)(11)[2],-22,add(22p)(11)[cp7] FISH: add(15p)= add(15p)(8,11) FISH: der(16)?= der(16)t(11;16)

New and **refined SKY** observations are shown in bold.

Table 4: Comparison of conventional cytogenetics and SKY in ALL cases

Case	Age (yr)	Sex	Diagnosis (FAB)	Conventional karyotype (ISCN, 1995)	SKY karyotype*
63	57	M	ALL	48,XY,t(9;22)(q34;q11)[1]/48,idem,del(6)(q22),t(7;22;13)(q11;q11;q33),+12,del(16)(q13),der(17)t(6;17)(q22;q25),+21[13]/48,idem,t(11;19)(p15;?p12),+12,+21[7]	46~48,XY,**der(6q)(10)**[3],der(7q)(13)[4],der(9q)(22)[7],der(11p)(19)[1],+12[5],der(13q)(22)[2],del(16)(q)[3],der(17q)(6)[4],+21[7],-22,der(22q)(7)[4][cp7]
64	63	M	ALL	55~64,X,-Y,+X,+1,+1,+2,+3,+5,+6,+6,add(7)(p1),add(9)(p1),add(9)(p2),+10,+11,+11,+12,-13,add(13)(p1),+14,-15,add(15)(p1),+18,-20,+21,+22,+22,+1~2mar[cp21]/46,XY[29]	55~64,X,-Y,+X,+1,+1,+2,+3,+5,+6,+6,der(7)(9),der(9p13)(15),der(9p22~23)(18),+10,+11,+11,+12,-13,i(13),+14,-15,+18,**+der(19)**,-20,+21,**+der(21),**+22[cp9]
65	76	M	ALL	46,XY,idic(17)(p11)[16]/48,XY,+Y,+?14[5]/46,XY[16]	46,XY,idic(17p)[2]/48,XY,+Y,+14[1]/46,XY[5]
66	14	M	ALL relapse	46,XY,der(9)t(9;18)(p13;q11.2),del(20)(q11q13)[10]/47,idem,+8[14]/46,idem,+8,-13[2]/46,XY[3]	46,XY,der(9p13)(18),**der(18q11)(9)**,del(20)(q)[7]/47,idem,+8[4]
67	12	F	cALL	48,XX,add(2)(p22),add(6)(q1?3),?der(7),der(8)?del(8)(p21p22)add(8)(q24),+10,del(12)(p11p13),?der(13),del(14)(q31q32),-15,add(17)(p10),+2mar[26]/46,XX[12]	48,XX,dup(2),der(6?q)(15),der(8q)(21),+10,der(12p)(21),del(14),**+der(15)**,der(17p)(6),**+21**[6]
68	29	F	cALL	46,XX,der(8)t(8;9)(q21;q31)t(9;22)(q34;q11),der(9)del(9)(p1?3)t(8;9)t(9;22),der(20)t(9;20)(p?2;p1?),der(22)t(9;22)[7]/47,idem,+8[7]/46,idem,i(8)(q10)[3]/46,idem,der(4)t(4;8)(q2?8;q1?2)[3]/46,XY[3]	46,XX,der(4?q)(8),der(8?q)(8::9::22),+der(8q)(9),der(9),der(20q)(9),del(22)[6]
69	11	M	cALL	43~48,XY,add(3)(q1),add(3)(q2?4),der(4)t(2;4)(q2?2;p16),add(6)(q15),add(8)(p12),der(9)del(9)(p22)?del(9)(q3),+10,?del(12)(p12p13),+del(22)(q1)[cp7] t(12;21) positive by RT-PCR	46~48,XY,der(3q)(6),der(3q)(7),der(4p)(2),der(6q)(22),**der(7)(3)**,der(8)dup(8),der(9),+10,**t(12;21),**del(22)[5]
70	16	F	T-ALL	46,XX,?add(5)(q3),add(7)(q3?1),t(7;14)(p13;q32),del(11)(q13q23),del(12)(p1?3),add(15)(p1),del(18)(p11)[31]	46,XX,**der(1p?32)(22)**,der(5q)(21),der(7p)(14),dup(7),del(11)(q),del(12)(p),der(14q)(7),der(15p)(12),del(18),**der(21p)(5)**[7]

New and **refined SKY** observations are shown in bold

Fluorescence in situ hybridisation (FISH)

FISH analysis was frequently used to confirm the chromosome aberrations identified using SKY, especially when small regions were involved. For this purpose, pBS whole chromosome paints [14] or commercially available whole chromosome paints (WCPs) (Eurodiagnostica, Arnhem, The Netherlands) and subtelomere-specific probes [15] (kind gift of Dr. L. Kearney) were hybridised to metaphase spreads following standard procedures. For investigating possible recurrent chromosome aberrations, locus-specific FISH probes were hybridised to metaphase spreads according to standard procedures. Hybridisation of 100 ng of each probe was performed overnight at 37°C in a moist chamber, and the slides were washed with 0.4xSSC at 72°C. Biotinylated probes were detected using FITC-labelled avidin, followed by biotinylated goat-anti-avidin and avidin-FITC incubation. Digoxigenin-labelled probes were detected using sheep-anti-digoxigenin-Rhodamine, followed by donkey-anti-sheep-Texas Red incubation. Slides were counterstained with DAPI. For each sample a minimum of 5-10 metaphases were analysed. Images were captured using an epifluorescence microscope (Axioplan 2, Zeiss) using MacProbe software (version 4.3, Applied Imaging, Newcastle upon Tyne, United Kingdom).

Scoring method for chromosome abnormalities

For description of the SKY karyotypes, we designed a short-hand notation of the chromosomal origins involved in an aberrant chromosome. For easy comparison to conventional karyotypes, we included the chromosome arm of the aberrant chromosome to which the identified chromosomal material was added, e.g. in case 9 an add(5)(q1?5) was observed using conventional cytogenetics, which SKY showed to be a der(5) with chromosome 3 material added to its long arm, noted as der(5q)(3). If it is noted as der(5)(3), the involved chromosome arm was not known. If it is specified as a derivative chromosome, e.g. der(8), the aberrant chromosome consists of the chromosome material indicated only. In case of deletions, the chromosome arm involved was specified if known, e.g. a deletion of the long arm of chromosome 5 was noted as del(5)(q). Known specific translocations, e.g. t(9;22)(q34;q11) were noted according to ISCN 1995 if both derivative chromosomes were observed using SKY. If only one of the derivatives was observed, the short-hand notation was used. Abnormalities, recurrent within our investigated group or newly identified using SKY (from apparently normal chromosomes) were noted following the short-hand notation, including the band designation if possible.

For evaluation of conventional cytogenetic chromosome involvement in the complex karyotypes, two categories of results were defined: numerical and structural aberrations. We defined five categories of SKY results. Here, we concentrated on structural abnormalities because the power of SKY is in the identification of structural rearrangements. The first category included those structural aberrations identified using conventional cytogenetics confirmed with SKY (SKY confirmed). Aberrations partly identified using karyotyping, which were further elucidated using SKY belonged to the next group (SKY further). The chromosome abnormalities newly identified with SKY were divided in two groups: those newly identified from apparently normal chromosomes (SKY new from app normal chrom) and those that elucidated the composition of

marker chromosomes (SKY new from mar). Finally, aberrations that appeared to be classified wrongly by the SKY View software were grouped separately in order to be able to determine the validity of SKY analysis (SKY wrong). Example given: an add(5)(q1?5), observed with conventional karyotyping (case 9), was classified as a structural aberration. SKY showed this add(5)(q1?5) to be a der(5q)(3). Therefore, the involvement of chromosome 5 was classified as SKY confirmed, whereas the involvement of chromosome 3 was classified as SKY further. In case of a reciprocal translocation, the involved chromosomes were counted twice, since they occur in both derivative chromosomes. The numbers for each chromosome for all cases within one group (i.e. AML, MDS or ALL) were added together. The karyotypes with specific known or simple aberrations (Table 1) were excluded from scoring the chromosome aberrations.

Results

Conventional cytogenetics

Ninety-three acute leukaemia cases had been analysed for routine diagnostic purposes at diagnosis using conventional karyotyping. Eight cases were included for SKY validation purposes because they showed specific chromosome aberrations (cases 1-8). In 35 other cases, simple karyotypes were observed, e.g. no (cases 18, 71-95) or mainly numerical aberrations, or (combined with) a single structural abnormality (cases 9-17) (Table 1). The other cases chosen showed complex structural rearrangements and marker chromosomes of which the composition could not be elucidated using conventional karyotyping (Tables 2, 3 and 4).

SKY validation

Eight cases with known translocations i.e. t(9;22)(q34;q11), t(9;11)(p21;q23), t(4;11)(q21;q23), t(8;21)(q22;q22), t(15;17)(q22;q21) and t(12;21)(p13;q22), the latter identified through FISH or RT-PCR, were used for validation of the SKY probe set (Table 1). In case of t(9;22), SKY showed only the der(9)t(9;22). In the three included cases with t(12;21) only the der(12)t(12;21) was identified. These results led us to use FISH and whole chromosome paints or subtelomere-specific probes when SKY showed small, seemingly unbalanced translocations to determine whether they were reciprocal. Two apparently normal chromosomes 19 were observed in case 6, of which SKY showed one to be a der(19)(11). Finally, FISH verification showed a SKY misclassification in case 1: a der(2p)(5) was identified, which FISH showed to be a der(2p)(18) instead.

SKY

To investigate chromosome abnormalities possibly present in the 50 cases with complex karyotypes, not detected using conventional cytogenetics, and to elucidate the composition of marker chromosomes, all cases were additionally analysed using SKY. SKY analysis clarified incompletely identified chromosome rearrangements and elucidated the composition of 88 marker chromosomes. The 25 AML/MDS cases with normal cytogenetics, included in SKY analysis to investigate the possible presence of cryptic rearrangements, showed no hidden

clonal structural abnormalities (Table 1). In the nine cases with relatively simple karyotypes, the partially identified aberrations could further be elucidated, but no hidden abnormalities were revealed using SKY (Table 1).

SKY on complex AML cases

In the AML group with complex karyotypes, conventional cytogenetics showed chromosomes 5, 7 and 17 to be frequently involved in numerical abnormalities (mostly losses) (Figure 1A). We observed a frequent structural involvement of chromosomes 1, 3, 5, 11 and 12 based on conventional cytogenetics only, which was confirmed using SKY. The composition of most markers observed with conventional karyotyping could be elucidated with SKY. However, the composition of a few marker chromosomes remained unclear (Figure 1A). SKY showed chromosomes 5 and 17 to be even more frequently involved in structural chromosome aberrations than could be seen from conventional karyotyping only. This augmented involvement mainly resulted from the elucidation of marker chromosomes (fig. 1A). Moreover, using SKY, we identified a new chromosome abnormality involving apparently normal chromosomes 2 and 3, the der(2q)(3) (ISCN nomenclature: der(2)t(2;3)(q3?;?)) (case 43) (Fig. 2A). A second new abnormality involving an apparently normal chromosome 7, der(7q)(17) (ISCN nomenclature: der(7)(t(7;17)(q?;?p)) was observed in case 26.

SKY on complex MDS cases

In the MDS group, chromosomes 5, 7, 15, 17 and 21 were frequently involved in numerical abnormalities, mainly losses, according to conventional cytogenetics, whereas a frequent structural involvement of chromosomes 1, 2, 3, 5, 7, 12 and 15 was observed, which was confirmed using SKY. SKY elucidated the composition of most marker chromosomes. Additionally, SKY showed that chromosomes 5, 17 and 22, and to a lesser extent chromosomes 2, 3 and 12, were more frequently involved in structural chromosome aberrations than could be seen from conventional karyotyping only. This involvement resulted from the elucidation of marker chromosome composition (Fig. 1B). We identified a new chromosome abnormality involving apparently normal chromosomes 10 and 20, the der(10p)(20) (ISCN nomenclature: der(10)t(10;20)(p1?;?)) in case 58 (Fig. 2B). A second new abnormality involving apparently normal chromosomes 11 and 16, the der(16?q)(11) (ISCN nomenclature: der(16)t(11;16)(?;?q)), was observed in case 62 (Figure 2B).

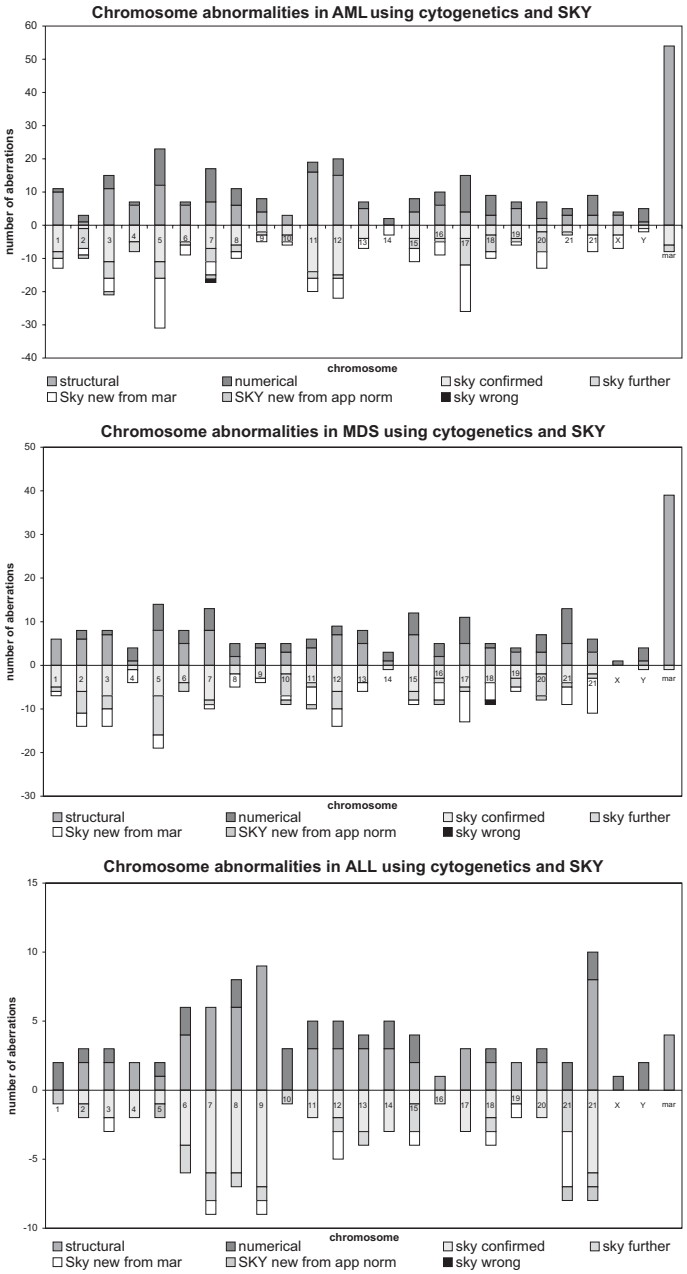

Figure 1: Comparison of conventional karyotyping and SKY. A) Comparison of conventional karyotyping and SKY analysis in 25 AML cases. On the X-axis the chromosome number is shown, whereas on the Y-axis the total number of abnormalities for each chromosome in 25 AML cases is shown. The positive part of the Y-axis shows the number of aberrations (both numerical and structural) observed with conventional karyotyping only; the negative part shows the number of structural aberrations found with SKY, divided into 5 categories; sky confirmed: structural aberrations already identified using conventional

cytogenetics, confirmed with SKY; sky further: aberrations partly identified using karyotyping alone and further elucidated using SKY; sky new from mar: chromosome abnormalities newly identified with SKY elucidating the composition of marker chromosomes; sky new from app norm: chromosome abnormalities newly identified with SKY from apparently normal chromosomes; sky wrong: aberrations classified wrongly by the SkyView software. B) Comparison of conventional karyotyping and SKY analysis in 17 MDS cases. The SKY results are grouped into the same categories described above. C) Comparison of conventional karyotyping and SKY analysis in 8 ALL cases. The SKY results are grouped into the same categories described above.

SKY on complex ALL cases

Numerical aberrations for the ALL group using conventional cytogenetics involved mostly chromosome 10 (gains) (Figure 1C). We observed a frequent structural involvement of chromosomes 7, 8, 9 and 22 based on conventional cytogenetics only, which was confirmed using SKY. Additionally, SKY showed that chromosome 21, and to lesser extent chromosomes 7 and 12, were more frequently involved in structural chromosome aberrations than could be seen from conventional karyotyping only, mainly resulting from the elucidation of marker chromosomes (fig 1C). Moreover, in case 70, we also identified 2 new chromosome abnormalities involving an apparently normal chromosome 1, the der(1p?32)(22) (ISCN nomenclature: der(1)t(1;22)(p3?2;q1?)), and 21, the der(21p)(5) (ISCN nomenclature: der(21)t(5;21)(?;p?)) (Figure 2C). The reciprocal der(5q)(21) in the SKY karyotype, had been partly identified using conventional karyotyping as ?add(5)(q3) (Table 4).

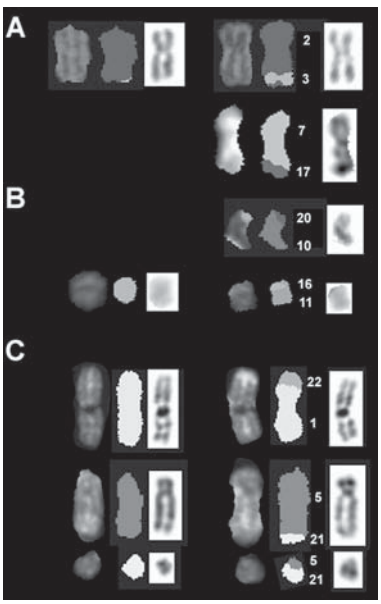

Figure 2: New structural chromosome aberrations identified using SKY in apparently normal chromosomes as observed with conventional karyotyping. A) SKY representation of the normal chromosome 2 and the der(2q)(3) (case 43) and der(7q)(17) (case 26) observed in AML. The SKY display image is on the left, classification image in the middle and the inverted DAPI image on the right of each chromosome series. B) SKY representation of the der(10p)(20) (case 58) and der(16?q)(11) (case 62) observed in MDS. C) SKY representation of the der(1p?32)(22) and der(21p)(5) observed in ALL (case 70).

Misclassifications and extremely complex cases

FISH confirmation showed the classification results to be wrong in very few cases. In the AML patient group, SKY misclassified chromosomal material only once (fig. 1A). In case 34, SKY identified a der(12p)(7), for which FISH showed that chromosome 17 was added instead of chromosome 7. In the MDS group, also one misclassification was observed (fig. 1B). In case 61, the add(16)(p1?1) observed with conventional karyotyping, was shown to be an ins(16)(16::18::16::18) using SKY. However, FISH showed for both locations that no chromosome 18 material was present in this derivative chromosome, but chromosome 20 instead. One ALL case with t(12;21)(p13;q22) showed misclassification (see SKY validation); within the complex ALL group, no chromosomal material was misclassified.

FISH verification showed some structural abnormalities to be even more complex. In AML case 38, a marker chromosome was observed, which SKY identified as a der(?6)(9::6::5::6::7). However, FISH showed this derivative to be a der(?6)(9::6::7::5::7::6::7) (Figure 3A). In the MDS group, conventional karyotyping identified an add(15)(p11) (case 56) and a marker chromosome (case 61), which SKY showed to be a der(?)(8::1::15) and ins(21;9). However, FISH showed that they were der(?)(8::1::8::20::15) and der(?)(9::21::9::21::20::21) respectively (Figure 3B).

Identification of recurrent abnormalities

Comparison of both conventional and SKY karyotypes for all cases showed several recurrent derivative chromosomes of (unbalanced) translocations. Recurrent in these cases was defined as observed in at least two cases of our patient group, e.g. der(9p13)(15) (cases 61 and 64), der(5q)(11)/der(5)(11) (cases 36 and 53) and the der(12p12)(20)/der(12p11~12)(20) (cases 11 and 53). In case 53, the der(5)(11) was originally classified as der(11)(5), but reinvestigation of the derivative chromosomes showed that it was in fact a der(5)(11) similar to case 36.

Some other abnormalities might be recurrent: der(12p12)(11)/der(12?q)(11) in case 25 and 51. Furthermore, der(5q23~31)(12)/der(5q13)(12) (cases 31 and 46) and der(5q12)(10)/der(5q23)(10) (cases 58 and 59) were observed. Finally, we observed a der(9p22~23)(18)/der(9p13)(18) in cases 64 and 66; for each of these abnormalities, the breakpoint of one of the chromosomes involved seemed to be cytogenetically identical in the two cases. However, the breakpoint of the second chromosome involved appeared cytogenetically different.

FISH using several locus-specific BAC clones was performed to investigate the similarity of the derivative chromosomes in the three (unbalanced) translocations in which both chromosome breakpoints seemed similar. However, we observed hybridisation differences between both cases in the der(9p)(15), der(12p)(20) as well as the der(5)(11), showing that the translocations are not identical. The derivatives of the other translocations, with one of both chromosomal breakpoints similar, could not further be investigated using FISH due to lack of material.

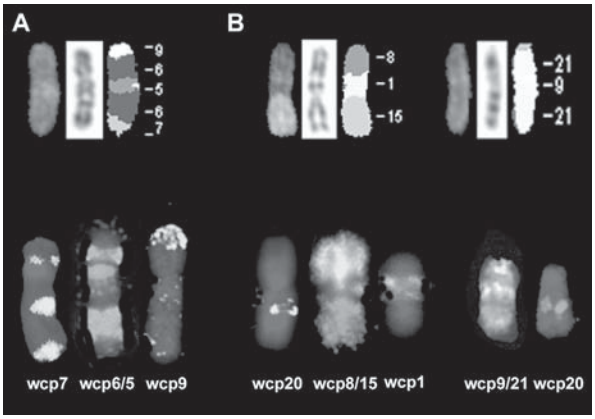

Figure 3: Complex chromosome aberrations observed using SKY and FISH. A) Upper panel: display, inverted DAPI and classified image of the der(?6)(9::6::5::6::7) observed using SKY in AML (case 38). Lower panel: FISH with whole chromosome paints (WCP) for chromosomes 5, 6, 7 and 9 identified even more complex composition, der(?6)(9::6::7::5::7::6::7), than anticipated after SKY. B) Upper panels: display, inverted DAPI and classified image of the der(?)(8::1::15) (case 56, upper left) and ins(21;9) (case 61, upper right) observed using SKY in MDS. Lower left panel: FISH with WCPs for chromosomes 1, 8, 15 and 20 identified der(?)(8::1::8::20::15), an even more complex composition than anticipated after SKY. Lower right panel: FISH with WCPs for chromosomes 9, 20 and 21 identified even more complex composition, der(?)(21::20::21::9::21::9), than anticipated after SKY.

Discussion

In this paper, we used SKY to identify new structural chromosome aberrations in 75 patients with acute leukaemia. In the total group, SKY identified 6 new chromosome aberrations involving apparently normal chromosomes. Additionally, SKY elucidated the composition of a total of 88 marker chromosomes in 30 cases. However, SKY was not completely accurate, since chromosomal material was misclassified 3 times in three different cases.

First, we determined the size that could be roughly detected using known translocations. The der(12)t(12;21)(p12;q22) was observed in the three selected ALL cases, but the der(21) could not be observed. However, both the der(12)t(12;21) and the der(21)t(12;21) were visible in a complex ALL case. SKY identified the der(9)t(9;22) in two ALL cases, but not the der(22)t(9;22). With the currently available probes, the minimum alteration detected using SKY ranges from 1-2 Mb [16], but the sensitivity is dependent on, besides the probe coverage of the chromosomes, the degree of condensation of the obtained chromosomes. The sizes of the rearranged fragments identified with SKY in this study differed from intermediate to quite large. However, as we were able to identify the t(12;21) in the complex ALL case 69, the sensitivity of this technique in our hands is comparable to the known sensitivity [11, 12].

SKY wrongly classified a der(12p)(17) as der(12p)(7) in AML, an ins(16)(16::20::16::20) as ins(16)(16::18::16::18) in MDS and a der(2p)(18) as der(2p)(5) in ALL, as was determined after FISH verification. These misclassifications can be explained when looking at the combinatorial table for the labels of the separate chromosomes in the SkyPaint™ kit (see appendix). At the

border of two combinatorial-labelled painting probes, the fluorescence interferes, resulting in new, different spectra, which are differently classified according to their own spectral information. If the translocated or inserted fragment is very small, only the interfering segment can be visible, resulting in misclassification, e.g. chromosome 16 is labelled with BD (B=Texas Red, D=FITC), whereas chromosome 20 is labelled with A (A=Rhodamine). Interference results in the combination ABD, which is the combination assigned to chromosome 18. So in total, SkyView software had problems with 3 out of 93 cases, and misclassifications from interfering spectra might be overcome by introducing more fluorescent labels for the combinatorial labelling. This might also overcome the inconclusive SKY results, through which structural rearrangements partly remained unclear and which could be resolved only using additional FISH.

In the material from the 25 AML/MDS patients with normal karyotypes we did not observe any clonal cryptic rearrangements using SKY. This is in agreement with the study of Mohr *et al.* [17], who also did not find any cryptic abnormalities in 19 AML/MDS cases with normal karyotypes. Using SKY and FISH, Hilgenfeld *et al.* [18] however identified clonal aberrations in 5/18 AML-M2 cases with a presumed normal karyotype. In addition, Zhang *et al.* [19] identified clonal aberrations in 2/28 analysed AML cases. Amongst the newly identified abnormalities in these two studies were aberrations that only occurred in minor subclones: e.g. -7, del(5)(q14q31), or -19/-21 in 10-14% of the metaphases. Furthermore these studies both identified a t(11;19)(q23;p13) case, present in 100% of metaphases. The patients selected for our SKY study were known to have no translocations involving *MLL* or *ETV6*. The absence of molecular cytogenetic abnormalities in the vast majority of AML cases with normal cytogenetics indicates that these cases probably carry other genetic abnormalities, which can not be detected using molecular cytogenetic techniques, such as *FLT3* mutations (ITD and TKD) [20, 21] or *C/EBPα* mutations [22, 23].

In the AML group with complex karyotypes, SKY showed that chromosomes 5 and 17 were more frequently involved in structural chromosome aberrations than could be seen from conventional karyotyping only, resulting from the elucidation of marker chromosomes. Structural rearrangements of chromosomes 5 and/or 17 in AML, mostly deletions and losses due to unbalanced translocations, have been reported previously [18, 24-28], suggesting that tumour suppressor genes in these regions are important for leukaemogenesis, but until now no specific gene(s) have been identified on chromosome 5. On chromosome 17p, *P53* might be a candidate gene, but no specific genes have been identified on 17q.

SKY showed that chromosomes 5, 17 and 22, and to a lesser extent also 2, 3 and 12, were more frequently involved in structural chromosome aberrations in the MDS group than could be seen from conventional karyotyping. Other groups also reported structural involvement of chromosomes 5, 17 and 22 in MDS [10, 24, 29, 30] and AML [24-28] using SKY or M-FISH. SKY additionally confirmed the involvement of the long arms of chromosomes 5 and 7. Losses of these chromosomes, resulting from unbalanced translocations frequently, have been observed before [24, 29].

For the ALL group, chromosomes 7, 8, 9 and 22 were frequently involved in structural aberrations. Additionally, SKY showed that chromosome 21, and to a lesser extent chromosomes 2 and

12, were more frequently involved in structural chromosome aberrations. Involvement of these chromosomes has been reported previously: Rowley *et al.* [9] observed del(9)(p) in three cases and del(12)(p) in one case, whereas Kerndrup and Kjeldsen [31] reported numerical involvement only of chromosome 21.

In three cases, derivative chromosomes turned out to be even more complex after verification with FISH than thought after SKY. Therefore, karyotypes can be even more complex than anticipated and although the SKY sensitivity is quite good, it is still necessary to confirm the findings using FISH, especially when dealing with very complex chromosomes. Additionally, it should be noted that SKY is unable to detect chromosomal inversions and small deletions.

In all three patient groups, so far non-recurrent new chromosome aberrations were identified. We identified a der(2q)(3) and a der(7q)(17) in AML, a der(10p)(20) and a der(16?q)(11) in MDS and a der(1p?32)(22) and der(21p)(5) in ALL. Attempts to clone the t(1;22) breakpoints of our der(1p?32)(22) case have been unsuccessful so far. Until now, we observed these new aberrations in only one case each, also when including all SKY investigated cases with other leukaemia subtypes. Cryptic translocations, involving different chromosomes, have been identified using SKY previously [18, 24, 26, 29, 31]. Mrozek *et al.* [26] identified a dic(10;20)(p11;q11.2); all other previously identified cryptic aberrations using SKY/M-FISH involved different combinations of chromosomes. The chromosomes involved in our cases were apparently normal in conventional karyotyping. Therefore, searching of Mitelman database [32] for the aberrations identified using SKY would not result in similar cryptic translocations. More general searches for add(2)(q?), add(10)(p?), add(16)(q), add(1)(p32) and add(21)(p?), resulted in many cases for each aberration [32]. Searches for the observed recurrent translocations showed that the translocations have been described, both with and without band designations, in different types of leukaemia cases [32]. Until the exact breakpoints in our cases are identified, it is hard to compare our cases to database results.

In conclusion, using SKY we did not observe any cryptic aberrations in the cases with normal cytogenetics. In those with more complex karyotypes we detected more frequent structural involvement of chromosomes 2, 5, 7, 12, 17, 21 and 22 in AML, MDS and ALL than can be observed with conventional cytogenetics only. In five of those cases we observed 6 rearrangements of apparently normal chromosomes: a der(2q)(3) and a der(7q)(17) in AML, a der(10p)(20) and der(16?q)(11) in MDS and a der(1p?32)(22) and der(21p)(5) in ALL. Future investigations elucidating the exact breakpoints of these translocations will show whether these abnormalities are recurrent and eventually identify the genes involved.

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Chapter 3

CGH analysis of complex chromosome aberrations in acute leukaemia

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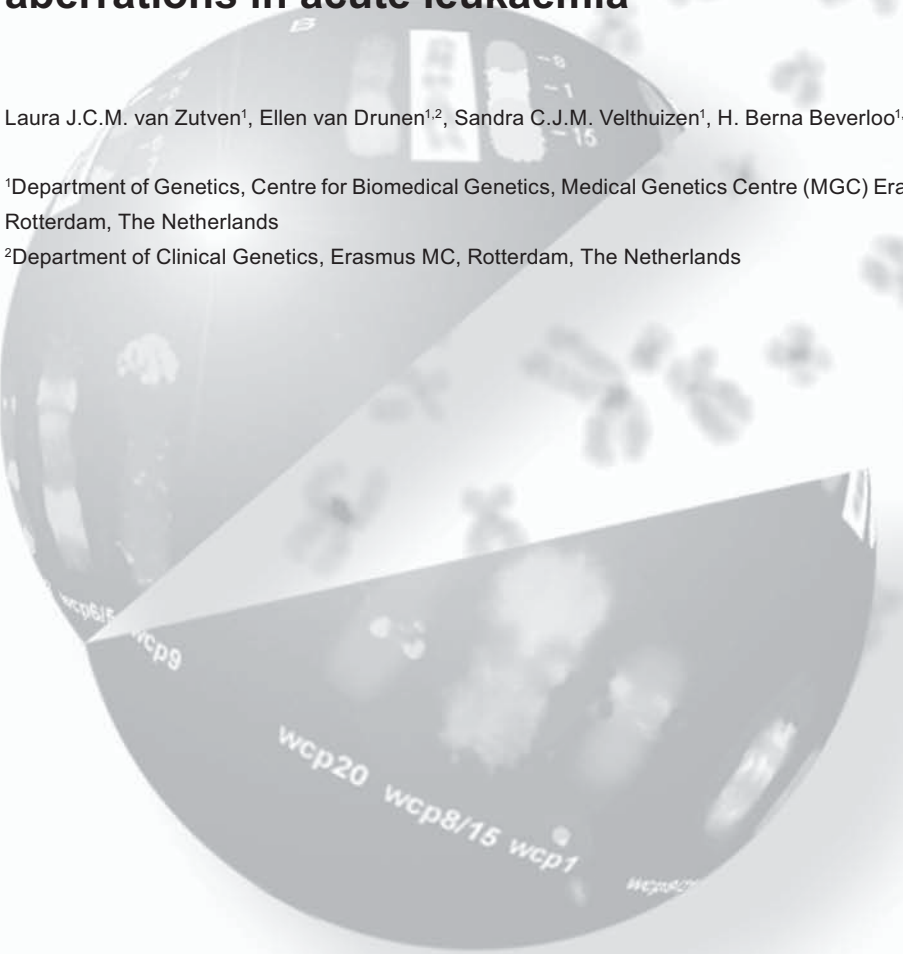


Chapter 3

CGH analysis of complex chromosome aberrations in acute leukaemia

Abstract

Chromosome aberrations are observed frequently in several types of leukaemia. Previously, we investigated 75 cases with acute myeloid leukaemia (AML), myelodysplastic syndrome (MDS) or acute lymphoblastic leukaemia (ALL) using spectral karyotyping (SKY) and fluorescence in situ hybridisation (FISH), and showed that chromosomes 2, 5, 7, 12, 17, 21 and/or 22 were more frequently involved in structural chromosome rearrangements than could be observed using conventional karyotyping only. In this study, metaphase comparative genomic hybridisation (CGH) was performed on cases with involvement of these chromosomes to investigate in more detail which regions were involved. Besides significant gains and losses, gains and losses were observed not reaching threshold values. FISH analysis showed that most of these latter gains/losses could be explained by rearrangements present in small subclones as shown by conventional and spectral karyotyping. Comparison of 40 cases in the overall group showed that regions 8q, 21q and 22q were frequently gained. Additionally, regions 3p, 5q, 7q, 12p and 17p were frequently lost. In 5 cases, high-level amplifications of 3q, 8q, 10q or 11q were observed. In the 18 AML cases, minimally lost regions were 3p24.2-p21.3, 3p21.1-p13, 5q31.2-q32, 7p22-q11.23, 7q22-q35, 12p13.3-p12.1, 16q22-q24, 17p13-p12 and 17q21.2-q21.3, and minimal gains were observed for 8q21.3-q24.3 and chromosome 22. In the 15 MDS cases 3p26-p14.3, 5q31.2-q32, 7q31.1-q36 and 17p13-p12 were the minimally lost regions, whereas 8q23-q24.1 and 21q22.1-q22.3 were gained. In the 7 ALL cases, gains of chromosomes 10, 12 and 21 were observed in more than one case. Most of the gained and lost regions corresponded to previously observed cytogenetic abnormalities, suggesting that genes present in these minimally lost or gained regions are important for leukaemogenesis.

Introduction

Chromosome abnormalities are frequently observed in haematological malignancies. The presence of these rearrangements constitutes a prognostic factor for the outcome of disease and has therapeutic consequences [1]. Molecular cytogenetic techniques can help to elucidate the composition of marker chromosomes and identify hidden chromosome abnormalities.

Conventional karyotyping and SKY require the presence of metaphases with sufficient chromosome quality, which is difficult to obtain in certain types of leukaemia. In addition,

especially smaller deletions are beyond the resolution of banding techniques. Comparative genomic hybridisation (CGH) is a quantitative double colour fluorescence in situ hybridisation (FISH) technique, which identifies and maps DNA copy number changes in a single hybridisation experiment. Total genomic DNA isolated from a tumour sample and from a reference individual with a normal karyotype are differently labelled, pooled and hybridised to normal human metaphase spreads. Differences in fluorescence intensities, visualized using digital imaging devices, reflect the DNA copy number changes in the tumour [2-4]. Thus, CGH analysis can be performed even if the malignant cells do not divide under laboratory conditions, provided that sufficient abnormal cells are present in the sample. CGH has been shown to be a useful tool for identification of genomic imbalances in a.o. haematological malignancies. In acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS), loss of 5q or 7q is frequently observed, but until now no specific genes, contributing to leukaemogenesis, have been identified in these two regions. CGH was useful to narrow down the minimally lost regions of these chromosomes [5, 6]. Additionally, amplification of 8q24 (*MYC*) [7] and 11q23 (*MLL*) [8, 9] has been observed in several studies, which had not been identified using conventional karyotyping only. In acute lymphoblastic leukaemia (ALL), CGH has shown a.o. the presence of cryptic 9p or 12p deletions, not identified with conventional karyotyping [5, 10].

Previously, we investigated a cohort of 75 patients with AML, MDS or ALL with normal cytogenetics or complex karyotypes. Using spectral karyotyping (SKY) and FISH, we showed that, amongst others, chromosomes 2, 5, 7, 12, 17, 21 and 22 were more frequently involved in structural chromosome aberrations than could be seen from conventional cytogenetics only (Chapter 2). Regions of most of these chromosomes are known to be frequently deleted in *de novo* and secondary AML and MDS, but no specific genes have been identified so far [5, 6]. To narrow down the regions involved in our cases, possibly resulting in the identification of underlying genes, we analysed 48 cases from the SKY-investigated cohort with involvement of chromosomes 2, 5, 7, 12, 17, 21 and 22 using CGH and compared them with the previously obtained SKY and karyotyping results.

Materials and Methods

Conventional cytogenetic analysis and patients selection

Bone marrow or blood samples of 54 acute leukaemia or MDS cases were previously analysed using conventional cytogenetics and SKY, and were selected for CGH analysis, because they showed aberrations of chromosomes 2, 5, 7, 12, 17, 21 and/or 22, chromosomes more frequently involved in structural abnormalities than observed with karyotyping only.

Conventional cytogenetic and SKY results have been previously described, and the case codes used here correspond to the earlier codes (Chapter 2). Remaining ethanol/acetic acid (3:1) fixed cell suspensions were stored at -20°C and remaining blood or bone marrow was viably frozen in 0.1 volume dimethylsulfoxide (DMSO)/0.9 volume foetal calf serum (FCS) and stored in liquid nitrogen until use. At diagnosis, informed consent of the patients and/or parents/guardians was obtained to use left-over material for research purposes.

Comparative genomic hybridisation (CGH)

Genomic DNA was isolated from cryopreserved bone marrow or blood according to standard procedures. When no bone marrow or blood was available, genomic DNA was isolated from fixed cell suspensions using the Qiagen DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. This latter DNA isolation method was validated using the RS4;11 cell line, using DNA isolated according to both isolation methods.

Of 48/54 cases (24 AML, 16 MDS and 8 ALL) sufficient viably frozen material or fixed cell suspension was available to isolate genomic DNA for CGH. The percentage of leukaemic cells in samples of these cases ranged from 8.5-90%. Patient DNA was labelled with Spectrum Green-dUTP (Vysis, Downers Grove, IL, USA) and reference DNA (pooled normal female DNA) with Alexa Fluor® 594-5-dUTP (Molecular Probes, Eugene, OR, USA) using the BioPrime® DNA Labelling System (Invitrogen, Breda, The Netherlands). An equal mixture of both patient and reference DNA was hybridised to normal male chromosomes. Probe DNA and chromosomes were simultaneously denatured at 73°C and hybridised at 37°C for at least 60 hours using the Hybaid OmniSlide System (Thermo Labsystems, Breda, The Netherlands). After this, the slides were washed using the Hybaid OmniSlide Wash Module (Thermo Labsystems) with 0.4xSSC/0.1% Tween pH 7.0 at 66.5°C for 2 minutes and with 1xPBS at 66.5°C for 3x5 minutes. Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted with anti-fade solution. For each case, at least 10 metaphases were captured using an epifluorescence microscope (Axioplan 2, Zeiss, Sliedrecht, The Netherlands) and analysed using Isis software (MetaSystems, Altlussheim, Germany). Losses were defined as chromosomal regions where the ratio green:red (threshold value) was below 0.8, while gains were defined as regions where the ratio green:red was above 1.2. High-level amplifications were defined as regions where the ratio green:red was above 1.5 [11].

Fluorescence in situ hybridisation (FISH)

FISH analysis was frequently used to investigate the observed high-level amplifications and to validate CGH results when gains and losses below threshold value were observed with CGH. For these purposes, locus-specific probes and whole chromosome painting (WCP) probes were hybridised to the patient's metaphase spreads as described in Chapter 2. For each sample with a high-level amplification at least 5 metaphases were analysed. For samples showing deviations from the ratio 1.0 line below threshold value, a minimum of 100 nuclei and 5 metaphases, if present, were analysed. Images were captured using an epifluorescence microscope (Zeiss) using MacProbe software (version 4.3, Applied Imaging, Newcastle upon Tyne, United Kingdom).

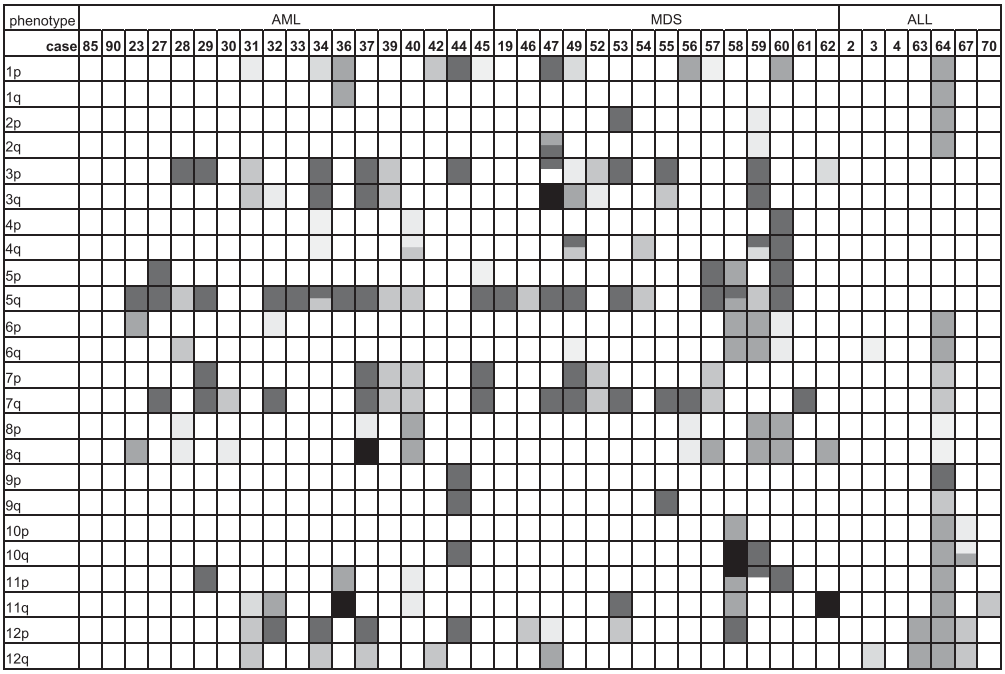
Results

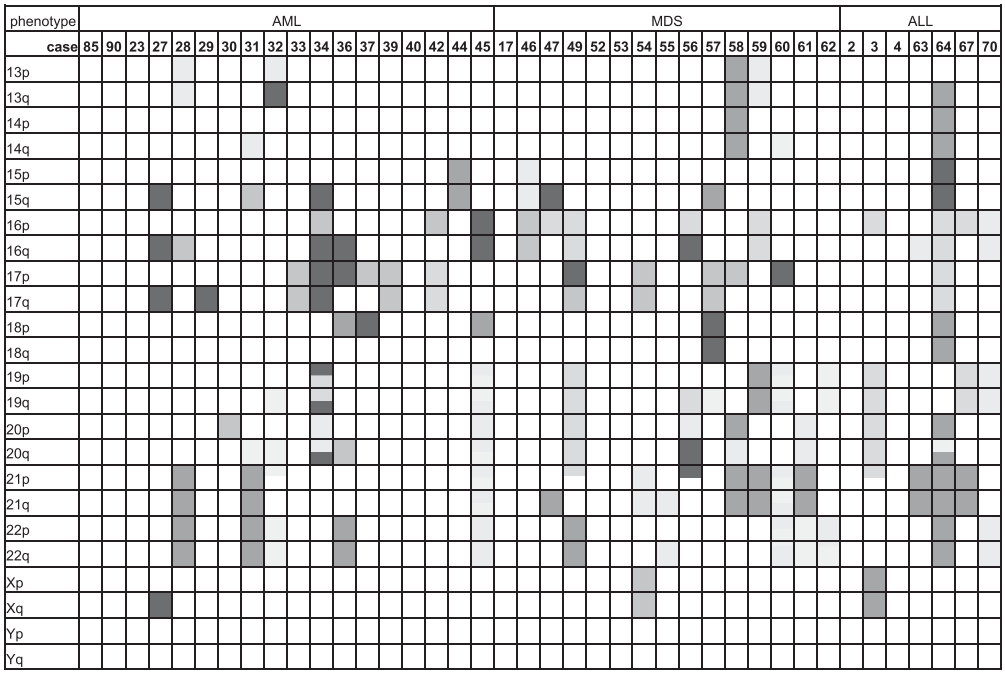
CGH

Seventy-five cases were previously analysed with conventional karyotyping and SKY to identify cryptic abnormalities and to elucidate the composition of markers and other unrecognised chromosomal material. SKY showed a more frequent involvement of chromosomes 2, 5, 7, 12, 17, 21 and/or 22 than observed with karyotyping in 54 of these cases (Chapter 2). CGH was performed to study the chromosomal regions involved in more detail, to more precisely identify the specific chromosomal segment, to determine whether the same region of the chromosomes was involved in different cases and to determine whether the complex karyotype led to gains, losses or a balanced situation. Of 40 cases (18 AML, 15 MDS and 7 ALL) with mostly complex karyotypes, CGH was successfully performed (Table 1).

For validation of the DNA isolation from fixed cell suspensions, CGH with cell line RS4;11 DNA isolated from either viable cells or a fixed cell suspension was performed. The analysis results from both isolation methods were concordant: the i(7)(q10) was clearly distinguishable in both profiles, as well as a small interstitial deletion in chromosome 9p. Additionally, Southern blot analysis, using a cDNA probe spanning the MLL breakpoint region, of both DNA samples showed an identical MLL breakpoint pattern. Also, self-hybridisation showed the expected normal CGH profile (data not shown).

Table 1: Overview of CGH results per chromosome arm





Colour codes used are: Red: loss; Pink: loss below threshold value (Category A); Salmon: loss below threshold that cannot be explained (Category B); Green: gain; Black: high-level amplification; Light green: gain below threshold value (Category A); Light blue: gain below threshold that cannot be explained (Category B).

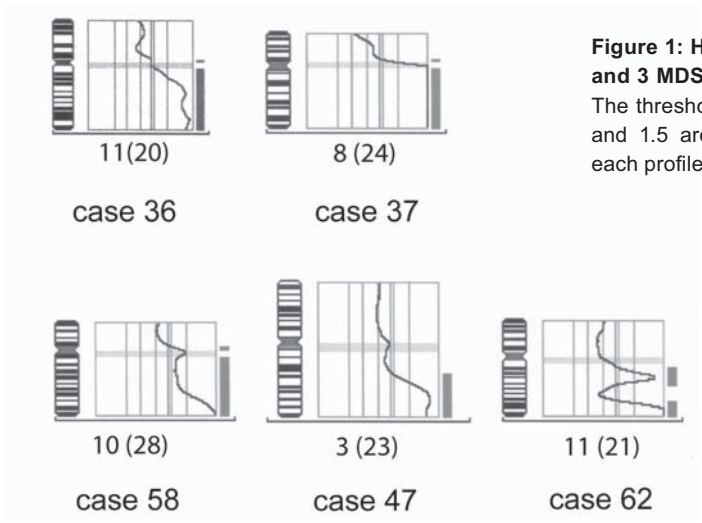


Figure 1: High-level amplifications in 2 AML and 3 MDS cases with complex karyotypes. The threshold values 0.5, 0.8 (red), 1.2 (green) and 1.5 are indicated by vertical lines within each profile frame.

In four of the 40 successfully analysed cases (2, 4, 85 and 90) a normal CGH profile was observed. These cases showed balanced translocations (case 2 and 4) or normal karyotypes (case 85 and 90) by SKY and conventional karyotyping. The CGH results rule out deletions or amplifications overlooked by the other methods. Six cases (cases 17, 27, 29, 33, 34 and 53) showed losses only, whereas one case showed only gains (case 3). CGH identified both losses and gains in 22/40 cases. Additionally, high-level amplifications (green:red ratio > 1.5) were observed in 5 of these 22 cases: enh(11)(q23q25) and enh(8)(q10q24.3) in AML (case 36 and 37), enh(3)(q13q29) and enh(10)(q24.3q26.3) in MDS (case 47 and 58) and two separate high-level gains of 11q in MDS case 62, enh(11)(q11q22.1) and enh(11)(q23.3q25) (Figure 1). FISH using WCPs confirmed the presence of all observed high-level amplifications (data not shown). The high-level amplified material was mostly present in (extra) derivative chromosomes observed with conventional cytogenetics as add, del or marker chromosomes, and its chromosomal origin had been elucidated using SKY (Table 2). Table 2 shows conventional and SKY karyotypes (as presented in Chapter 2) of the cases worked out in detail in this chapter.

Deviations of the ratio 1.0 line not reaching threshold values

In 22 cases showing gains and losses above threshold values ("true"), also deviations from the 1.0 ratio line just below threshold values were observed. These types of CGH results were present as "sole" abnormality in 7/40 cases (cases 30, 39, 42, 46, 52, 54 and 70) (Table 1). Comparison of the CGH results to conventional and SKY karyotyping in these cases (detailed description below) distinguished two categories of gains/losses just below threshold value: those explained by unbalanced rearrangements present in small cytogenetic subclones or in samples with a low percentage of leukaemic cells (category A) and those that could not be explained by the karyotype (category B). FISH was used to investigate these gains/losses and confirmed the CGH results within category A in each case tested. Examples of category A were the diminished signals just not reaching the threshold value for chromosome 5q and for chromosome 7 in cases 39 and 40 (Figure 2). Conventional karyotyping and SKY in case 39 showed a der(5)t(5;17) (15/21 metaphases) and loss of chromosome 7 (11/21 metaphases). In case 40, conventional karyotyping showed a.o. loss of chromosome 7 (18/21 metaphases) and several marker chromosomes, which SKY showed to be a der(1)t(1;7), a der(20)t(5;20) and a der(5)t(1;5). FISH showed loss of RP11-21J3 (5q31) signals in 70% and 48% of the interphase nuclei in case 39 and 40 respectively. Additionally, loss of 7pter signals (855a6, D7S481) was observed in both cases as well, in 70% and 19% of interphase nuclei respectively. Thus, the derivatives identified in both cases had shown the presence of chromosome 5 and/or chromosome 7 material, but did not represent the whole chromosome, leading to a loss visible with CGH as diminished signals not reaching threshold values, because the abnormality was present in a minority of cells. FISH did not confirm the CGH findings in most of the cases tested within category B. An example of category B was present in patient 70. In this case, most of the aberrations observed with CGH could be explained by the conventional/SKY karyotype, but the enh(16) and enh(19) could not. FISH using subtelomeric probes did not confirm the enh(19), but showed and extra 16qter signal in both metaphases and (in 88% of) interphase

nuclei, indicating the presence of a cryptic aberration involving at least part of chromosome 16q, which had not been observed by SKY.
Five of the seven cases with only gains/losses not reaching threshold value belonged to category A, whereas the remaining cases contained both category A and B gains and losses. Of the 24 cases showing both true abnormalities and those below threshold, the gains/losses not reaching threshold values belonged to category A in 9 cases, to category B in 1 case and to both A and B in 14 cases. The findings from category A were included in the comparison described below, but those from B were excluded.

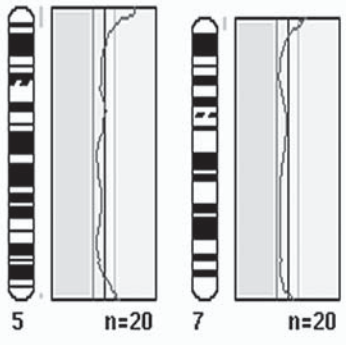


Figure 2: CGH profile of chromosomes 5 and 7 (case 40) showing losses not reaching threshold values (category A). The threshold values 0.8 (red) and 1.2 (green) are indicated by vertical lines within each profile frame.

Minimally overlapping lost or gained regions

We compared the CGH results of all 40 cases successfully investigated to identify the regions most commonly gained or lost. Loss of chromosome 3p (12 cases), 5q (22 cases), 7q (17 cases), 12p (9 cases) and 17p (11 cases) occurred frequently, while the most frequently observed gains were of chromosome 8q (10 cases), 21q (11 cases) and 22q (8 cases) (Table 1). The minimally lost regions included 3p21-p14.1, 5q31.2-q32 (figure 3A), 7q11.1-q11.23, 7q33-q35 (figure 3B), 12p12.3-p12.1 and 17p13-p12, whereas the minimally gained regions were 8q23-q24.1, 21q22.1-q22.3 or the complete long arm and 22q13.2-q13.3 (Table 3). Chromosome 2, which we showed (Chapter 2) to be more frequently involved in structural aberrations than observed with conventional karyotyping, was neither lost nor gained frequently using CGH, suggesting this chromosome is involved mostly in balanced structural rearrangements.
We investigated the gains and losses per disease group as well (Table 1). Among 18 AML cases, frequent losses of 3p24.2-p21.3, 3p21.1-p13, 5q31.2-q32, 7p22-q11.23, 7q22-q35, 12p13.3-p12.1, 16q22-q24, 17p13-p12 and 17q21.2-q21.3 were observed. Gains were observed for 8q21.3-q24.3 and chromosome 22. Within the MDS group (n=15), we observed losses of 3p26-p14.3, 5q31.2-q32, 7q31.1-q36 and 17p13-p12, whereas gains were observed for 8q23-q24.1 and 21q22.1-q22.3. In the ALL group (n=7), only gains of chromosomes 10, 12 and 21 were observed.

Table 2: Comparison of conventional cytogenetics and SKY of the cases worked out in detail in Chapter 3

Case	Age (yr)	Sex	Diagnosis (FAB)	Conventional karyotype (ISCN, 1995)	SKY karyotype*
3	56	F	ALL	47~49,XX,+X,t(9;22)(q34;q11),inc[cp3]/46,XX[58]	46,XX,der(9)t(9;22)(q34;q11)[1]/46,XX[3][a]
27	69	F	AML-M1	44~46,X,-X,?der(2)(p)[3],der(3)t(3;15)(p?21;q1?5),?der(4)(p)[6],-5,add(7)(q2?2),-15[17],-16[17],-17[17],?der(18)(p)[4],-22,+mar1,+mar2,+mar3[14],+mar4[11],+mar5[7],+mar6[2],+mar7[14],+1-2mar[10][cp18]	43~45,X,**der(Xq)(17)**[4],**i(Xp)**[3],**del(X)(p)[**3],der(3p)(15),**der(5p)(22)**,der(7?q)(3),**del(16)**[7],**del(17)**[5],**der(?)(22)**[cp10]
28	59	F	AML-M1	43,XX,del(3)(p21p26),-4,-5,add(6)(?q10),-7,+8,add(16)(q21),-17,-18,+mar1[5]/45,XX,del(3)(p21p26),-5,-6,-7,add(16)(q21),-17,+idic(22)(p11),+psudic(?;6)t(?;6)(?;q11)t(?;5)(?;q13),+mar1[5]/45,XX,del(3)(p21p26),-5,-6,idic(11)(p11),add(16)(q21),-17,+psudic(?;6)t(?;6)(?;q11)t(?;5)(?;q13),+mar1[5]/43~49,XX,del(3)(p21p26),add(6)(?q10),+8[2],add(16)(q21),-17,+mar1,+mar2[cp3]/46,XX[1]	45~48,XX,del(3)(p),-4,**der(5q)(?)**,der(6q)(6::17::5)[2],der(6q)(17)[9],-7[5],+8[5],dic(11p)[4],der(16q)(21),-17,-18[5],+21[2],+22[4][cp11] FISH: der(5q)(?)= der(5)t(5;17)
30	60	F	AML-M1/M2	46,XX,del(11)(q13q25),+add(11)(p10),-16,-20,+mar1[12]/46,idem,del(7)(q21q31)[11]/47~49,idem,+X[1],del(7)(q21q31),+8,+8[2],+mar[1][cp10]/46,XX[2]	46,XX,del(11)(q),der(16p)(11),**der(20p)(16)**[4]/46,idem,del(7)(q)[5]/46,idem,del(7)(q),+8,**del(17)(q)**,**+del(17)(q)**[2]
33	60	F	AML-M2	45,X,add(X)(q13),add(4)(q28)[27],del(5)(q1?4q3?2),-17[27],-19[4],add(19)(q13)[7],del(19)(p13)[12][cp30]/66~85,idemx2[2]	45,X,der(Xq)(9),der(4),del(5)(q),-17,der(19q)(17),**der(20p)(17)**[5]
36	62	F	AML-M4	47,XX,+1,add(5)(q1?2),+add(11)(p1?1),-16,add(17)(p12),add(18)(p11),-20,+mar[4]/48~52,XX,+1,add(5)(q1?2),+9[2],add(11)(p1?1),+add(11)(p1?1),-16,add(17)(p12),add(18)(p11),-20,-22[30],+idic(22)(q1?2),+idic(22)(q1?2)[22],+idic(22)(q1?2)[5],+idic(22)(q1?2)[3],+idic(22)(q13),+mar[cp31]	48~50,XX,+1,der(5q11)(11),ins(11p)(18),+der(11p)(18),**del(16)**[6],**der(16?q)(5)**[3],der(17p)(17::5::20),der(18p)(20),-20,der(22),+i(22),+der(22)[7],+der(22)[1][cp11]
37	69	M	AML-M5	41~52,XY,-3,-5,-7,idic(8)(p1?2),+idic(8)(p1?2)[10],?der(10)(q),-12,-14,-17,-18,+mar1,+mar2,+mar3,+2-9mar[cp23]/72~130,idemx2-3,inc[3]/46,XY[2]	44~46,XY,-3,der(5)(12::5::12::17)[6],der(5)(5::12::17)[5],-7,idic(8q)[10],+idic(8q),der(8),+der(8)[6],+der(8)[4],der(12q)(12::5::17),del(14)(q)[10],-17,der(18p)(?::14::15::22::18)[6][11]
39	67	M	AML-M6	43~45,XY,-3,-5[4],der(5)t(5;17)(q1?3;q2?1)[15],-7,i(8)(q10)[11],-11[13],-17,ider(19)(q10)add(19)(q13)[18],add(20)(q1?3)[13],+mar1[17],+mar2[15],+1-2mar[5][cp19]/46,XY[2]	43~46,XY,**del(3)**,-5[2],der(5q)(17)[8],-7,i(8q)[4],-11[2],**del(11)**[7],**der(13)(6)**[2],-17,**der(17)**[2],**der(19)**,der(19)[6],der(20)(11)[8][cp11]
40	61	M	AML-M6	45~48,XY,der(1)del(1)(p13p31)inv(1)(p13q24)add(1)(q3?2)[18],add(4)(q?12)[17],-5[19],-7[18],del(7)(p1?2p2?2)[18],+8[18],+11[14],-17[18],-20,+mar1[18],+mar2[17],+mar3[14],+2-5mar[cp19]/46,XY[2]	47,XY,der(1q)(7),del(4)(q),**der(5q)(1)**,-7,del(7)(p),+8,+11,**der(?1)(17::1::17::1)**,**der(20q)(5)** or **ins(20q;5)**[cp12]

42	1	M	AML-M7 relapse	46,Y,t(X;1)(q24;q23)[3]/46,idem,t(3;12)(q22;p13)[22]/46,idem, t(3;12),t(9;16)(q34;q12)[7]/46,idem,der(3)t(3;12),der(7)t(7;12) (q22;p11)t(3;12),der(12)t(7;12)t(3;12)[3]/46,XY[11]	44~47,Y,t(X;1),t(3;12),?del(16)[a]
44	70	F	t-AML-M0	44,XX,del(3)(p11p21),?add(4)(q3?4),del(5)(q32q33),der(6)t(6;9) (q2?5;q34),-7,-9,add(10)(q22),add(10)(q23),-11,-12,del(15) (q2??2q2?4),add(18)(p11),der(19)t(12;19)(q1?3;p13.3),add(21) (p11),+mar1,+mar2[15]/46,XX[6]	44,XX,del(3)(p),der(4)dup(4),del(5)(q),der(6q)(9),**+der(6)(6::7::9)**,-7,-9,der(10q)(7),der(10q)(7),**der(11)**,-12,del(15)(q),der(18p) (10),der(19q)(12),der(21p)(15)[10]
46	54	F	CMMol	43~45,XX,add(2)(q36),der(5)t(5;12)(q13;q14),-7,-12,-15,-16,-17, ?der(19)t(7;19)(p13;q10),add(20)(q12~13),der(?)t(?;7)(?;q11) [16],+r,+mar1[15],+mar2[15][cp18]/46,XX[1]	42~46,XX,der(2q)(12)[4],der(5q13)(12)[5],**+der(7)**[3],der(7p) (7::19::17),-12[5],**der(12q)(2)**[3],**r(15)**[6],**del(16)**[3],-16[4],-17, der(19q)(7),**der(20q)(17)**[cp8]
47	1	M	JMML	46,XY,del(1)(p3?43?6),del(2)(q3?5q3?),add(5)(p1?2),add(5) (q3?3),add(6)(q2?5),add(7)(q2?1),add(11)(q23),add(15)(q2?5) [41]	46,XY,der(1p)(21),der(2q)(12),der(5p)(11),der(5q)(2),der(6q)(3), der(7q)(12),der(11q)(5),der(15q)(3)[12]
52	61	M	RAEB	44,XY,-2,add(3)(p12),add(3)(q11),-5,-7,add(11)(p14),der(12) t(2;12)(q2?3;p12),add(17)(p11.2),+mar[26]/46,XY[6]	44,XY,**der(2q)(12)**[7],der(3p)(5),ins(3q)(5),-5,-7,der(11p)(?)[2], der(12p)(2)[7],der(17p)(2)[7][cp8] FISH: der(11)(?)= der(11)(12)
54	68	F	RAEB-T	45,X,-X,-5,-16,-17,-21,+mar1,+mar2,+mar3,+mar4[27]/45,idem, +X,-15[3]/46,idem,+X[2]	45,XX,-X[10],**del(5)ins(5q)(16)**,**der(16p)(17)ins(16q;5)**,-17,**+21** [7],**+i(21q)**[3][12]
58	55	F	RAEB-T	49~56,XX,add(5)(q1?2),+add(5)(q1?2)[2],+6,+8,-10,?der(10) (p),+11,del(12)(p11p13),+13,+14,add(17)(p11),add(20)(q1?1), +add(20)(q1?1),+21,+21[3],+22[2][cp21]	47~58,XX,der(5q12)(10),+der(5q12)(10),+6[4],+8,-10,**der(10p) (20)**,+11,del(12)(p),+13[4],+14,der(17p)(10),der(20q)(10)[4], +der(20q)(10)[2],+21,+21[2],+22[1][cp7]
62	73	M	t-RAEB-T	41~45,XY,add(8)(q23)[12],-11,add(11)(p11)[11],-15[11],add(15) (p11)[12],-17[10],?der(20)[5],add(22)(p11)[cp22]/46,XY[2]	39~45,XY,der(8q)(15)[2],-11[6],add(15p)(8)[2],**der(16?q)(11)**[2], add(17p)(11)[2],-22,add(22p)(11)[cp7] FISH: add(15p)= add(15p)(8,11) FISH: der(16)?= der(16)t(11;16)
70	16	F	T-ALL	46,XX,?add(5)(q3),add(7)(q3?1),t(7;14)(p13;q32),del(11) (q13q23),del(12)(p1?3),add(15)(p1),del(18)(p11)[31]	46,XX,**der(1p?32)(22)**,der(5q)(21),der(7p)(14)dup(7),del(11)(q), del(12)(p),der(14q)(7),der(15p)(12),del(18),**der(21p)(5)**[7]

[a] no conclusion possible about t(9;16)

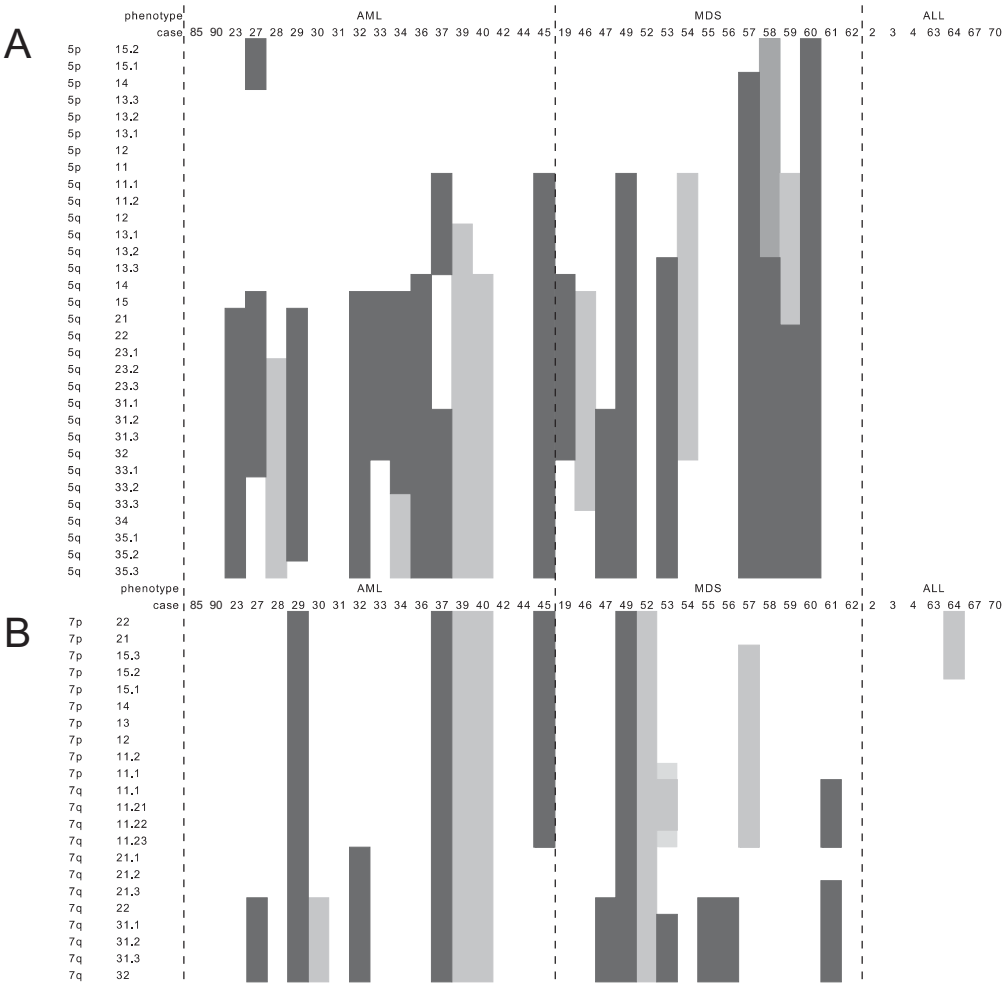


Figure 3: Graphic overview of gains and losses of chromosomes 5 and 7. A) CGH results of chromosome 5. Red represents losses, pink losses below threshold value (Category A) and salmon losses below threshold value that cannot be explained (Category B). Green are gains, light green gains below threshold value (category A) and light blue gains below threshold value that cannot be explained (Category B). B) CGH results of chromosome 7. Colour codes are as in figure A.

Case	Gains	Losses	Gains below threshold	Losses below threshold
17	-	dim(5)(q14q32)	-	-
23	enh(8)(q21.3q24.3)	dim(5)(q21q35)	-	-
27	-	dim(5)(p14p15.2) dim(5)(q15q33.1) dim(7)(q22) dim(17)(q21.2q23)	-	-
28	enh(21) enh(22)	dim(3)(p21.3p24.3)	enh(8)	dim(5)(q23.2)
29	-	dim(3)(p26p10) dim(5)(q21q35.2) dim(7)	-	-
30	-	-	enh(8)(q12q24.3)	dim(7)(q22q35)
31	enh(21) enh(22)	-	-	dim(3)(p26p11) dim(12)(p13.2q11) dim(12)(q14q23)
32	-	dim(5)(q15q35) dim(7)(q21.1q36) dim(12)(p13p11)	-	-
33	-	dim(5)(q15q32)	-	dim(17)
34	-	dim(3) dim(5)(q15q33.2) dim(12)(p13p12.1) dim(17)(p13q21.3)	-	-
36	enh(22)	dim(5)(q14) dim(17)(p12)	-	-
37	enh(8)	dim(3)(p24.2q29) dim(5)(q11q13.3) dim(5)(q31.2q35) dim(7) dim(12)(p13p11.2)	-	dim(17)(p13p12)
39	-	-	-	dim(3)(p21.2p13) dim(3)(q13.2q23) dim(5)(q13) dim(7) dim(17)(p13q21.3)
40	enh(8)	-	-	dim(5)(q14) dim(7)
44	-	dim(3)(p21.1p12) dim(12)(p10)	-	-
45	-	dim(5)(q11q35) dim(7)(p22q11)	enh(22)	-
46	-	-	-	dim(5)(q15q33)
47	enh(21)	dim(5)(q31.2q35) dim(7)(q22q36)	enh(3)(q11q13)	-
49	-	dim(5)(q11q35) dim(7) dim(17)(p13p11)	enh(22)	dim(17)(p11q25)
52	-	-	-	dim(3)(p26p14) dim(7)
53	-	dim(3)(p25p12) dim(5)(q13.3q35) dim(7)(q31.1q36)	-	dim(7)(q11q11.23) dim(12)(p13p11)
54	-	-	enh(21)	dim(5)(q11q32) dim(17)(p13q12)
55	-	dim(3)(p24.1p14.3) dim(3)(p11.2q13.3) dim(3)(q24q25) dim(7)(q22q36)	enh(21)(q22q22.3) enh(22)(q13.2q13.3)	-
56	-	dim(7)(q22q36)	enh(8)(q23q24.1)	
57	enh(8)(q11q24.3)	dim(5)(p14q35)	-	dim(7)(p15q11) dim(17)(p13q11.1)
58	enh(8) enh(21)	dim(5)(q13.2q35) dim(12)(p13p11)	-	dim(17)(p13p12)
59	enh(8) enh(21)	dim(3)(p26q11) dim(5)(q21q35)	-	dim(5)(q11q21)
60	-	dim(5) dim(17)(p13p11)	-	dim(17)(p11q25)
61	enh(21)	dim(7)(q11.1q11.2) dim(7)(q21.2q35)	-	-
62	enh(8)(q23q24.3)	-	enh(22)	-
63	enh(21)	-	-	-
64	enh(21) enh(21)	-	-	dim(7)
67	enh(21)	-	-	dim(12)(p13p11)
70	-	-	enh(22)	-

Table 3: Chromosome regions most frequently gained or lost as assessed by CGH

Gains and losses are those regions where the ratio green:red was above 1.2 and below 0.8 respectively. Gains and losses below threshold are those regions where the ratio green:red deviated from the ratio=1 line, but did not reach the threshold values mentioned above. - no losses or gains observed using CGH

Comparison of CGH with conventional and SKY karyotyping

We compared the CGH results with the findings of conventional cytogenetics and SKY to investigate, which regions of chromosomes involved in (complex) aberrations were still present and which regions were numerically aberrant in each case.

The conventional and SKY karyotypes of the cases worked out in detail are shown in Table 2. In 4 cases (cases 2, 4, 85 and 90) we observed a normal CGH profile, indicating the presence of normal or a balanced situation in these cases. These were cases with normal conventional and SKY karyotypes or with a single specific translocation, which had been included in CGH to investigate the possible presence of cryptic deletions or amplifications. In case 3, we only observed gain of chromosome X, which confirmed the karyotypically observed +X. Additionally, CGH showed that the t(9;22)(q34;q11) did not contain cryptic deletions within the resolution of the technique.

In 6 cases, we observed losses only. Using CGH, a dim(5)(q15q32) was observed in case 33, and a dim(17) both not reaching the threshold value (category A). Conventional and spectral karyotyping showed a del(5)(q1?4q3?2) and -17. Additionally, we observed a der(19q)(17), corresponding to the add(19)(q13) in the conventional karyotype, and der(20p)(17) with SKY. Using SKY, the der(20p)(17) was observed in the same cells as the der(19q)(17). As the add(19)(q13) was found in 7 cells and the -17 in 27 cells of the conventional karyotype, the dim(17) below the threshold value represents the cells with -17 that did not have the der(19q)(17) nor the der(20p)(17). In case 27, using CGH we observed dim(5)(p15.2p14), dim(5)(q15q33.1), dim(7)(q22), dim(15)(q14q24), dim(16)(q12), dim(17)(q21.2q23) and dim(X)(q21.1q27). With SKY and conventional karyotyping, we observed a.o. der(5p)(22), der(7?q)(3), der(3p)(15), del(16), del(17), der(Xq)(17), i(Xp) and del(X)(p). Probably, in formation of the der(5p)(22), der(7?q)(3), der(3p)(15), del(17) and der(Xq)(17) sequences from 5p15.2-p14, 5q15-q33.1, 7q22-q36, 15q14-q24 and 17q21.2q23 were lost. As was observed with CGH, only 16p13-q12 sequences were present in the del(16). Using CGH, we did not observe loss of chromosome 22, as was identified using conventional karyotyping, so this chromosome is, most likely, completely present in the der(5p)(22) found with SKY. CGH showed that Xq21.1-q27 was lost; thus, the 3 derivative chromosomes containing X are likely to contain the remaining, non-lost, sequences of the X chromosome. For the other 4 cases with losses only (cases 17, 29, 34, and 53), we compared the CGH results with (SKY) karyotypes likewise.

In 22 cases CGH identified both losses and gains. Examples of comparing CGH with SKY results are given for 4/22 cases (28, 36, 40 and 44). All other cases were investigated similarly. In case 44, CGH identified dim(1)(p36), dim(3)(p21.1p12), dim(9)(p31q23), dim(10)(q25), dim(12)(p10) and enh(15)(p13q15), whereas conventional and SKY karyotyping showed a.o. del(3)(p), der(4)dup(4), der(6q)(9), +der(6)(6::7::9), -7, -9, der(10q)(7), -12, der(18p)(10) and der(19q)(12). Thus CGH confirmed the del(3)(p). SKY and CGH showed that the -9 was partly present in the der(6q)(9) and +der(6)(6::7::9), resulting in a loss of sequences between 9p23 and 9q31. 10q25-q26.3 was lost and not present in the derivatives containing chromosome 10 material. Only the long arm of chromosome 12 was present in the der(19q)(12). No chromosome 6, 7, 18 or 19 material was lost or gained, indicating the 6q material absent in the der(6q)(9) is present in the der(6)(6::7::9) and the -7 is completely incorporated in the der(6)(6::7::9) and

der(10q)(7). Chromosome 10 and 12 material was added to chromosome 18p and 19q without loss of any 18p and 19q material.
In case 28, CGH confirmed the gain of chromosomes 21 and 22 and the loss of 3p24.3-p21.3. The gains and losses not reaching the threshold value in this case could be explained by the presence of unbalanced rearrangements in small cytogenetic subclones.
In case 40, +8 was confirmed using CGH. The add(4)(q?12) from conventional karyotyping, which SKY had shown to be a del(4), was confirmed to be dim(4)(q24q32). CGH results below threshold value could mostly be explained by the presence of a small subclone with unbalanced translocations. Case 36 showed enh(1) and enh(22), also observed with conventional and SKY karyotyping as +1 and idic(22)(q)/i(22)/der(22). Although SKY showed chromosome 5 to be present in der(5)t(5;11), der(16?q)(5) and der(17p)(20::5::17), sequences from 5q14 to 5q35 were lost. Also in the derivatives containing chromosome 16 or 17, only part of the sequences was present, as CGH showed diminished signals for 16q and 17p. On the other hand, 11p12-q25 and 18p11.3p11.32 were gained; these regions are probably present in der(5)t(5;11), ins(11p)(18) and +der(11p)(18).

Discussion

In this chapter, we compared the results obtained using conventional cytogenetics, SKY and FISH (Chapter 2) with the CGH results. The four cases, that had been included in CGH analysis to investigate the presence of cryptic deletions or amplifications, showed a normal CGH profile, confirming the presence of normal or a balanced situation in these patients and showing that no aberrant clones were present that did not divide under laboratory conditions. Therefore, the observed conventional karyotype most probably reflects the genomic situation present in these cases.
(Partial) gains and losses of chromosomes, observed with different molecular cytogenetic techniques, have been reported frequently in AML: del(5)(q), -7, del(7)(q), +8, +10, del(12)(p), del(16)(q), -17, del(17)(p), del(17)(q), -18, +20 and partial gains involving 8q, 11q, 21q and 22q [6, 12-17]. For MDS too, (partial) losses and gains have been reported frequently: -5, del(5)(q), -7, del(7)(q) and del(17)(p) [6, 17-19], as well as gains involving chromosome 8 and 11q [6, 17-19]. Our findings in AML (n=18) and MDS (n=15) are consistent with the earlier publications, but additionally we observed loss of chromosome 3p in both AML (3p24-p21.3 and 3p21.1-p13) and MDS (3p26-p14.3). Although we narrowed down the minimally overlapping lost/gained regions in our cases, the intervals still encompass several cytogenetic bands, containing many genes, including some leukaemia-associated genes, like *ARHGAP26, AF5q31* and *IL3* on 5q31, *ETO, MYC* and *NBS1* on 8q, *TEL* on 12p13, *CBFβ* on 16q22, *AML1* on 21q22 and *BCR* on 22q11 [9]. In addition, the AML and MDS groups in this study showed overlapping losses and gains: del(3)(p), del(5)(q), del(7)(q), del(17)(p) and gain of 8q. As MDS is sometimes called a pre-leukaemic disorder, which can progress into AML, those cases of AML with losses/gains frequently observed in MDS as well, might have evolved from MDS. This would imply the losses and gains to be relatively early events in the tumourigenic evolution.
In the ALL group (n=7), only gains of chromosomes 10, 12 and 21 were observed in more than

one case. Using CGH, gains have been observed previously in ALL cases with hyperdiploid karyotypes, mostly of chromosomes 21, 10, 6, 14, 18, X, 17, 4 (mentioned in order of decreasing frequency) [11, 20-22], whereas only one hyperdiploid case was present in our ALL group. To our knowledge, gain of chromosome 10, 12 or 21 in nonhyperdiploid karyotypes has been reported in one previous study [22]. Deletions of 6q, 9p and 12p have also been reported [17]. In our 7 ALL cases, we did not observe these deletions in more than one case each. However, as the number of ALL patients investigated in our study is small, extending the study with additional cases might yield results comparable to the earlier publications.

In several cases, we observed gains and losses just below the threshold value for gain or loss. This could be explained in two ways; firstly, a small amount of abnormal leukaemic cells is present in between many normal bone marrow cells [11, 23]. Secondly, several, cytogenetically slightly different, leukaemic clones exist next to each other, a well-known phenomenon [5, 23]. Additionally, different abnormalities in each individual clone may sometimes balance each other to some extent (e.g. 47,XY,+7[5]/45,XY,-7[7]), making detection difficult [23]. The minimal percentage of cells with a certain chromosome abnormality to be detected using CGH varies in literature from 35% [24] to 50% [5, 23], depending on the threshold values chosen or calculated. Thresholds chosen at 0.8 and 1.2 theoretically account for a minimum of 40% abnormal cells and a maximum of 60% normal cells in the sample. As CGH uses total DNA of all cells or cell types present in the sample, either explanation results in a lower load for the cytogenetic aberration studied. Depending on the amount present, this might be visible as deviations not reaching threshold values. Both explanations could be true for most cases in category A (CGH results could be explained by conventional/SKY karyotype), when the aberration explaining the CGH result below threshold is present in only a minority of the cells (percentage of leukaemic cells in samples of patients falling within this category: 8.5-70%). Gains/losses of category B (CGH results not explained by the conventional/SKY karyotype) could indicate the presence of additional clones that did not divide under laboratory conditions (percentage of leukaemic cells in samples of patients falling within this category: 14-40%). FISH analysis confirmed the presence of category A losses/gains, but confirmed only very few of the category B losses/gains. However, the resolution of chromosomal CGH leaves some uncertainty regarding the borders of gained/lost regions. The FISH probes used for investigating the CGH results could therefore be located outside the actual gained/lost region, resulting in discrepancies between CGH and FISH results. Furthermore, category B losses/gains frequently involved chromosome 16, 19 and 22, regions known to be less reliable because of their high GC contents. CGH analysis of normal samples show characteristic deviations from the ratio 1.0 line for these regions as well as for peri-centromeric, heterochromatic and telomeric regions [23], which are automatically excluded from analysis by the software used (grey bars in figure 1). Kirchhoff *et al.* [26] developed a high-resolution CGH technique using a standard reference interval, which is especially wide at these less reliable regions. For each sample, the 99.5% confidence interval of each mean ratio profile value is compared to a corresponding 99.5% standard reference interval based on normal cases. If no overlap exists between the intervals, an aberration is present. The comparison of the intervals can be automated if few aberrations are present, but the presence of many aberrations in the sample

makes this impossible and comparison has to be performed manually [26]. This technique and/or other higher resolution studies, e.g. array-CGH, are needed to distinguish false positive results in the less reliable regions from true positive results and for better characterisation of true category B losses/gains eventually present.

Additionally, we identified high-level amplifications in 5 cases: enh(10)(q24.3q26.3) (case 58) and enh(3)(q13q29) (case 47) in MDS, enh(8)(q10q24.3) (case 37) and enh(11)(q23q25) (case 36) in AML, and two separate high-level amplifications in case 62 (MDS), enh(11)(q11q22.1) and enh(11)(q23.3q25). These amplifications were also (partly) observed with conventional and spectral karyotyping and comprise relatively large regions, each of which contains several genes involved in haematological malignancies [9]. On chromosome 10, *NFKB2* and *ADD3*, both normally expressed in the bone marrow, are located within the amplified region. *NFKB2* is frequently rearranged in many forms of lymphomas, whereas *ADD3* is fused to *NUP98* in the t(10;11)(q24;p15.5) in ALL. Within the amplified region of 3q, *EIF4A2* and *BCL6*, normally expressed in bone marrow and involved in non-Hodgkin lymphoma, and *MLF1, EVI1* and *GMPS*, involved in AML/MDS, are located. In AML, *GMPS* is fused to *MLL* in the t(3;11)(q25;q23) and in both AML and MDS *MLF1* is fused to *NPM1* in the t(3;5)(q25;q34). *EVI1,* normally not expressed in bone marrow, is involved in several rearrangements, all resulting in inappropriate (elevated) *EVI1* expression, causing alteration of terminal differentiation of bone marrow progenitors to mature granulocytes, erythrocytes and megakaryocytes [27]. As *EVI1* is within the highly amplified region on 3q in our case, its expression might be altered, thus contributing to the development of leukaemia. The highly amplified region of chromosome 8 contains three genes involved in haematological malignancies, *ETO, MYC* and *NBS1. NBS1* is implicated in the Nijmegen breakage syndrome, a chromosome instability syndrome and cancer-prone disease at risk for non-Hodgkin lymphomas [28]. Additionally, missense mutations in *NBS1* have been associated with childhood acute lymphoblastic leukaemia [29]. However, as disruption of *NBS1* is associated with Nijmegen breakage syndrome and leukaemia, this gene is not a likely candidate to play a role in leukaemogenesis if present in a highly amplified region. *ETO* is the fusion partner of *AML1* in the t(8;21)(q24;q22) in AML. The fusion protein retains the ability to recognize the *AML1* consensus binding site and to dimerise with the *CBTB* subunit, probably causing altered transcriptional regulation of normal *AML1* target genes [30]. *MYC* is frequently involved in t(8;14)(q24;q11) observed in Burkitt's lymphoma. Additionally, amplification of *MYC* has been described in non-Hodgkin lymphomas and many other solid tumour types as well, suggesting enhanced *MYC* expression to be a likely candidate for oncogenesis in our case with high-level amplification [7]. Many leukaemia-associated genes are located within the high-level amplified region of chromosome 11q, including *MLL, PLZF, CALM, DDX10, LARG* and *NUMA1*. Of these genes, *MLL* is involved in many translocations with different partner genes and has been shown to be amplified in some cases [8, 17]. As amplification of *MLL* and surrounding genes has been reported previously [8], it might have contributed to the development of leukaemia in our case.

In conclusion, CGH was performed on those cases with aberrations, which we detected using SKY and karyotyping to occur frequently in complex rearrangements. Several regions, some reported previously though some with larger intervals, were specifically lost, whereas

others were gained or highly amplified. All highly amplified regions, as well as the minimally gained/lost regions, need to be studied in more detail. The resolution of CGH can be increased significantly using matrix/array-CGH [31, 32]. Whole-genome arrays and microarrays specific for a disease or chromosomal region of interest are being developed. As the sequence of the clones on the arrays is known, future investigations using these arrays and/or FISH will hopefully identify genes in these regions contributing to leukaemogenesis.

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Chapter 4

Two dual-colour split signal fluorescence *in situ* hybridisation assays to detect t(5;14) involving *HOX11L2* or *CSX* in T-cell acute lymphoblastic leukaemia

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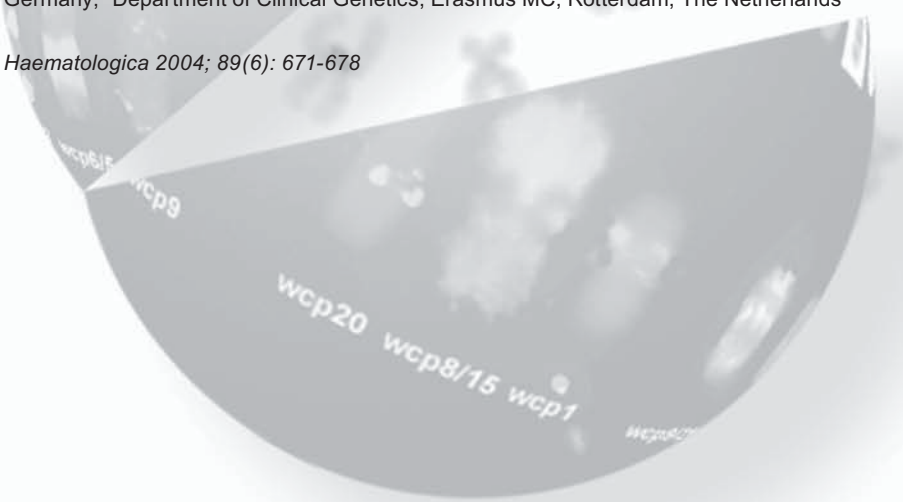

Chapter 4

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Abstract

Background and objectives. The t(5;14)(q35;q32) is a novel cryptic translocation in paediatric T-cell acute lymphoblastic leukaemia (T-ALL), involving *HOX11L2* or *CSX* on 5q35. The 14q32 breakpoints are heterogeneous. Because the t(5;14)(q35;q32) is hard to detect using conventional karyotyping, it is easily missed in routine diagnostics. Here we describe the development and application of split signal FISH assays for both *HOX11L2* and *CSX*, for detection of t(5;14) possibly present in T-ALL patients.

Design and methods. We developed and validated two split signal FISH assays for metaphase and interphase detection of t(5;14) in T-ALL patients. We also investigated the involvement of *IGH* on 14q32. In addition, *HOX11L2* and *SIL-TAL1* expression were studied using RT-PCR.

Results. The FISH assays were validated on cell lines and T-ALL patients. We did not identify cases with a t(5;14)(q35;q32) involving *CSX*, but we did identify 5 t(5;14) cases involving *HOX11L2* out of 32 T-ALL cases; in each case the 14q32 breakpoint was found to be centromeric of the *IGH* region. All 5 positive cases showed *HOX11L2* expression, as well as 1 case without t(5;14)(q35;q32). Cases with t(5;14)(q35;q32) involving *HOX11L2* did not show *TAL1* abnormalities, whereas 5 *HOX11L2* negative cases did.

Interpretation and conclusions. Using the newly developed and validated FISH probe sets, we identified 5 new t(5;14) cases involving *HOX11L2* both on metaphases and interphases. The incidence of the t(5;14)(q35;q32) involving *CSX* is probably low. RT-PCR results suggest that *TAL1* and *HOX11L2* expression, or *TAL1* aberrations and the t(5;14)(q35;q32) involving *HOX11L2* are mutually exclusive.

Introduction

Acute lymphoblastic leukaemia (ALL) is a worldwide disease, with an incidence of approximately 1.3 cases per 100,000 inhabitants per year in The Netherlands. Chromosomal rearrangements are found in many cases of ALL. Some of these rearrangements are specifically associated with certain subtypes of ALL. The presence of certain chromosome rearrangements constitutes an important prognostic factor for the outcome of disease and has therapeutic consequences.

In up to 30% of T-cell acute lymphoblastic leukaemia (T-ALL) patients, aberrations of the *TAL1*

gene (1p32) are found [1]. The majority are submicroscopic deletions of all coding exons of the *SIL* gene and the 5' untranslated region of *TAL1*. As a result, the *TAL1* coding region is placed directly under the control of the *SIL* promoter, leading to ectopic *TAL1* expression in precursor T-cells [2-5]. In another 3% of T-ALL patients ectopic *TAL1* expression is caused by translocations involving the 5' untranslated region of the *TAL1* gene [3, 6-9].

A novel cryptic translocation occurring mainly in childhood T-ALL is the recently described t(5;14)(q35;q32) [10, 11]. Fluorescence in situ hybridisation (FISH) demonstrated that the 5q breakpoint is heterogeneous. In the majority of patients the breakpoint is located within or downstream of *RANBP17*. This locus is also involved in the t(5;14)(q35;q11), with the breakpoint on 14q11 involving *TCRD* [12]. A second breakpoint on 5q35, located 2 Mb telomeric of *RANBP17*, was recently found in the T-ALL cell lines PEER and CCRF-CEM immediately upstream of a homeobox gene, *NKX2-5* or *CSX* [13]. Thus far, the breakpoints on chromosome 14q seem to be very heterogeneous as well. Bernard *et al.* [10] and Hélias *et al.* [11] found that the 14q breakpoint was centromeric to the *IGH* region. Additionally, Bernard *et al.* [10] narrowed the breakpoint region down to 700 kb between the *TCL1* and *AKT1* loci. No single clone was found so far encompassing the breakpoints in all patients tested; however, the breakpoints in the patient samples were all shown to be downstream of *BCL11B* [10], thus resembling the location of the 14q32 breakpoints in the cell lines HPB-ALL [14], CCRF-CEM and PEER [13]. *BCL11B* lies approximately 6.6 Mb centromeric of *IGH*.

The *RANBP17* gene does not seem to be deregulated as a result of the t(5;14)(q35;q32). However, another gene in the near vicinity*, HOX11L2* (also called *TLX3*), was ectopically expressed in these patients [15], and in the paediatric T-ALL cell line HPB-ALL, which carries the same translocation [14]. Ferrando *et al.* [16] and Ballerini *et al.* [17] showed that *HOX11L2* expression is associated with a poor prognosis. However, Cavé *et al.* [18] recently showed that patients with *HOX11L2* expression did not have a significantly different clinical outcome than patients without this expression.

Because the t(5;14)(q35;q32) is cryptic and thus hard to detect using conventional karyotyping, it escapes detection by routine diagnostics. Therefore the incidence is underestimated. Here we describe the development and application of two dual colour split signal FISH assays for detection of t(5;14)(q35;q32) involving *HOX11L2* or *CSX*. We included the very recently in T-ALL cell lines described *CSX* variant [13] to investigate whether this variant also occurs in primary samples. The probe sets were validated in a series of 32 T-ALL cases. To further study additional heterogeneity of the 14q32 breakpoint in our cases, we used a split signal probe combination for the possible involvement of *IGH,* which is a well-known partner gene in many different translocations occurring in acute leukaemia. In addition, we investigated whether the t(5;14) involving *HOX11L2* or *CSX* and *TAL1* aberrations can coexist.

Design and methods

Cell lines and patient samples

The cell lines PEER, DU.528, CCRF-CEM, MOLT16, and HPB-ALL, established from patients with paediatric T-ALL, were obtained from the DSMZ (Braunschweig, Germany) (details given

in Drexler [19] and at www.dsmz.de). They were cultured according to standard protocols, and cytogenetically prepared as described elsewhere [20]. The published karyotype, modified after FISH, for CCRF-CEM [13] is 46,XX, der(5)t(5;14)(q35.1;q32.2),t(8;9)(p11;p24),der(9)del(9) (p24)del(9)(q11q13~21),ins(14;5)(q32.2;q35.1q35.1),+20. The cell line used in this study had become near-tetraploid, as was also reported by Drexler [19]. This tetraploidisation was also observed for HPB-ALL.

Bone marrow or blood of 32 T-ALL patients was obtained at diagnosis and cultured and harvested using standard cytogenetic protocols. For each patient 20-32 metaphase cells were analysed using both Q- and R-banding. The chromosome aberrations observed were described according to the International System for Human Cytogenetic Nomenclature (ISCN) (ISCN, 1995). The patients' karyotypes are shown in Table 1. Methanol/acetic acid fixed cell suspensions were stored at –20°C. At diagnosis, informed consent of the patients and/or parents/guardians was obtained to use left-over material at diagnosis for research purposes.

HOX11L2, CSX and *TAL1* specific split signal probes

End-sequenced bacterial artificial chromosome (BAC) clones for the *HOX11L2* and *CSX* loci were identified by analysis of contig sequences using the TIGR STC-BAC program http://www.tigr.org/tdb/humgen/bac_end_search/bac_end_search.html and the UCSC Genome Bio informatics website http://genome.ucsc.edu. The DNA sequence of the different loci was assembled using Lasergene (DNASTAR inc. Madison, USA). For *HOX11L2* sequences AC021077, AC016574, AC010306, AC091980, AC093246, AC011400, and AC022426 were used; for *CSX,* the published sequences AC110011, AC008429, AC008378, AC008412, AC106731, AC016573, AC008632, AC008663, AC010339, AC008674, and AC093275 were used. These analyses identified BAC clones with 5´- and 3´- end-sequences >200 bp in length displaying > 94% identity.

For the t(5;14)(q35;q32) involving *HOX11L2*, breakpoints on the der(5)t(5;14) have been described between *RANBP17* exon 20 and *HOX11L2* exon 1, while one T-ALL patient had a translocation breakpoint downstream of *HOX11L2* exon 1 [10]. These results were used to design a split signal FISH assay (DakoCytomation, Glostrup, Denmark) for translocations involving *HOX11L2* by selecting one BAC clone located < 1kb centromeric to *RANBP17* exon 19 that spans 192 kb of DNA (HOX11L2-U) and three BAC clones spanning 269 kb located telomeric to the breakpoint region (HOX11L2-D) (Figure 1A). A 121 kb gap separates the HOX11L2-D and HOX11L2-U probes. For the t(5;14)(q35;q32) involving *CSX*, which is transcribed in a telomeric-centromeric direction, the described breakpoints on the der(5) mapped upstream of *CSX* exon 1 in cell lines PEER and CCRF-CEM [13].

Analysis of BAC clones telomeric of these breakpoints revealed 3 partially overlapping BAC clones spanning 523 kb (CSX-U), whereas analysis of the BAC end sequenced clones centromeric of the breakpoint region revealed one clone with an end-sequence located 36 kb downstream of exon 2 of the *CSX* gene and an intermediate clone with a gap of less than 1 kb (CSX-D*,* covering 330 kb) (Figure 2A). The CSX-U and CSX-D probes (DakoCytomation, Glostrup, Denmark) are separated by a 117 kb gap. Both *HOX11L2* and *CSX* FISH probe sets are available via DakoCytomation (Dr. T.S. Poulsen, Department of Probe Application, Produktionsvej 42, DK-2600, Glostrup, Denmark).

Table 1: Karyotypes, FISH and RT-PCR results of 32 T-cell lymphoblastic leukaemia (T-ALL) cases and 5 T-ALL cell lines

Case/ cell line	Age(y) /sex	Karyotype	*HOX11L2* FISH	*CSX* FISH	*IGH* FISH	*HOX11L2* RT-PCR	*TAL1* FISH[#]	*SIL-TAL1* RT-PCR
1	18/F	46,XX[32]	Split	N	der(5)	+	N	-
2	5/M	46,XY,ins(5)(q3?p1?4p1?2)[15]/46,XY[4]	Split	der(14)	Metaphases N	+	Nd	-
3	6/M	Diagnosis: 46,XY,del(11)(q2?1q2?3)[21]/46,idem,add(9)(q11)[4] Relapse: 46,XY,der(6)t(6;8)(q2?6;q24),del(8)(q2?4),del(9)(p21p21),der(9)del(9)(p21p21)add(9)(q11),del(11)(q2?1q2?3)[6]/46,idem,der(3;9)(p10;q10),der(6)t(3;6)(q2?9;p2?5),+del(9)[8]/46,XY[11]	Split Split	der(14) der(14)	der(5) der(5)	+	Nd	-
4	8/F	46,XX[61]	Split	der(14)	der(5)	+	N	-
5	14/M	46,XY,t(7;9)(p1?3;p2?2)[30]/46,XY[4]	Split	der(14)	der(5)	+	Nd	-
6	7/M	46,XY[74]	N	N	N	Nd	Nd	Nd
7	5/F	46,XX,t(5;16)(p10;q10)[8]/46,XX[15]	N	N	N	+	Nd	-
8	12/M	46,XY[22]	N	N	Nc	-	Nd	+
9	10/M	46,XY,del(9)(p13p23),t(11;14)(p12;q31)[14]/46,XY[10]	N	N	N	-	Nd	-
10	11/F	46,XX[20]	N	N	N	-	Nd	-
11	14/M	Diagnosis: 46,XY[26] Relapse: 46,XY[26]	N N	N N	N N	-	Nd	-
12	9/M	46,XY[22]	N	N	N	Nd	Nd	Nd
13	2/M	46,XY,+10[20]/46,XY[7]	N	N	N	-	Nd	-
14	12/M	Diagnosis: 46,XY,t(1;13;9)(q2?4;q1?4;p2?1),add(7)(q22),der(10)t(7;10)(q22;p12),del(11)(q23q25),t(14;16)(q3?2;q2?3)[21]/46,XY[10] Relapse after BMT: 45,XY,t(1;13;9)(q2?1;q13;p2?1),add(3)(q13),add(7)(q22),der(10)t(7;10)(q22;p12),der(11)add(11)(p1?3)del(11)(q23q25),t(14;16)(q3?2;q2?3),add(17)(p11),-18,+1~2mar[cp7]/45,idem,-X,+t(X;12)(p10;p10),-Y,t(1;8)(p10;p10),add(3)(p21),add(4)(q2?6),add(7)(q22),-der(9)t(1;13;9),+der(9)t(1;13;9)ins(9;?)(p2?1;?),-der(11),+add(11)(p15),add(11)(q14),-12,-der(13)t(1;13;9),+add(13)(q1?3),der(13)t(13;?;Y)(q13;?;q11),t(18;20)(p10;p10)[13]/46,XX(donor)[1]	Nd N	Nd N	Nd N	Nd Nd	Nd Nd	Nd -
15	12/M	47,XY,+mar1[9]/48,idem,+mar2[7]/46,XY[8]	N	N	N	-	Nd	-
16	2/M	46,XY[25]	N	N	N	-	Nd	-

17	20/M	45,XY,-7,der(9)t(7;9)(q11;p12~13)[14]/46,XY[11]	N	N	N	-	*SIL* deletion	+
18	16/F	46,XX,add(5)(q3),add(7)(q3?1),t(7;14)(p13;q32),del(11)(q13q23),del(12)(p1?3),add(15)(p1),del(18)(p11)[31]/46,XX[2]/ after FISH: 46,XX,der(1)t(1;22)p?32;?),(der(5)t(5;21),add(7)(q3?1),t(7;14)(p13;q32),del(11)(q13q23),del(12)(p1?2p1?3),der(15)t(12;15)(p1?3;p1),del(18)(p11),der(21)t(5;21)(?;p?)	N	Loss of 1 fused signal	der(7)	-	N	-
19	24/F	47-48,XX,?der(3),?der(11),del(12)(p1?1),+14,+21,+21,inc[cp11]/47-48,?der(3),?der(11),i(12)(q10),+21,+21,inc[cp8]/46,XX[1]	N	N	3 fused signals	-	N	-
20	10/F	45,XX,der(9)t(9;14)(p13;q1?2),del(12)(p12p13),-14,idic(17)(p11)[36]/46,XX[2]	N	N	N	-	*SIL* deletion	+
21	4/M	45,XY,del(9)(p13),der(13;14)(q10;q10)c[3]/45,idem,del(6)(q1?5q2?4)[13]/45,idem,del(6)(q?16q2?2)[10]/45,XY,der(13;14)(q10;q10)c[39]	N	N	der(13;14)c	-	Nd	-
22	33/M	44-46,Y,der(X)t(X;15)(p11;q1?)[16],del(5)(q23q34)[5],der(10)t(X;10)(p11;p11)[2],add(15)(q2?2)[6],del(17)(p11p13)[cp17]/46,XY[3]	N	N	N	-	Nd	Nd
23	71/F	46,XX,del(3)(q2?1q2?6)[9]/46,XX[12]	N	N	No metaphases	Nd	N	-
24	8/M	46,XY[32]	N	N	N	-	*SIL* deletion	+
25	8/F	46,XX,?add(9)(p1?)[6]/46,XX[30]	N	N	N	-	Nd	+
26	2/F	46,XX,t(6;11)(q27;q23)[10]/47,idem,+8[6]/46,XX[8]*	N	N	N	-	Nd	-
27	8/F	46,XX,add(2)(q1?),add(6)(q2?3),del(9)(q1?3~2?1q3?1),?del(11)(q?),der(21)t(2;21)(q11~12;q22)[10]/46,XX[14]	N	N	N	-	Nd	-
28	12/M	Diagnosis: Nd, Relapse: 46,XY[34]	N	N	N	-	Nd	-
29	4/F	46,XX[28]	N	N	N	-	Nd	-
30	55/M	45,XY,add(2)(p2?3),add(7)(q21),-9,-15,-16,del(17)(p11),+mar1,+mar2[17]/46,XY[6]	N	N	N	-	Nd	-
31	14/M	46,XY,der(1)t(?;1)(?;p3?3)t(?;6)(?;p11),add(6)(p11),der(?)t(?;1)(?;p3?3)[9]/46,XY[16]/ after FISH: 46,XY,der(1)t(1;6)(p3?3;p11)ins(1;14)(p33;q11q32),der(6)t(6;14)(p11;q32),der(14)t(1;14)(p3?3;q11)	N	N	Split	-	Nd	-
32	9/M	46,XY,add(1)(p31)[2],del(6)(q2q2)[3],inc[cp5]/46,XY[18]	N	N	N	-	Nd	-

Case/ cell line	Age(y) /sex	Karyotype	*HOX11L2* FISH	*CSX* FISH	*IGH* FISH	*HOX11L2* RT-PCR	*TAL1* FISH[#]	*SIL-TAL1* RT-PCR
HPB-ALL		Karyotype according to literature**: 46,XY,der(1)t(1;16)(q22;p11~12)add(16)(p13),del(2)(p24),del(3)(p11),der(5)t(1;5)(q22;q32~33),r(16)(?p12?q12)/45,idem,-del(3),-3/94,idemx2,+del(21)(q12)x2/ after FISH***: der(1)(1pter→q22::16q22→q22::14q32.2→qter),del(2)(p24),del(3)(p11),der(5)(5pter→q35::1q22→qter),der(14)(pter→q32.2::5q35→5q35::16p11.2→p13.1::16q23→qter),der(16)r(16)(p11~q11q22)del(16)(q11.2q12~21)	Split	N	der(1), der(1)	+	N	Nd
PEER		Karyotype according to literature**: 42-47,XX,ider(4)(q10)del(4)(q2?8q3?1),del(5)(q22q31),del(6)(q13q22),del(9)(p11p22),del(9)(q22)/ after FISH****: 46,XX,ider(4)(q10)del(4)(q2?8q3?1),der(5)del(5)(q22q31)t(5;14)(q35.1;q32.2),del(6)(q13q22),del(9)(p11p22),del(9)(q22),ins(14;5)(q32.2;q35.1q35.1)	N	CSX-U deletion	Nd	-	N	Nd
CCRF-CEM		Karyotype according to literature**: 88-101,XX,-X,-X,t(8;9)(p11;p24)x2,der(9)del(9)(p21~22)del(9)(q11q13~21)x2,+20,+20; sideline with +5,+21,add(13)(q3?3),del(16)(q12)/ after FISH****: 46,XX,t(8;9)(p11;p24),der(5)t(5;14)(q35.1;q32.2),der(9)del(9)(p24)del(9)(q11q13~21),ins(14;5)(q32.2;q35.1q35.1),+20	N	CSX-U deletion	Nd	-	*SIL* deletion	Nd
DU.528		Karyotype according to literature**: 46,XY,+del(1)(p33),+del(1)(q11),t(1;14)(p33;q11),del(13)(q14),-14	N	N	Nd	Nd	t(1;14)(p32;q11)	Nd
MOLT16		Karyotype according to literature**: 43-47,XX,t(3;11)(p21;p13),der(7)t(7;7)(qter→p15::q11.2→qter),t(8;14)(q24;q11),-9,dup(9)(pter→p13::p24→qter),-15,der(15)t(15;19)(qter→p1?1::q1?1→qter)	N	N	Nd	-	*SIL* deletion	Nd

Abbreviations and symbols used: M male; F female; age: age at diagnosis in years; N normal hybridisation pattern; Nd not done; + a band of the expected size was observed in RT-PCR; - no band was observed in RT-PCR; Nc: non conclusive result; [#] patients' *TAL1* FISH described in Van der Burg *et al.* [21]

* t(6;11)(q27;q23) with *MLL-AF6* fusion

** details given at www.dsmz.de/ and in Drexler [19]

*** recent karyotyping results described in MacLeod *et al.* [14]

**** recent karyotyping results described in Nagel *et al.* [13]

For the investigated cell lines, the split signal FISH assay for *TAL1* aberrations was modified from that published by van der Burg *et al.* [21], in that the downstream TAL1-D probe, a single PAC, was replaced by 3 BAC clones comprising 566 kb (DakoCytomation, Glostrup, Denmark). The patients' *TAL1* FISH analysis was performed as described by van der Burg *et al.* [21] and results were described in that publication. All clones were further verified by end sequencing, restriction endonuclease digestion and FISH. As probes for the *IGH* locus on 14q32, α [22] (kindly provided by dr. H. Döhner, Heidelberg, Germany) and IGH [23] (kindly provided by Dr. H. Riethman, Wistar Institute, Philadelphia) were used.

Fluorescent in situ hybridisation (FISH)

Growth of clones and DNA purification were performed according to the manufacturer's instructions using QIAGEN 500 tips (Qiagen, Hilden, Germany). Each probe set, designed to flank likely breakpoints, was labelled by nick translation with either Texas Red-dCTP (CSX-D, HOX11L2-U, SIL-U) or FITC-dCTP (CSX-U, HOX11L2-D, TAL1-D) and either biotin-16-dUTP (α) or digoxigenin-11-dUTP (IGH). For FISH, freshly prepared metaphase spreads from –20°C stored methanol/acetic acid cell suspensions were used.

HOX11L2, *CSX* or *TAL1* probe mixtures consisted of 100 ng of each probe in 10 µl hybridisation buffer (45% formamide, 300 mM NaCl, 5 mM phosphate, 10% dextransulphate, blocking PNA). Repetitive sequences were suppressed using blocking PNA (DakoCytomation, Glostrup, Denmark). Chromosomes and probes were denatured simultaneously for 5 minutes at 80°C, and hybridised overnight at 45°C in a moist chamber. The slides were stringently washed in 0.2x SSC/0.1% Triton X-100 at 65°C for 10 minutes, passed through a wash buffer (TBS) for 1 minute, dehydrated through an ethanol series (70%, 85%, and 96%) and mounted with antifade containing 4'6-Diamino-2-Phenyl Indol (DAPI) as counterstain.

For *IGH* split signal FISH, slides were pre-treated with RNAse and pepsin, and post-fixed with formaldehyde. Hybridisation of 100 ng of each probe was performed overnight at 37°C in a moist chamber, and the slides were washed with 0.4xSSC at 72°C. Biotinylated probes were detected using FITC-labelled avidin, followed by biotinylated goat-anti-avidin and avidin-FITC incubation. Digoxigenin-labelled probes were detected using sheep-anti-digoxigenin-Rhodamine, followed by donkey-anti-sheep-Texas Red incubation. Slides were counterstained with DAPI. For each sample a minimum of 100 interphase cells were scored, as well as 5-10 metaphases if present. Images were captured using a Zeiss epifluorescence microscope using MacProbe software (version 4.3, Applied Imaging, Newcastle upon Tyne, UK).

RT-PCR

The presence of *SIL-TAL1* fusion transcripts was determined by RT-PCR as described elsewhere [24]. *HOX11L2* RT-PCR was performed using primers F-HOX11L2-EMC (5'-GGTTCCAAAACCGGAGGAC-3') and R-HOX11L2-EMC (5'-TGCAGACAGAGCGGGTCAG-3'), resulting in a 154 bp PCR product. The reaction mixture, consisting of 1x Gold buffer, 1 U TaqGold, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 10 pmol of each primer, was run on the ABI 9600 PCR machine (Applied Biosystems, Foster City, CA, USA) according to the following program: 7 minutes at 95°C, followed by 35 cycles consisting of 30 seconds at 94°C, 45 seconds at 60°C and 90 seconds at 72°C, with a final extension at 72°C for 10 minutes.

Results

Verification and validation of *HOX11L2, CSX,* and *TAL1* probes

Verification of the clones using five different enzymes separately showed that the restriction endonuclease band patterns for all clones were identical to those predicted *in silico*. For *HOX11L2* and *CSX,* the BAC end-sequences of the clones showed >98% identity compared to the already published sequences at TIGR. Using the dual colour split signal probes, FISH for *HOX11L2, CSX* and *TAL1* was subsequently tested on five T-ALL cell lines PEER, DU.528, CCRF-CEM, MOLT16, and HPB-ALL (Table 1). As expected, only HPB-ALL was positive for the *HOX11L2* translocation [14], whereas the remaining cell lines were negative. PEER and CCRF-CEM (Figure 2D, 2E) showed an abnormal hybridisation pattern using the *CSX* probe set [13]. These cell lines, both having an unbalanced der(5)t(5;14)(q35;q32) and a concomitant ins(14;5)(q32.2;q35.1q35.1) [13], showed a deletion of CSX-U. The remaining three cell lines were negative. DU.528 showed a t(1;14)(p32;q11) [19], while MOLT-16 and CCRF-CEM showed deletion of sequences between *SIL* and *TAL1* [19]. PEER and HPB-ALL showed no *TAL1* rearrangements by FISH.

To validate the new diagnostic probe sets further, the cut-off values were determined for each set. For this purpose, those cases without the translocation involving the gene studied were selected (n=16 for *HOX11L2*; n=36 for *CSX*). The cut-off values for each probe set were defined as the mean plus three times the standard deviation. This, for the split-signal pattern, resulted in cut-off values of 3% for *HOX11L2* and 2% for *CSX*. These values are far lower than all percentages observed in all positive cases. For loss of a complete fused signal, the cut-off values were 4% for both probe sets. We did not observe deletion of either the green (HOX11L2-D or CSX-U) or the red (HOX11L2-U or CSX-D) signal in any of the cases without the t(5;14)(q35;q32).

Since these results showed that these new diagnostic probe sets for *HOX11L2* and *CSX* hybridise to the expected chromosomal localization (Figures 1B and 2B) and demonstrated a split signal in both interphases and metaphases in case of a translocation (data not shown; Figures 2D and 2E) in the cell lines, the probes were used to analyse 32, mostly childhood, T-ALL patients.

Cytogenetics and t(5;14) FISH results of 32 T-ALL cases

The patients' karyotypes and FISH results for the different t(5;14) split signal assays are shown in Table 1. Using conventional cytogenetics, 11/32 cases were normal while the remaining 21 cases showed abnormal karyotypes, 9 of which were complex (>3 structural aberrations). All cases were tested for the presence of the t(5;14) using the newly developed probe combinations. Using the *HOX11L2* probe combination, both metaphase and interphase nuclei from 5 out of 32 cases (cases 1-5) showed one colocalised HOX11L2-D and HOX11L2-U signal, as well as a separate green (HOX11L2-D) and a separate red signal (HOX11L2-U), indicating a break in this region (Figures 1D, 1E). In the metaphases, the fused signal was located on the normal chromosome 5; the HOX11L2-U signal was visible on the der(5), whereas the HOX11L2-D signal was translocated to the der(14) (Figure 1D).

When investigating all cases with the split signal probe combination for *CSX*, no translocations involving *CSX* were found. In metaphases of cases 2, 3, 4 and 5 (positive for split *HOX11L2* signals) one colocalised CSX-D and CSX-U signal was observed on the normal chromosome 5, whereas the other fused signal was translocated to the der(14). These results are consistent with the fact that *HOX11L2* is located centromeric to *CSX*. In interphase nuclei this change could of course not be observed, since the *CSX* signals do not split due to the centromeric breakpoint in *HOX11L2*. In case 1, no metaphases were present for confirmation of translocation of the fused *CSX* signal to the der(14). In case 18, we observed loss of one fused *CSX* signal, as can probably be explained by the presence of an unbalanced t(5;21) observed in the karyotype after FISH and SKY.

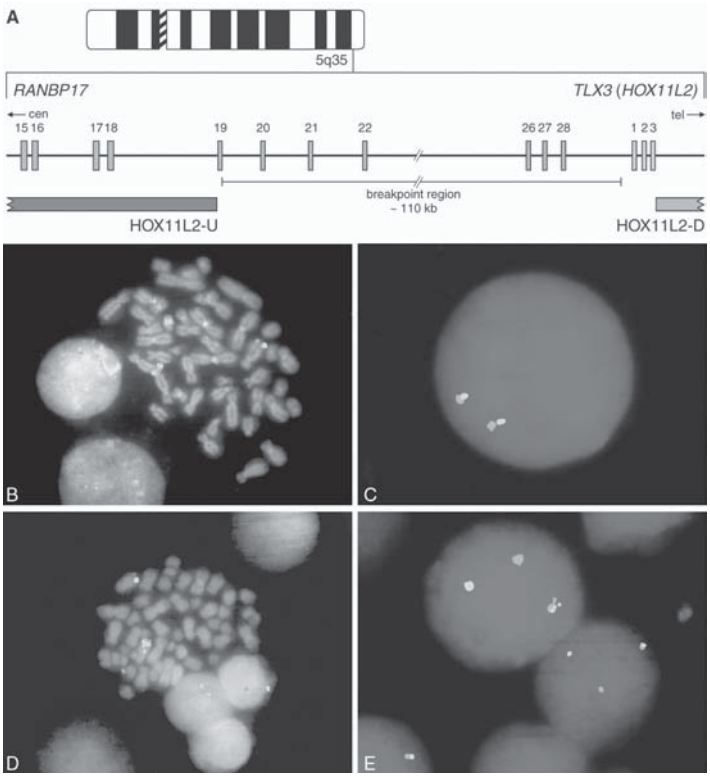

Figure 1: Split signal FISH using *HOX11L2* probes A) Schematic representation of (part of) the *RANBP17* and *HOX11L2* genes on chromosome region 5q35. The *HOX11L2* translocation breakpoint region is indicated with the thin bar. The HOX11L2-U FISH probe consisted of one and the HOX11L2-D probe of three BAC clones. B) Metaphase spread of a healthy donor, depicted in inverted DAPI staining showing the colocalised signals on chromosomes 5. C) Interphase nucleus of a healthy donor, showing two fused signals of the normal chromosomes 5. D) Metaphase spread of t(5;14) (case 5), depicted in inverted DAPI staining showing one fused signal on the normal chromosome 5 as well as a separate green (HOX11L2-D) signal on the der(14) and red (HOX11L2-U) signal on the der(5). E) Interphase nucleus depicting t(5;14) (case 5), showing one fused signal (normal chromosome 5), as well as separate green (HOX11L2-D) and red (HOX11L2-U) signals.

Next, we also analysed the 14q32 breakpoint using probes for the *IGH* gene (Table 1). In 1 of 32 cases (case 31), an abnormal hybridisation pattern was found in interphase nuclei. This case showed one fused signal as well as one separate green (α) and red (IGH) signal, indicating a break in the *IGH* gene. Additionally, in 4 of the 5 cases showing the split signal for *HOX11L2* (cases 1, 3, 4 and 5), one fused α and IGH probe signal was visible on the der(5), indicating that the 14q breakpoint in these t(5;14) cases is indeed centromeric to *IGH*. Case 18 showed two fused signals, one on chromosome 14 and one on a der(7)t(7;14), whereas case 21 showed fused signals, one on chromosome 14 and one on the Robertsonian der(13;14)(q10;q10). Finally, case 19 showed 3 colocalised signals on 3 copies of chromosome 14. For case 2, which showed the separate HOX11L2-D and HOX11L2-U signals and the *CSX* fused signal on a der(14), the metaphases found in the slide hybridised for *IGH* showed fused signals on chromosomes 14. Probably, the percentage of abnormal metaphases in this slide was too low to detect a metaphase with the t(5;14), although we cannot fully exclude the possibility that the 14q32 breakpoint is telomeric to the *IGH* gene.

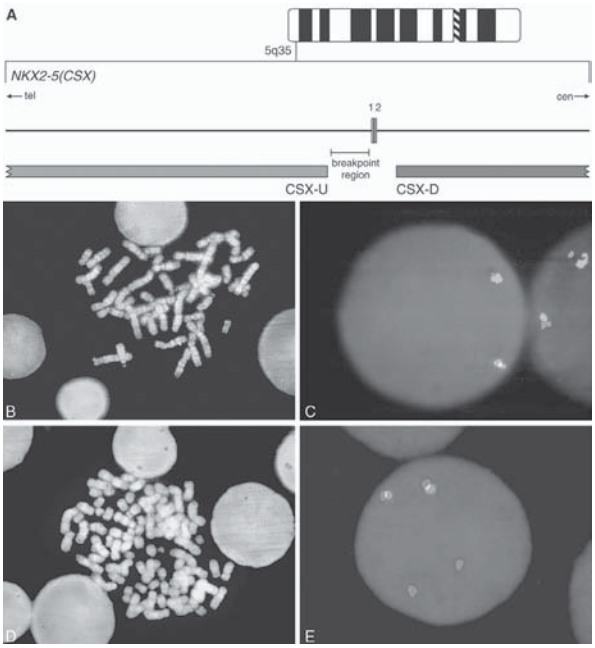

Figure 2: Split signal FISH using *CSX* probes A) Schematic representation of the *CSX* gene on chromosome region 5q35. The *CSX* translocation breakpoint region is indicated with the thin bar. The CSX-D FISH probe consisted of two and the CSX-U probe of three BAC clones. B) Metaphase spread of a healthy donor, depicted in inverted DAPI staining showing the fused signals on chromosomes 5. C) Interphase nucleus of a healthy donor, showing two fused signals of the normal chromosomes. D) Metaphase spread of cell line CCRF-CEM, depicted in inverted DAPI staining showing the unbalanced t(5;14). E) Interphase nucleus of cell line CCRF-CEM, showing the unbalanced t(5;14).

HOX11L2 and *SIL-TAL1* expression

From 28/32 cases sufficient material was available to investigate *HOX11L2* expression. Six cases, among which all 5 t(5;14)+ cases (cases 1-5, Table 1), showed a transcript using RT-PCR, the other 22 cases were negative. The sixth PCR positive case was case 7, in which no translocation was detected with the split signal *HOX11L2* and *CSX* probe sets.

Ferrando *et al.* [16] showed that expression of *TAL1* and *HOX11* (to which *HOX11L2* is closely related) are mutually exclusive within leukaemic samples. Furthermore, analysis of expression in the here used human T-ALL cell lines indicated that the presence of a *SIL-TAL1* fusion might be a negative predictor for the presence of the t(5;14)(q35;q32) involving *HOX11L2*. In cell line CCRF-CEM both a *SIL-TAL1* fusion and a t(5;14)(q35;q32) involving *CSX* did occur. If sufficient material was available, *SIL-TAL1* fusions had been searched for at diagnosis, using RT-PCR on a routine basis, and/or FISH. cDNA of 29 cases was available for *SIL-TAL1* RT-PCR (Table 1); 5 cases (8, 17, 20, 24 and 25) showed a fusion between *SIL* and *TAL1*, 3 of which (17, 20 and 24) also showed an abnormal FISH hybridisation pattern using the *TAL1* probe set (Table 1). Of the other 2 cases (case 8 and 25) no methanol/acetic acid fixed cell suspension was available for additional *TAL1* FISH analysis. All 5 cases were negative for the t(5;14) involving *HOX11L2*, indicating that *SIL-TAL1* fusions and the t(5;14) involving *HOX11L2* are mutually exclusive. Unfortunately, as no patients with *CSX* involvement were identified, we could not draw definite conclusions about the coexistence of *CSX* and *TAL1* aberrations.

Discussion

Here we describe two new dual colour split signal FISH assays for the detection of t(5;14)(q35;q32) involving *HOX11L2* or *CSX*, or other translocations involving these genes, e.g. t(5;14)(q35;q11). These t(5;14)(q35;q32) are cryptic translocations and thus may escape detection using routine diagnostic methods even when augmented by chromosome painting. Single locus FISH is a helpful technique to identify juxtapositional rearrangements and gene fusions with variable or widely displaced breakpoints or partner genes, which may be laborious to detect using molecular methods such as RT-PCR. The wide dispersal of 14q32 breakpoints in the far downstream region of *BCL11B* together with its inconsistent expression effectively rule out PCR-based methods for detecting t(5;14).

The split signal FISH assays we developed for *HOX11L2* and *CSX* should fill a niche in identifying these translocations in new patients. The assays were first validated on T-ALL cell lines known to have a t(5;14)(q35;q32). The cut-off values calculated for the 2 probe sets were 3% for *HOX11L2* and 2% for *CSX*. Using these newly developed diagnostic sets, 32 T-ALL cases were analysed for the presence of both types of t(5;14) involving either *HOX11L2* or *CSX*. To study the breakpoint on chromosome 14q32, a split signal FISH assay for *IGH* was used as well. We identified 5 cases with a t(5;14) involving *HOX11L2*, whose conventional karyotypes did not show this translocation. In 3 of our 5 cases, (complex) chromosomal aberrations were observed, indicating that the t(5;14) does occur when other abnormalities are present. For the t(5;14) involving *HOX11L2*, we observed an incidence of 15% in our total series and 19% (5/27) in childhood and adolescent T-ALL. These percentages resemble

those published by Bernard *et al.* (16.7 and 22% respectively) [10]. Direct comparison with data of Berger *et al.* [25] and of Cavé *et al.* [18] is impossible as they pooled their t(5;14) and *HOX11L2* expressing cases. However, unlike Berger *et al.* [25], we did not observe the t(5;14) in our 3 adult patients investigated. In our investigated group, no cases were identified with a t(5;14)(q35;q32) involving *CSX*, indicating that the incidence of this alternative 5q35 translocation is probably low. However, at this point we cannot exclude the possibility that this variant is cultural in origin. The 14q breakpoint was found centromeric to the *IGH* locus in our patient group; in 4 of 5 t(5;14) cases, the fused α and IGH probe signal translocated to the derivative chromosome 5. These results confirm the findings of Bernard *et al.* [10] and Hélias *et al.* [11]. Therefore, studying the region downstream of *BCL11B* in our t(5;14) positive cases in more detail could help delineating the 14q32 breakpoint more precisely. Activation of *HOX11L2* expression through these different 14q loci could contribute to leukaemic transformation of normal bone marrow cells, e.g. through regulatory elements of *BCL11B* – a gene which has recently been shown to control thymocyte development and survival [26]; another possibility however, is that disruption of negative regulatory regions at 5q35 (*HOX11L2* is not expressed during T-cell development) mediates this effect. The hypothesis, which best explains the extraordinary recurrence of t(5;14)(q35;q32) in T-ALL posits ectopic activation of *HOX11L2* following juxtaposition with regulatory elements present in the far downstream region of *BCL11B*. We did not observe the t(5;14)(q35;q11) in our series. However, the here used probe set would detect this translocation as well, as the 5q breakpoint described for these cases is situated within the same region as for the t(5;14)(q35;q32) involving *HOX11L2* [12].

It has been shown that cases with a t(5;14)(q35;q32) involving *HOX11L2* exhibit *HOX11L2* expression [10, 14] whereas precursor B-ALL, adult T-ALL and 40% of childhood T-ALL cases mostly do not [15]. However, more recently t(5;14)(q35;q32) and/or *HOX11L2* expression was observed in 13% of adult T-ALL cases by Berger *et al.* [25]. Our 5 cases having the t(5;14) involving *HOX11L2* did show *HOX11L2* expression. Additionally, we observed *HOX11L2* expression in one case without the t(5;14) by FISH, showing that the presence of *HOX11L2* expression does not always require the presence of a t(5;14)(q35;q32). Mauvieux *et al.* [15] also reported this observation for one of their cases. Therefore FISH is needed to make an accurate diagnosis of t(5;14)(q35;q32). In first instance, an unexplained correlation of *HOX11L2* expression with male sex was observed by Mauvieux *et al.* [15]. They observed *HOX11L2* expression only in male t(5;14)(q35;q32) cases, whereas only 1 female case with *HOX11L2* expression was observed by others [10] until recently. In our series, we identified 5 new t(5;14) cases, including 2 female cases, both showing *HOX11L2* expression. Berger *et al.* [25] identified 67 t(5;14)/*HOX11L2* positive cases (defined as cases demonstrating either a 5q35 breakpoint involving *HOX11L2* (47 cases) by FISH or *HOX11L2* expression (55 cases) by RT-PCR). They did not observe any male preference in their series. However, direct comparison with their data is difficult as they pooled the translocated and *HOX11L2* expressing patients. This also holds true for the 35 t(5;14)/*HOX11L2* positive cases, among which were 7 females, that were described by Cavé *et al.* [18].

Expression of *TAL1* and *HOX11* (to which *HOX11L2* is closely related) has been shown to be mutually exclusive within leukaemic samples and expression of these genes may be used to

stratify different prognostic subgroups [16]. Furthermore, analysis of human T-ALL cell lines indicated that the presence of *TAL1* aberrations might be a negative predictor for the presence of the t(5;14)(q35;q32) involving *HOX11L2*. In our series, no cases having the t(5;14), involving *HOX11L2*, showed *TAL1* abnormalities, whereas 5 other cases were positive for the *SIL-TAL1* fusion product. These results suggest that *TAL1* expression and *HOX11L2* expression, and therefore also *TAL1* expression and the t(5;14)(q35;q32) are very unlikely to coexist in one patient. This is in line with similar findings in the very recent studies by Berger *et al.* [25] and Cavé *et al.*[18].

In conclusion, we describe two robust new dual colour split signal FISH assays for the detection of t(5;14)(q35;q32) involving *HOX11L2* or *CSX*, or variant translocations involving these genes for use in both interphase and metaphase cytogenetics. Using these assays, we did not identify patients with a t(5;14)(q35;q32) involving *CSX*, indicating that the incidence of this alternative 5q35 translocation is probably low. However, we did identify 5 new cases with a t(5;14) involving *HOX11L2* with the 14q32 breakpoint centromeric of the *IGH* region. All five of our positive cases showed *HOX11L2* expression, as well as 1 case without the t(5;14)(q35;q32), showing that *HOX11L2* expression may occur by non-cytogenetic means. Additionally, our results suggest that *TAL1* aberrations and the t(5;14)(q35;q32) translocation involving *HOX11L2* are highly unlikely to coexist in one patient, thus confirming the idea that these aberrations reflect oncogenetically different T-ALL subgroups.

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Chapter 5

Identification of *NUP98* abnormalities in acute leukaemia: *NCL* (2q37) as a potential new partner gene

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Submitted

Chapter 5

Identification of *NUP98* abnormalities in acute leukaemia: *NCL* (2q37) as a potential new partner gene

Abstract

Chromosome rearrangements are found in many cases with acute leukaemia. As a result of chromosome aberrations, genes located at the breakpoints can be disrupted and fusion genes can be formed. One of the genes involved in several chromosome aberrations in haematological malignancies is *NUP98* (11p15). As *NUP98* is located close to the 11p telomere, small translocations might easily be missed. Using a *NUP98* specific split signal fluorescence in situ hybridisation (FISH) probe combination, we analysed 84 cases with acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL) or myelodysplastic syndrome (MDS) with either normal karyotypes, or 11p abnormalities to investigate whether rearrangements exist that have not been identified yet. Neither *NUP98* translocations nor deletions were identified in patients with normal karyotypes, indicating that these aberrations may be very rare in this group. However, *NUP98* deletions were observed in 4 patients with unbalanced 11p aberrations, indicating that the breakpoint is centromeric of *NUP98*.

In two patients, both showing 11p abnormalities in the diagnostic karyotype, rearrangements of *NUP98* were identified: in a 60-year old woman with AML-M0 a t(4;11)(q1?3;p15) with expression of the *NUP98-RAP1GDS1* fusion product was detected. In a 1-year old boy with AML-M7 an add(11)(p15) with a der(21)t(11;21)(p15;p13) was observed cytogenetically. *NCL* was identified as the fusion partner of *NUP98* using 3' RACE. *NCL*, located on 2q37, codes for nucleolin, a nucleolar phosphoprotein with numerous functions, amongst which ribosome biogenesis. It is the first time *NCL* was found as a partner gene in leukaemia.

Introduction

Chromosome rearrangements are found frequently in patients with acute myeloid leukaemia (AML) or myelodysplastic syndrome (MDS), and to a lesser extent in lymphoblastic leukaemia (ALL). Some of these rearrangements are particularly associated with a specific type of leukaemia according to the French-American-British (FAB) and the World Health Organisation (WHO) classification. The presence of certain chromosome aberrations might constitute an important prognostic factor for the outcome of disease and has therapeutic consequences. For example, t(8;21)(q22;q22), t(15;17)(q22;q21) or inv(16)(p13q22) in AML are associated

with good prognosis, and are currently used for clinical stratification of therapy, whereas t(9;22)(q34;q11) or t(4;11)(q21;q23) in ALL are associated with poor outcome [1]. As a result of the chromosome aberration, genes located at the breakpoints can be disrupted and if the two genes subsequently fuse in frame on the derivative chromosome(s), fusion genes might be formed. Consequently, normal function of these genes can be disturbed, which can act as a contributing factor for leukaemogenesis [2, 3].
One of the genes involved in several different chromosome aberrations in haematological malignancies, is *NUP98,* located on 11p15. NUP98 is part of a nuclear pore complex, and it is involved in RNA export from the nucleus [4-6]. To date 16 different translocations and one inversion involving this gene have been described, and in all cloned cases fusion genes with different partner genes were formed [7-26]. Most *NUP98* translocations have been reported in AML, MDS and therapy-related AML or MDS (t-AML/t-MDS) [7-11, 13-18, 21-26], but two translocations occurred in T-ALL [12, 19, 20]. The most frequently occurring rearrangement involving *NUP98* is the t(7;11)(p15;p15), fusing *NUP98* to *HOXA9, HOXA11* or *HOXA13* [27]. As *NUP98* is located very close to the end of the short arm of chromosome 11, small translocations can be easily missed. To search for novel rearrangements, we analysed 84 leukaemia cases (AML, MDS, ALL) with 11p aberrations or normal karyotypes using *NUP98*-specific fluorescence in situ hybridisation (FISH) probes.

Materials and methods

Patients

Eighty-four patients with different haematological malignancies suspected of acute leukaemia, cytogenetically investigated at diagnosis between 1996 and 2004, were included in this study. Thirty-four leukaemia cases were included because 11p aberrations were observed (Table 1), and the other 50 cases with normal karyotypes or non-specific aberrations were selected for leukaemia phenotypes (AML, MDS, t-AML, t-MDS) often observed to have *NUP98* rearrangements (Table 2). At diagnosis, informed consent of the patients and/or parents/guardians was obtained after approval of the Medical Ethical Committee of the Erasmus MC, Rotterdam.

Conventional cytogenetic analysis

Bone marrow or blood of the investigated cases was obtained at diagnosis, cultured and harvested according to standard cytogenetic protocols. For each case 20-32 metaphase cells were analysed using both QFQ- and RBA-banding. The chromosome aberrations observed were described according to the International System for Human Cytogenetic Nomenclature (ISCN 1995) [28]. Remaining methanol/acetic acid (3:1) fixed cell suspensions were stored at -20°C. Remaining blood or bone marrow was viably frozen in 0.1 volume dimethylsulfoxide (DMSO)/0.9 volume foetal calf serum (FCS) and stored in liquid nitrogen until further use.

Table 1: Thirty-four leukaemia cases included for 11p aberrations

	BM[a]/HA[b]	*Sex*	*Age (years)*	*Leukaemia subtype*	*11p aberrations in karyotype **	*NUP98 FISH results*
1	BM	F[c]	70	AML[e]	der(11)(?)	Normal
2	BM	F	52	AML	**t(8;11;21)(q22;p14~15;q22)**	#11, der(21) both fused
3	BM	M[d]	71	AML	**add(11)(p1?5)**	#11, add(11) or #11, add(11), both fused
4	BM	M	77	AML	**der(11)?dup(11)(q23q24)t(11;11)(p15;q23)**	Normal
5	BM	M	2	AML	**t(4;11)(p12;p15)**	Normal
6	BM	F	60	AML-M0	**t(4;11)(q1?3;p15)**	**Split**, der(11), der(4)
7	BM	F	33	AML-M3	t(6;11)(p12;p14)	Normal
8	BM	F	21	AML-M3	**inv(11)(p15q13)**	Normal
9	BM	M	14	AML-M4	**del(11)(p1?2p15)**	Normal
10	BM	F	62	AML-M4	add(11)(p1?1)	Normal
11	BM	M	66	AML-M5A	trp(11)(pter->q25::q25->q14::q11->qter)	Normal
12	BM	M	1	AML-M6	add(11)(p14)	Normal
13	BM	M	1	AML-M7	**add(11)(p15),der(21)t(11;21)(p15;p13)**	**Split**, add(11), der(21)
14	HA	M	73	AML-M7	+add(11)(p1?4)	1 fused signal
15	BM	M	35	CML[f]	**add(11)(p1?2~p15)**	Normal
16	HA	M	53	CML BC[g]	?der(11)(p)	Normal
17	BM	F	58	MDS[h]	**del(11)(p12p15)**	Normal
18	BM	F	68	MDS	add(11)(p1?4)	Normal
19	BM	M	70	MDS	add(11)(p1?)	Normal
20	BM	F	54	MDS	**dic(11;18)(p15;p11)**	Normal
21	BM	M	66	PCP[i]	-11x2, +der(?)t(?;11)(?;q1?0)x2	1 fused signal
22	BM	M	33	RA[j]	?der(11)(p)	Normal
23	BM	M	61	RAEB[k]	add(11)(p14)	Normal
24	BM	F	74	RAEB	**add(11)(p1?5)**	Normal
25	BM	M	66	RAEB	**der(11)t(11;12)(p15;q1?3)**	#11, der(12), both fused
26	BM	M	66	RAEB-T[l]	**dup(11)(p13p15)**	1 fused signal
27	BM	M	69	RAEB-T	**der(11)t(11;22)(p1?5;q11)**	Normal
28	BM	M	20	ALL[m]	**t(3;11)(q22;p15)**	Normal
29	BM	F	16	Ph+ ALL	add(11)(p1?4)	Normal
30	BM	M	7	PreB-ALL	der(11)inv(11)(p?q),+der(11)?inv(11)	3 fused signals
31	BM	M	57	PreB-ALL	**t(11;19)(p15;?)**	Normal
32	BM	F	2	PreB-ALL	?der(11)(p)	Normal
33	BM	F	53	T-ALL	t(11;12)(?;?), t(11;17)(?;p12)	Normal
34	BM	M	13	T-ALL	**add(11)(p15)**	1 fused signal

Only 11p part of karyotype shown, with aberrations of 11p15 in bold; Abbreviations used: [a] bone marrow; [b] blood; [c] female; [d] male; [e] acute myeloid leukaemia; [f] chronic myeloid leukaemia; [g] blast crisis; [h] myelodysplastic syndrome; [i] pancytopenia; [j] refractory anaemia; [k] refractory anaemia with excess of blasts; [l] refractory anaemia with excess of blasts in transformation; [m] acute lymphoblastic leukaemia

Table 2: Fifty leukaemia cases included for leukaemia subtypes often observed with *NUP98* rearrangements

	BM[a]/HA[b]	*Sex*	*Age (years)*	*Leukaemia subtype*	*Karyotype*	*NUP98 FISH results*
35	BM	F[c]	67	AML[e]	Normal	Normal
36	BM	F	68	AML	Normal	Normal
37	HA	F	82	AML	Normal	Normal
38	BM	F	52	AML	Normal	Normal
39	BM	F	52	AML	Normal	Normal
40	BM	M[d]	64	AML	+11 [2/21]	Normal
41	BM	M	64	AML from MDS[f]	Complex	Normal
42	BM	F	70	AML-M1	Complex	Normal
43	BM	M	64	AML-M1	del(5)(q?21q3?3), t(X;17)(q2?7;p11)	Normal
44	BM	F	59	AML-M1	Normal	Normal
45	BM	F	59	AML-M1	Normal	Normal
46	BM	F	55	AML-M1	Normal	Normal
47	BM	F	57	AML-M1	Normal	Normal
48	BM	F	73	AML-M1 from MDS	Normal	Normal
49	BM	M	60	AML-M2	inv(7)(q22q32)	Normal
50	BM	F	39	AML-M2	Normal	Normal
51	BM	M	55	AML-M2	Normal	Normal
52	BM	F	29	AML-M2	Normal	Normal
53	BM	M	43	AML-M2	Normal	Normal
54	BM	M	55	AML-M2	Normal	Normal
55	BM	F	70	AML-M2	Normal	Normal
56	BM	F	46	AML-M4	Normal	Normal
57	BM	F	60	AML-M4	Normal	Normal
58	BM	M	52	t-AML-M4	Normal	Normal
59	BM	M	51	AML-M5	Normal	Normal
60	BM	F	78	Anaemia	Normal	Normal
61	BM	F	32	Anaemia	Normal	Normal
62	BM	F	31	LC[g]/TC[h]	Normal	Normal
63	BM	M	66	MDS	Normal	Normal
64	BM	M	49	MDS	Normal	Normal
65	BM	M	74	MDS	-Y	Normal
66	BM	M	72	MDS	Normal	Normal
67	BM	F	64	MDS	Normal	Normal
68	BM	M	69	MDS	Normal	Normal
69	BM	F	43	MDS	Normal	Normal
70	BM	F	48	MDS	Normal	Normal
71	BM	F	65	MDS	Normal	Normal
72	BM	F	58	MDS	Normal	Normal
73	BM	F	63	MDS	Normal	Normal
74	BM	M	60	MPS[i]	Normal	Normal
75	BM	M	58	PCP[j]	Normal	Normal
76	BM	M	78	RA[k]	-Y	Normal
77	BM	M	39	RAEB[l]	Normal	Normal
78	BM	M	62	RAEB	Normal	Normal
79	BM	F	75	RAEB	Normal	Normal
80	BM	F	68	RAEB	Normal	Normal
81	BM	M	59	t-RAEB-T[m]	-5,-7,del(12)(p12p13)	Normal
82	BM	F	42	RARS[n]	Normal	Normal
83	BM	M	50	TC	Normal	Normal
84	BM	M	72	TCP[o]	?der(20)(p)	Normal

Abbreviations used: [a] bone marrow; [b] blood; [c] female; [d] male; [e] acute myeloid leukaemia; [f] myelodysplastic syndrome; [g] leucocytosis; [h] thrombocytosis; [i] myeloproliferative syndrome; [j] pancytopenia; [k] refractory anaemia; [l] refractory anaemia with excess of blasts; [m] refractory anaemia with excess of blasts in transformation; [n] refractory anaemia with ringed sideroblasts; [o] thrombocytopenia

FISH

For FISH analysis, freshly prepared metaphase/interphase preparations from -20°C stored methanol/acetic acid fixed cell suspensions were used. For identification of *NUP98* rearrangements BAC clones RP11-120E20 (184 kb) and RP11-348A20 (165 kb) (BAC/PAC Resource Centre, Children's Hospital, Oakland, CA, USA), both covering *NUP98*, were used. RP11-120E20 is located more telomeric compared to RP11-348A20, whereas the two probes have an overlap of 44 kb (Figure 1A). DNA purification of clones was performed using the QIAGEN plasmid maxi kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Probes were labelled by nick translation with either biotin-16-dUTP (RP11-120E20) or digoxigenin-11-dUTP (RP11-348A20). Since both clones are adjacent and have a small overlap, two fused signals are expected in a *NUP98* non-rearranged case. When a break occurs in *NUP98*, one of the fused signals is expected to split and separate green and red signals will be observed. Hybridisation of 100-150 ng of each probe to a sample and detection was performed as described elsewhere [29]. For each sample a minimum of 200 interphase cells were scored, as well as 5-10 metaphases if present.
The cases without *NUP98* abnormalities were used to calculate the cut-off value for this probe combination for use in interphase nuclei. The cut-off value was defined as the mean plus three times the standard deviation. The cut-off value for the split signal pattern was 2 %, whereas the cut-off value for the presence of 3 fused signals (gain of *NUP98*) was 3.5% and for the presence of 1 fused signal (loss of *NUP98*) was 7.7%.
Additionally, whole chromosome paints (WCPs) for chromosomes 11 and 21 and locus-specific FISH probes for 11pter (220a2) [30], *WT1* (WT1, 11p13; kindly provided by N. Groot, Department of Clinical Genetics, AMC, The Netherlands), *MLL* (MLL-D, 11q23) [31], 11qter (cos2072c1) [30], *AML1* (C0664, 21q22) [32] and a ribosomal satellite probe (R521, kindly provided by B. Bakker, Department of Clinical Genetics, LUMC, The Netherlands) were used for further detailed investigations of case 13 and 26.
Possible *NUP98* deletions were investigated using probes RP11-21N2 (telomeric of *LMO1;* approximately 4.3 Mb centromeric of *NUP98*), RP11-141F21 (*LMO1),* RP11-79E12 (approximately 7 Mb centromeric of *NUP98*), RP11-88C11 (*LMO2*, 11p13; approximately 30 Mb centromeric of *NUP98*), RP11-489C13 *(CRNA10),* RP11-113A5 (approximately 1.5 Mb telomeric of *NUP98*) and RP11-371C18 (*BRSK2*; 11p subtelomeric, approximately 2.3 Mb telomeric of *NUP98*). Images were captured using an epifluorescence microscope (Axioplan 2, Zeiss, Sliedrecht, The Netherlands) using MacProbe software (version 4.3, Applied Imaging, Newcastle upon Tyne, UK).

RT-PCR of *NUP98-RAP1GDS1* fusion transcript

Detection of the *NUP98-RAP1GDS1* fusion transcripts using RT-PCR was performed as described previously [19], with a small modification of the *NUP98* forward primer (NUP98-1252F). Primers used are shown in table 3. The nucleotide sequence of the *NUP98-RAP1GDS1* fusion transcripts was determined using an automated ABI377 sequencing station (Applied Biosystems, Foster City, CA, USA).

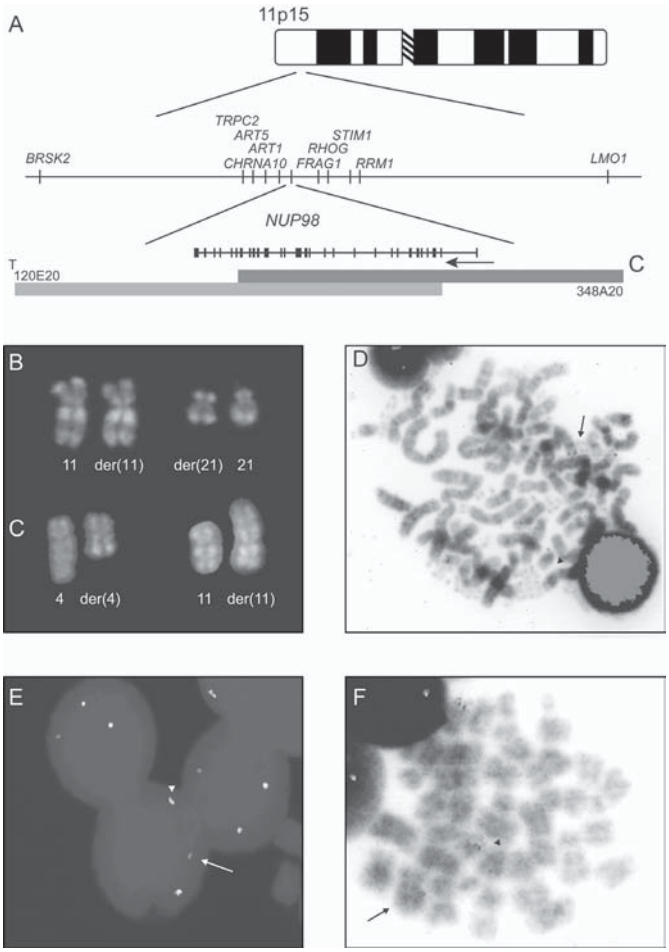

Figure 1: FISH probe localisation and results A) Schematic overview (not drawn to scale) of location of clones RP11-120E20 and RP11-348A20 relative to *NUP98*. T: telomeric side; C: centromeric side. The direction of transcription is indicated with an arrow. B) Case 13: R-banded chromosomes 11, 21 and derivative chromosomes. C) Case 6: R-banded chromosomes 4, 11 and derivative chromosomes. D) Case 13: Metaphase spread of FISH using RP11-120E20 (green) and RP11-348A20 (red), depicted in inverted DAPI staining, showing one fused signal on the normal chromosome 11 as well as separate green on the der(21) (arrowhead) and red signal on the add(11) (arrow). E) Case 6: Interphase nuclei showing one fused signal and a separated green (arrowhead) and red signal (arrow). F) Case 6: Metaphase spread of FISH using RP11-120E20 (green) and RP11-348A20 (red), depicted in inverse DAPI staining, showing one fused signal on the normal chromosome 11 as well as separate green on the der(4) (arrowhead) and red signal on the der(11) (arrow).

3' RACE-PCR and cloning of *NUP98* breakpoint in case 13

One microgram of total RNA (kindly provided by Dr. M.L. den Boer, Department of Paediatric Oncology/Haematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands)

was reverse transcribed to cDNA using an anchored hexamer primer [33, 34]. This primer was used because the exact *NUP98* breakpoint location was unknown. For amplification of the cDNA, NUP98-14F1 and the reverse anchor primer were used, followed by a semi-nested PCR using NUP98-14F2 and the reverse anchor primer (Table 3). PCR products obtained were cloned into the TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA). Nucleotide sequences of the clones were determined by automatic sequencing (Baseclear, Leiden, The Netherlands). Sequences were analysed and compared with GenBank sequences by means of the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). RT-PCR is currently performed to confirm the *NUP98-NCL* fusion using the primers shown in Table 3.

Table 3: PCR primers used

Primer (gene-primer)	Sequence (5´→ 3´)	Direction	Position	Gene accession number
t(4;11)(q21;p15.5)				
NUP98-1252F	CCTACTACATTTGGAAGCAG	Forward	1252-1268	U41815
RAP1GDS1-90R	CAGACAATCCAAGCATCCTTC	Reverse	69-90	X63465
3'RACE-PCR				
Random hexamer	GACCACGCGTATCGATGTCGACNNNNNN	Forward	-	-
Reverse anchor	GTCGACATCGATACGCGTGGT	Reverse	-	-
NUP98-9F1	GGACAGCTACAAACACCAGC	Forward	1286-1305	NM_016320
NUP98-9F2	GGAACAGGTCTCTTTGGGCAG	Forward	1327-1347	NM_016320
NUP98-12F1	GTGCTGGACAGGCATCTTTG	Forward	1565-1584	NM_016320
NUP98-12F2	ACTACGACAGCCACTTTGGG	Forward	1654-1673	NM_016320
NUP98-14F1	CCAGAAGGCTCTTACTACACC	Forward	1860-1880	NM_016320
NUP98-14F2	GAACCATCCCTAGCCAAT	Forward	1987-2006	NM_016320
RT-PCR				
NUP98-14F1	CCAGAAGGCTCTTACTACACC	Forward	1860-1880	NM_016320
NUP98-14F2	GAACCATCCCTAGCCAAT	Forward	1987-2006	NM_016320
NUP98-16R1	CCTGCTGGTGATTCTCATCAAC	Reverse	2161-2182	NM_016320
NUP98-16R2	CCCATTTCGCAAAGCAGCAC	Reverse	2321-2400	NM_016320
NCL-3F1	GATGACGAGGACGAGGATGA	Forward	616-635	NM_005381
NCL-3F2	GACGAAGATGATGAGGATGACG	Forward	715-736	NM_005381
NCL-4R1	CAGCCACGTTCTTGGCTTTC	Reverse	822-841	NM_005381
NCL-5R1	CAGGTGCTTCTTTGACAGGC	Reverse	954-973	NM_005381
NCL-6R1	GAAAGCCGTAGTCGGTTCTG	Reverse	1043-1062	NM_005381
NCL-12R1	CAGTAACTATCCTTGCCCGAAC	Reverse	1927-1948	NM_005381

Results and discussion

Blood or bone marrow of 84 patients with haematological malignancies suspected of acute leukaemia (AML, MDS, ALL and other) was analysed for *NUP98* rearrangements using FISH. Using the 120E20/348A20 probe combination (Figure 1A), two AML patients with 11p15 aberrations in the diagnostic karyotype showed separated FISH signals (cases 6 and 13). One patient (case 30) showed an extra fused signal and four cases showed loss of one fused signal in 10-41% of the investigated interphase cells (cases 14, 21, 26, and 34). In the other 77 cases the pattern expected for non-rearranged *NUP98* genes was observed. Split FISH signals were only observed in two cases, indicating that the incidence of *NUP98* rearrangements was very

low in the total investigated group (2%). In the group of patients with karyotypic abnormalities of 11p, this percentage was 6% (11% in group with 11p15 aberrations). Kobsev *et al.* [35] identified seven AML/MDS cases with *NUP98* translocations in a screening on 46 cases with 11p15 abnormalities (15%), resulting in a total number of 55 reported *NUP98* rearranged AML/MDS cases worldwide (57 when including our two cases, described below in more detail).

Cytogenetically visible 11p15 abnormalities without *NUP98* involvement

In three of the 16 cases with karyotypic 11p15 abnormalities but without a *NUP98* translocation, one fused signal was observed on the normal chromosome 11, while the other was located on a derivative partner chromosome (cases 2, 3 and 25), showing that the breakpoint on the der(11) was centromeric to *NUP98.* In the other 13 patients, one fused signal was observed on the normal chromosome 11 and one on the der(11). In case 3, two different der(11) were observed in different cell clones, noted as der(11) and der(11) in table 1. In both clones, two fused signals were visible; one on the normal and one on the derivative chromosome 11.

In the above-mentioned cases without involvement of *NUP98*, other genes in the region may be involved. Genes located at 11p15 include *FANCF* and *H-RAS*, genes known to be implicated in different types of cancer. *FANCF* is implicated in Fanconi anaemia, a chromosome instability syndrome/cancer prone disease, at risk of leukaemia and squamous cell carcinoma, and might thus represent a likely candidate tumour suppressor gene in the 11p15 region [36]. Other genes, closely located to *NUP98*, are *CHRNA10, ART1, ART5* and *TRPC2* on the telomeric side, and *FRAG1, RHOG, STIM1* and *RRM1* on the centromeric side and olfactory receptor genes on either side of *NUP98*. Of these genes, *STIM1* is thought to be a tumour suppressor gene, being involved in different tumour types, and may additionally play a role in haematopoiesis in mediating attachment to stromal cells (http://www.ncbi.nlm.nih.gov/entrez). In the three cases with the 11p15 breakpoint centromeric to *NUP98*, *STIM1* constitutes a good alternative candidate gene for the 11p15 breakpoint. If disrupted by the aberration, the tumour suppressor function of *STIM1* and its possible role in haematopoiesis could be abolished, contributing to the development of leukaemia.

In 1/34 patients with 11p aberrations (case 30), we observed gain of one fused *NUP98* signal both in interphases and metaphases. Using conventional karyotyping, a der(11)inv(11)(p?q) and a +der(11)?inv(11) were observed. In the metaphases, the three fused signals were visible on the p-arm of chromosome 11 and both der(11). If *NUP98* had been involved in the inversion, separate signals would be expected on both the long and short arms of the derivative chromosome. Thus a complete extra copy of *NUP98* was present in this case, and the breakpoint of the presumed inversion was centromeric to *NUP98*.

NUP98 deletions

In 4/34 (8.5%) cases (cases 14, 21, 26 and 34) with 11p aberrations in the diagnostic karyotype (Table 1), we observed loss of one fused *NUP98* signal in 10-41% of the investigated interphases cells. In 2/4 cases with 11p aberrations, an add(11)(p1?4-15) was observed cytogenetically. In the third case, showing a near-tetraploid karyotype with loss of two copies of the normal chromosome 11, two copies of a der(?)t(?;11)(?;q1?0) were observed and in the fourth case

a dup(11)(p13p15). Additional FISH experiments in this latter case (case 26) showed that instead of duplication of 11p13-p15, addition of 11q23-11qter to 11p had occurred with loss of *NUP98,* but not *WT1* (11p13). In the case with the near-tetraploid karyotype (case 21), besides the two copies of der(?)t(?;11)(?;q1?0), two normal chromosomes 11 were observed. As only one fused *NUP98* signal was observed, a cryptic *NUP98* deletion occurred in one of these chromosomes.

Patients with *NUP98* translocations

Both patients (cases 6 and 13) with separated FISH *NUP98* signals, indicative for the presence of a translocation, belonged to the patient group selected for 11p abnormalities.

Patient 6 was a 60-year-old woman, presenting with complaints of fatigue, dyspnoea when exercising and palpitations. Physical examination showed anaemia, otherwise no abnormalities. Laboratory examination: Hb 9.0 g/dl, platelet count 31 × 10^9/L, WBC 3.9 × 10^9/L with 59% blasts. Bone marrow aspirate showed 90% of peroxidase negative blasts, CD34+, CD33+, CD13+, CD133+(weak), CD117 (partially), CD7+ and HLADr+, corresponding to a diagnosis of AML-M0. After informed consent, the patient was treated with two cycles induction chemotherapy (including idarubicin and cytarabin) according to the HOVON-SAKK AML-42 protocol (www.hovon.nl). Only after the second cycle a complete remission was obtained. As no allogeneic sibling donor was available for the patient, consolidation therapy consisted of busulphan and cyclofosfamide, followed by autologous peripheral blood stem cell transplantation. Two months later, the patient showed a relapse, for which no curative therapy was available and the patient subsequently died, 8 months after initial diagnosis. Her diagnostic karyotype was 46~47,XX,t(1;17)(q22;q11),t(4;11)(q1?3;p15),?der(8)(p)[7],del(11)(p12p1?3)[6], del(12)(p11p13),add(14)(q3?2)[7],+15[3],del(15)(q1?3q2?2)[cp22]/46,XX[4] (partly shown in figure 1C).

Using FISH, we observed one fused signal, one separate green and one separate red signal in both interphases and metaphases (Figure 1E + 1F). In metaphases, the fused signal was visible on the normal chromosome 11 or the del(11)(p12p1?3), the green signal (RP11-120E20) on the der(4)t(4;11) and red signal (RP11-348A20) on the der(11)t(4;11), indicating a break in *NUP98* (Figure 1F). The observed abnormality looked similar to the t(4;11)(q21;p15.5) described in T-ALL, in which *NUP98* is fused to *RAP1GDS1* [12, 19]. RT-PCR and sequencing showed the presence of two different *NUP98-RAPGDS1* fusion products. The smaller band matched a product fusing *NUP98* exon 10 with *RAP1GDS1* exon 2, which resulted in the fusion of NUP98 amino acids 1-391 to RAP1GDS1 amino acids 2-558. The larger band matched a product fusing *NUP98* exon 10 with *RAP1GDS1* exon 2, but from a cryptic donor splicing site in intron 11, located 51 nucleotides after the donor site. At the protein level, this product results in the in frame fusion of NUP98 amino acids 1-391 to RAP1GDS1 amino acids 2-558 with additional 17 amino acids encoded by *NUP98* intron 11 nucleotide sequence. In both cases, the *RAP1GDS1* fusion point was identical to the one published in T-ALL [12, 19]. However the breakpoint in *NUP98* was different than the ones reported in T-ALL patients [12, 19]. This is the first time that a *NUP98-RAP1GDS1* is observed in an AML patient. Like in all translocations described so far, the fusion transcript contains the 5' portion of *NUP98*, which is in this patient fused in frame to *RAP1GDS1*.

Patient 13, a 1-year-old boy, presented in April 2000 with pallor, bruises on legs and forehead, weight loss, and no hepatosplenomegaly or extramedullary disease. At presentation, Hb was at 6.5 g/dl, platelet count 138 × 10^9/L, and white blood cell count (WBC) was 8.4 × 10^9/L with 1-2% blasts. Hypocellular bone marrow revealed 40% of blasts, morphologically, cytochemically and immunologically corresponding to a diagnosis of AML-M7 according to FAB criteria. Treatment according to Dutch Childhood Oncology Group (DCOG)/MRC AML12 treatment protocol ANLL-97 (including cytarabine (Ara-C), methotrexate, prednison, mitoxantron, etoposide) was started. In July 2000, the patient reached complete remission, and remained in continuous complete remission at the last examination in August 2004. At diagnosis, clonal cytogenetic abnormalities were found, i.e. 46,XY,der(1)t(1;13)(p36;q1?4),add(11)(p15),der(13)t(1;13)(p36;q1?2),der(21)t(11;21)(p15;p13)[31]/ 46,XY[7] (karyotype partly shown in figure 1B).

Using FISH, we observed one fused signal on the normal chromosome 11 and a separate green signal (RP11-120E20) on the der(21)t(11;21) and a red signal (RP11-348A20) on the der(11) (figure 1D). Using conventional karyotyping, it was not possible to determine the origin of the added material on the der(11). We investigated this translocation in more detail using FISH with whole chromosome paints (WCPs) for chromosomes 11 and 21 and locus-specific probes. Chromosome 11 material translocated to the der(21), but no chromosome 21 material could be detected on the der(11). Consequently, the t(11;21) was indeed an unbalanced translocation, as suggested cytogenetically. Additionally, the signal for 11pter translocated to the der(21), and a combination of RP11-120E20 and a probe for *AML1* (21q22) showed that chromosome 11p material was indeed present on the short arm of this der(21). Using the ribosomal satellite probe R521, in total 8-9 signals could be detected on the acrocentric chromosomes and a C-group chromosome, identified by DAPI banding as chromosome 12. This chromosome was cytogenetically normal, but FISH showed this cryptic abnormality. The ribosomal satellite sequences residing at 21p were present only on the normal 21, but no R521 signal could be detected on the der(21) (Figure 2).

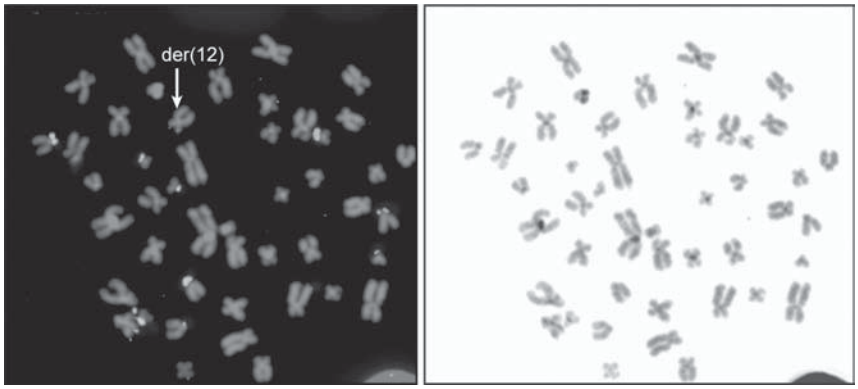

Figure 2: Additional FISH analysis in case 13. Metaphase spread and corresponding inverted DAPI image of case 13, showing absence of R521 signal (red) and presence of RP11-120E20 (green) signal on the der(21).

3' RACE-PCR using *NUP98* forward primers and a reverse anchor primer showed products of different sizes on agarose gel (data not shown). The PCR products were cloned, sequenced and analysed. Several clones were obtained with sequences corresponding to the normal *NUP98*. A band smaller than the normal *NUP98* band in the other clones, matched the 5' end of *NUP98* (exon 14) fused with the coding region of exon 4 of nucleolin (*NCL*). The fusion maintained the reading frame of *NCL*. *NCL* is located on chromosome 2q37.1 and encodes a nucleolar phosphoprotein with numerous functions, including a role in ribosome biogenesis [37]. Another *NUP98* translocation involving chromosome 2, the recurrent t(2;11)(q31;p15), fusing *NUP98* and *HOXD11,* has been described in myeloid malignancies [17]. Thus far, no translocations involving *NCL* have been described in haematological malignancies. Nucleolin is associated with proliferation, cell cycle control and apoptosis, all cellular processes deregulated in cancer [37]. Elevated levels of nucleolin expression are generally related to malignancy and predict a poor prognosis in many types of cancer [38]. Sequencing of the 3'RACE-PCR product showed that the fusion transcript contains the 5' portion of *NUP98*, as in all other known *NUP98* fusion transcripts. The Phe-Gly (FG) repeats in the N-terminal part of NUP98, which are presumed contact sites for soluble nucleocytoplasmic transport factors carrying different kinds of cargo, have been shown to activate transcription [39]. The *NCL* sequence present started with exon 4. Therefore, the nuclear localisation signal, the 4 RNA recognition motifs (RRMs) and the C-terminal domain of NCL are probably present in the fusion protein encoded by the fusion gene. These domains are thought to play a role in interaction with multiple RNA targets [37]. The N-terminal domain of NCL, likely to be absent in the fusion protein, contains acidic stretches, which have been proposed to be responsible for the capacity of nucleolin to modulate DNA condensation in chromatin. In addition, this N-terminal domain is thought to be required for efficient shuttling between the nucleus and cytoplasm [37]. Fusion of DNA stretches encoding the RRMs and C-terminal domain of *NCL* to the 5' portion of *NUP98* could provide binding specificity for targets not normally binding NUP98*,* and can determine downstream targets of the NUP98-NCL fusion protein, causing delocalisation and deregulation of these targets [39]. Additionally, it could cause delocalisation of NCL itself*,* resulting in disruption of normal NCL function.

Conclusion

Our results showed that the probe combination used (RP11-120E20 and RP11-348A20) is useful for detection of *NUP98* rearrangements, both on metaphases and interphase nuclei. In the study presented here, we identified two patients with *NUP98* translocations (2% of the total investigated group): a patient with AML-M0 who showed the t(4;11)(q1?3;p15), a translocation previously published in literature, and a boy with AML-M7 who had an unbalanced add(11)(p15) and a der(21)t(11;21)(p15;p13) resulting in a *NUP98-NCL* fusion product. Additionally, we observed subclonal *NUP98* deletions in four patients with unbalanced 11p aberrations. In general, *NUP98* rearrangements continue to be very rare, as confirmed by our findings. Large-scale screening will provide more *NUP98* rearranged cases, allowing more accurate estimations of the incidence, since we used a selected group of cases. When more patients with *NUP98* aberrations are identified, the prognostic value of these rearrangements and deletions can be estimated more properly.

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Chapter 6

CDKN2 deletions have no prognostic value in childhood precursor-B acute lymphoblastic leukaemia

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Chapter 6

CDKN2 deletions have no prognostic value in childhood precursor-B acute lymphoblastic leukaemia

Abstract

Rearrangements or deletions of the *CDKN2* locus on chromosome 9p21 are frequently found in acute lymphoblastic leukaemia (ALL). Therefore, inactivation of this locus is thought to be important in leukaemogenesis and could have important clinical significance. However, publications on the prognostic value of *CDKN2* inactivation in acute leukaemia are inconsistent. As previous studies grouped cases with T and B lineage ALL, the prognostic value of *CDKN2* deletions in c/pre-B-ALL is unknown. We analysed, in a single centre study, 109 diagnostic childhood c/pre-B-ALL cases for loss of *CDKN2* using fluorescence in situ hybridisation (FISH). We observed *CDKN2* deletions in 37/109 (34%) cases, whereas the other 72/109 (66%) cases were normal for this locus. Deletions observed were hemizygous (19%), homozygous (40%) or a mixture of both hemizygous and homozygous deletions (40%). We did not observe a significant difference in long-term clinical outcome between cases with and without *CDKN2* deletions using both uni and multivariate Cox regression analysis of disease free and overall survival. No difference in *CDKN2* status between diagnosis and relapse was observed in 13/17 diagnosis-relapse couples. The relatively high incidence of *CDKN2* deletions suggests that genes at this locus play a role in leukaemogenesis not only in T-ALL, but also in childhood c/pre-B-ALL.

Introduction

The *CDKN2* locus on 9p21 encodes three genes involved in cell cycle regulation, $p16^{INK4A}$, $p15^{INK4B}$ and $p14^{ARF}$. $p15^{INK4B}$ and $p16^{INK4A}$ together encode INK4, the inhibitor of kinase 4 that specifically inhibits cyclin dependent kinases (CDK) CDK4 and CDK6. CDK4 and CDK6 normally trigger retinoblastoma (RB) phosphorylation; INK4 thus prevents RB phosphorylation. RB phosphorylation triggers the E2F transcriptional program, resulting in progression into S-phase. As a result of INK4 expression, unphosphorylated RB keeps E2F sequestered, leading to G1 arrest (reviewed in [1-3]). $p14^{ARF}$ links the RB and p53 pathways of cell cycle regulation. It shares exons 2 and 3 with $p16^{INK4A}$, but has an alternative exon 1 (1β) and an alternative open reading frame. The $p14^{ARF}$ protein is unrelated to INK4. It sequesters MDM2, p53 negative regulator, upon E2F stimulation, resulting in p53 stabilization and accumulation, leading to G1 arrest (reviewed in [2, 4]). All three genes are thought to act as tumour suppressor genes,

and therefore, inactivation or loss of these genes would be an expected observation in tumour cells. Indeed, rearrangements and/or deletions of the *CDKN2* locus are frequently found in different tumour types, including acute leukaemia [5, 6].
In acute leukaemia, *p15*/*p16 (CDKN2)* deletions have been found with high frequencies in both adult and childhood acute lymphoblastic leukaemia (ALL) (>30%). The highest frequency occurs in T-ALL (>50%), but deletions are observed in precursor-B-ALL as well (>20%) [7]. In acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML) and myelodysplastic syndromes (MDS) very low frequencies of *p15/p16* deletions have been observed (0-1%) [7]. However, the rates of *p15* promoter hypermethylation, another frequent mechanism of *p15/p16* inactivation, are very high in AML (70%), MDS (40%) and also precursor-B-ALL and T-ALL (40%) [7]. Therefore, inactivation of this locus is thought to play an important role in leukaemogenesis and could have important clinical significance.
The publications on the prognostic value of *CDKN2* inactivation in acute leukaemia are inconsistent. Some studies describe a significantly worse prognosis for childhood ALL cases with *p15/p16* inactivation [8-14], whereas others do not find a significantly different outcome for patients with *p15/p16* inactivation by either deletions [15-18] or promoter hypermethylation [19]. Additionally, one study showed a difference in prognostic value between homozygous and hemizygous deletions [20]. In most of these studies however, no difference was made between childhood ALL cases with B- and T-cell phenotype. One study investigated the prognostic significance in childhood T-ALL cases only and reported a significantly worse outcome for cases with homozygous *CDKN2* deletions [12]. As the clinical relevance of *CDKN2* deletions is still subject of debate, we investigated, in a single centre study using fluorescence in situ hybridisation (FISH), the prognostic significance of *CDKN2* deletions in 109 diagnostic childhood common/precursor B (c/pre-B)-ALL cases.

Materials and Methods

Patients

Between 1992 and 2002, 155 childhood acute leukaemia cases from the Erasmus MC, Sophia Children's Hospital, analysed for the presence of cytogenetic aberrations, were analysed for *CDKN2* deletions using FISH analysis. Of these 155, 129 cases were diagnosed with c/pre-B-ALL, 20 with T-ALL and 6 with AML. For evaluation of the prognostic value of *CDKN2* loss, only those c/pre-B-ALL cases successfully analysed for *CDKN2* deletions, were included (n=111), as the T-ALL and AML groups were to small to obtain statistically significant results. The remaining 18 c/pre-B-ALL cases were excluded due to lack of material for FISH or bad hybridisation quality. Male gender and leukocyte count below 50 x 10^9/l occurred statistically more often in this latter group than in the group of 111 successfully analysed cases; the distributions of age and chromosome abnormalities were the same in both groups. Additionally, 17/111 ALL c/pre-B-ALL and 3/20 T-ALL cases were also analysed at relapse, and 1/111 c/pre-B-ALL case developed into a secondary AML. Patients were treated with either one of 4 protocols: 19/111 (17.1%) cases were treated according to Dutch Childhood Oncology Group (DCOG) treatment protocol ALL-8, 90/111 (81.1%) according to ALL-9, 1/111 (0.9%) according

to CZS8406 and 1/111 (0.9%) was included in the Interfant 99 study. The latter two cases were excluded for further analysis. At diagnosis, informed consent of the patients and/or parents/guardians was obtained to use left-over material for research purposes.

Conventional cytogenetic analysis

Bone marrow or blood obtained at diagnosis was cultured and harvested using standard cytogenetic protocols. For each case 20-32 metaphase cells were analysed using both QFQ- and RBA-banding. The chromosome aberrations observed were described according to the International System for Human Cytogenetic Nomenclature (1995) [21]. Methanol/acetic acid (3:1) fixed cell suspensions were stored at -20°C. Remaining blood or bone marrow was viably frozen in 0.1 volume dimethylsulfoxide (DMSO)/0.9 volume foetal calf serum (FCS) and stored in liquid nitrogen until further use.

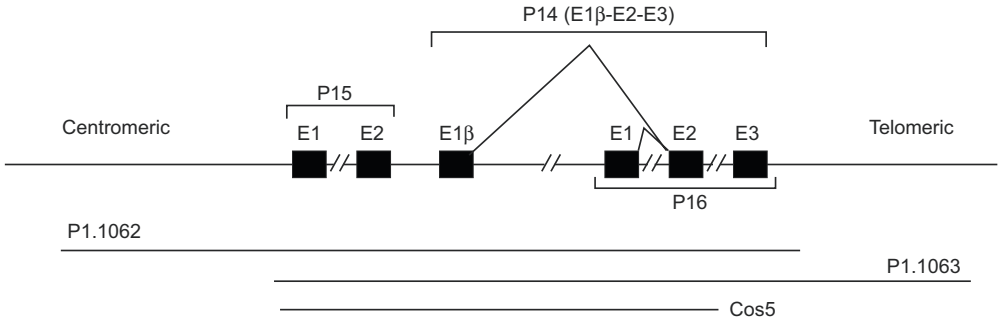

Figure 1: Localisation of FISH probes covering the *CDKN2* locus. Schematic localisation of P1.1063, P1.1062 and cos5 regarding the *CDKN2* locus. *p15, p16* and *p14* exons (E) are shown by blocks. The schematic drawing is not to scale. Modified from Kamb *et al.*, Stone *et al.* and Shapiro *et al.* [1, 5, 22]

FISH

Loss of *CDKN2* was analysed using P1.1063 (53 kb), which spans most of the locus, covering the region from upstream exon 2 of *p15* to distal to exon 2 of *p16*, including the coding sequence of *p14* (Figure 1) [5]. The P1.164 probe containing the complete coding sequence of *ETO* (*CBFA2T1*) (8q22) was used as a control [23]. If a deletion was observed, it was further characterized using a combination of P1.1062 and cos5. These probes cover both *p15* and *p16*, but differ from P1.1063 in that P1.1062 (51 kb) starts upstream of *p15* and ends immediately downstream of *p16*. Cos5 encompasses *p15* and exons 1-2 of *p16* and *p14*, and because of it's smaller size (23 kb), smaller deletions may be detected [5]. DNA purification of clones was performed using the QIAGEN plasmid maxi kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Each probe was labelled by nick translation with either biotin-16-dUTP or digoxigenin-11-dUTP. For FISH, freshly prepared metaphase spreads from -20°C stored methanol/acetic acid cell suspensions were used. Probes were hybridised to these metaphase spreads and detected as described previously [24]. Slides were counterstained with 4'6-Diamino-2-Phenyl Indol (DAPI). For each hybridisation a minimum of 200 interphase cells were scored, as well as 5-10 metaphases if present. Images were captured using an epifluorescence microscope (Axioplan 2, Zeiss, Sliedrecht, The Netherlands) using MacProbe

software (version 4.3, Applied Imaging, Newcastle upon Tyne, UK). The cut-off values for each probe were defined as the mean plus three times the standard deviation as observed in 3800 control interphase nuclei of 19 ALL cases without deletions. If losses were observed below these cut-off values, the sample was considered normal for the *CDKN2* locus. In addition, cases were screened using FISH for 11q23 rearrangements, t(9;22)(q34;q11) and t(12;21)(p13;q22).

Data classification

Diagnostic cytogenetic results were classified as shown in Table 1. For survival analyses, we divided the cytogenetic results into karyotypes with poor prognostic specific chromosome aberrations (t(9;22)(q34;q11) and 11q23 rearrangements) and other karyotypes (Table 2).

Table 1: Cytogenetic findings in 109 c/pre-B-ALL and 20 T-ALL cases

	c/pre-B-ALL		T-ALL	
	Number	Percentage	Number	Percentage
Total	109	100	20	100
Cytogenetic abnormalities				
None	12	11	6	30
Numerical	31	28	1	5
Structural	45	42	8	40
Complex structural*	20	18	4	20
Karyotype failure	1	1	1	5
(Specific) chromosome aberrations				
11q23 rearrangements	2	2	1	5
t(9;22)(q34;q11)	2	2	0	
t(1;19)(q23;p13.3)	3	3	0	
t(12;21)(p13;q22)	25	23	0	
Hyperdiploid (> 50 chromosomes)	41	38	0	
CDKN2 deletion				
None	72	66	5	25
Deletion	37	34	15	75
Hemizygous	7		1	
Homozygous	15		14	
Mixed hemi-/homozygous	15		0	

* >3 structural abnormalities

In addition to investigating the presence or absence of *CDKN2* deletions, the type of deletion present was recorded. Hemizygous deletions were defined as loss of the *CDKN2* FISH signal on one chromosome 9, with the signal on the other chromosome 9 being retained. These deletions of the *CDKN2* locus were considered present if >12% of interphase nuclei showed only one P1.1063 signal. For the combination of P1.1062 and cos5 this cut-off value was 15% of interphase nuclei. Homozygous deletions are defined as loss of *CDKN2* FISH signals on both chromosomes 9. These deletions were considered present if >4% of interphase nuclei showed loss of both P1.1063 FISH signals. For the P1.1062/cos5 combination this cut off value was 2%. Mixed hemizygous/homozygous deletions were present if two or more clones with

both types of deletions were present in one sample, taking into account the determined cut-off values.

Statistical analysis

Fisher's exact test was used to test for differences in the occurrence of *CDKN2* deletions and age, leukocyte count at diagnosis (WBC), gender, treatment protocol and the presence of poor prognostic specific chromosome aberrations (t(9;22) and 11q23 rearrangements). Cox proportional hazard analysis was used to perform univariate and multivariate analysis of disease free survival (DFS) and overall survival (OS). DFS was defined as the time from first diagnosis until a leukaemia-related event (non-response or relapse). OS was defined as the time from first diagnosis until death. All survival times are given in years. Survival curves were estimated using the Kaplan-Meier method. Data were considered statistically significant if $p \leq 0.05$.

Table 2: Patient characteristics in 109 c/pre-B-ALL cases and 20 T-ALL cases

	c/pre-B-ALL		T-ALL	
	Number	Percentage	Number	Percentage
Total	109	100	20	100
Gender				
Male	66	61	14	70
Female	43	39	6	30
WBC at diagnosis				
< 50 x 10^9/l	93	85	5	25
≥ 50 x 10^9/l	16	15	15	75
Median	7.9		138	
Range	0.8-684.0		5.0-533.0	
Age				
< 10 years	85	78	17	85
≥ 10 years	24	22	3	15
Median	5.0		6.1	
Range	0.8 - 16.8		1.5-13.8	
Chromosome aberrations in karyotype				
Poor prognostic*	4	4	1	5
Other	104	95	18	90
Karyotype failure	1	1	1	5

WBC: leukocyte count at diagnosis; * t(9;22)(q34;q11) and 11q23 rearrangements

Results and discussion

Conventional cytogenetics

Cytogenetic abnormalities were observed in 96/109 (88%) c/pre-B-ALL cases and 13/20 T-ALL cases. The remainder had apparently normal karyotypes, except for one c/pre-B-ALL and one T-ALL case with karyotype failure due to lack of metaphases. We also investigated the karyotypes for the presence of specific structural abnormalities of known prognostic significance. The cytogenetic findings are summarized in Table 1. Since the numbers of prognostic important translocations were too few to use the individual translocations as a factor

in survival analysis, the known poor prognostic aberrations, t(9;22) and 11q23 rearrangements, were grouped together (Table 2).

FISH results

Loss of *CDKN2* (both *p15* and *p16*) was observed in 37/109 (34%) c/pre-B-ALL cases using P1.1063 and the combination of P1.1062 and cos5. Of these, 7/37 (19%) cases had hemizygous deletions, whereas homozygous deletions were observed in 15/37 (40%) cases. Additionally, we observed the presence of both hemi- and homozygous deletions in 15/37 (40%) cases (Table 1). In seven cases, both types of *CDKN2* deletions were observed in 20-25% of the nuclei each. In three cases, the percentage of cells with a homozygous deletion was much higher than that of hemizygous deletions (58-61% versus 18-20% respectively), whereas in three other cases hemizygous deletions were observed in higher percentages than homozygous deletions (50-74% versus 17-21% respectively). In two cases, both hemizygous and homozygous deletions were observed in 40-50% of the nuclei. The group with mixed deletions shows that different clones can occur next to each other. This also demonstrates the heterogeneity in leukaemic samples. The percentage of deletions (34%) observed in our study is higher than that reviewed in Drexler [7] (23% for both *p15* and *p16*), but comparable to other studies in which *CDKN2* deletions have been observed in ~30% of B-lineage ALLs [18, 25].
In T-ALL, 15/20 (75%) cases investigated showed *CDKN2* deletions, 14 homozygous and 1 hemizygous. This percentage is comparable to other studies [7, 12]. The high incidence of homozygous deletions (93%) in this deleted T-ALL group confirms our earlier results [12], showing that most T-ALL cases with *CDKN2* deletions had homozygous deletions, although the percentage in that study (69%) was lower than currently observed.

Table 3: Overview of cytogenetic results of 109 c/pre-B-ALL cases regarding 9p and *CDKN2* FISH results

			***CDKN2* FISH**		Total
			Normal	**Deletion**	
9p by karyotyping	**Normal**		68	**23**	91
	Abnormal	9p21 abnormal	**3**	2	5
		Other	1	**12**	13
		Total	72	37	109

Bold: discrepant cases

The FISH results were compared to the corresponding karyotypes (Table 3). In total, only 18 c/pre-B-ALL cases had cytogenetically visible aberrations of the short arm of chromosome 9 (Table 4). Five of these cases had breakpoints in 9p21 (cases 1-5), whereas the remaining 13 cases showed breakpoints in other bands of 9p (cases 6-18). Only 2/5 cases with 9p21 breakpoints showed *CDKN2* deletions with FISH (cases 4 and 5), resulting either from deletions or unbalanced translocations as observed in the karyotype. The other 3/5 cases, having cytogenetically visible deletions or a translocation involving 9p21, did not have *CDKN2* deletions (cases 1-3). In addition, 12/13 cases with abnormalities of the short arm of chromosome 9 other than 9p21, showed *CDKN2* deletions (cases 7-18). Among the

cytogenetically visible aberrations were dic(9;20)(p11-13;q11) in four cases (cases 7, 12, 15 and 16), a dic(9;10)(p13;p1?1) (case 8), unbalanced translocations with breakpoint in 9p13 or 9p2? in two cases (cases 13 and 18), additions to 9p1?2, p2?2 or p1 in three cases (cases 9-11), a del(9)(p?) in case 14 and a ?der(9)(p) in case 17. In 6/12 cases, homozygous *CDKN2* deletions were observed. Deletion of the second allele has occurred, which was not expected based on the karyotypic information. The remaining case in this category with a dic(9;10)(p?13;q?21) had no *CDKN2* deletion (case 6). The other 23/37 cases with *CDKN2* deletions showed no aberrations of 9p, indicating that *CDKN2* deletions mostly are cryptic abnormalities. Thus, *CDKN2* deletions occurred in patients with abnormal and normal karyotypes with and without cytogenetic 9p abnormalities. This was also observed for the T-ALL group: 15 cases showed *CDKN2* deletions, but only three of these cases showed cytogenetically visible aberrations of 9p, none of them in 9p21.

Table 4: *CDKN2* FISH results of c/pre-B-ALL cases with cytogenetically visible 9p aberrations

Case	Age (years)	Karyotype at diagnosis	*CDKN2* deletion status
1	13.6	46,XX,del(9)(p1?3p2?1)[7]/46,XX,?der(9)(p)[5]/46,XX[19]	No
2	5.2	46,XX,t(1;12)(p3?5;q1?2),?der(21)(q)[1]/46,idem,t(9;13)(p21;q1?3)[23]/46, idem,-X,t(X;7)(q1?2;q3?5)[12]/46,t(1;12),inc[15]/46,XX[27]	No
3	2.5	95,XXYY,add(7)(q2?2)x2,del(9)(p21)x2,del(11)(q22)x2,add(12)(p12)x2,+3mar [75]/46,XY[34]	No
4	11.3	46,XY,del(9)(p13~21p22)[1]/46,idem,t(12;21)(p13;q22)[12]/46,idem,?der(12)(p), ?der(21)(q)[9]/46,XY[21]	Homozygous
5	4.3	48,XY,add(7)(p11),der(9)t(9;14)(p21;q22),add(12)(p11),add(14)(q22),?der(21), +?21,+mar,inc[28]/46,XY[5]	Homozygous
6	15	34,<1n>,X,+1,+5,+6,+8,+9,+10,+11,+18,+19,+21,+22[4]/34,idem,+9,dic(9;10) (p?13;q?21)[23]/46,XY[6]	No
7	2.0	47,XX,dic(9;20)(p11;q11),+21,+mar[4]/48,idem,+21[12]/46,XX[14]	Hemizygous
8	0.8	45,XY,dic(9;10)(p13;p1?1),t(13;15)(q1?4;q2?4)[28]/46,XY[3]	Homozygous
9	3.8	45,XY,t(1;6)(p21;q26~27),add(2)(p?),-9,add(9)(p2?2),add(11)(q1?4),add(12) (p12),inc[37]	Homozygous
10	2.2	47,XY,i(9)(q10),t(9;22)(q34;q11),+der(22)t(9;22)(q34;q11)[14]/46,XY,add(9)(p1) , t(9;22)(q34;q11)[4]/46,XY[3]	Homozygous
11	16.7	49~51,XY,+X,del(1)(p3?3p3?5),+7[9],add(9)(p1?2),del(11)(q1?3q2?2),+14[10], ?der(20),+21[10][cp38]/46,XY[59]	Homozygous
12	3.4	46,XX,+X,dic(9;20)(p13;?q11)[16]/46,XX[1]	Homozygous
13	3.8	55~56,XX,+?X,+4,+5,+6,der(9)t(9;14)(p2?;q?1),+?15,+17,+21,+21,+1-2mar[4] [cp29]/46,XX[3]	Homozygous
14	9.1	46,XY,?del(2)(q?)[9]/46,idem,del(9)(p?)[9]/46,XY[15]	Mixed
15	1.8	45,XX,dic(9;20)(p12;q11)[7]/44,idem,-X[20]/46,XX[9]	Mixed
16	1.3	45,XY,dic(9;20)(p1?3;q1?1)[7]/46,XY[9]	Mixed
17	1.8	46,XY,add(6)(q?2),del(7)(p1?2p2?2),del(12)(p1?1p1?3)[14]/46,idem,?der(9)(p) [14]/46,idem,?der(1)(p),?der(9)(p)[5]/46,XY,?der(9)(p)[1]/46,XY[1]	Mixed
18	1.3	45,XY,inv(1)(p35q32),-7,der(9)t(7;9)(q11;p13)[31]/46,XY[17]	Mixed

We also investigated the association of *CDKN2* deletions with specific cytogenetic abnormalities. The two diagnostic 11q23 rearranged c/pre-B-ALL samples showed no *CDKN2* deletions, but a childhood secondary AML case with t(1;11)(p3?2;q23) and a T-ALL case with

t(6;11)(q27;q23) involving *MLL* did. Maloney *et al.* [26] did not observe *CDKN2* deletions in infant, *MLL* rearranged, ALL samples, whereas Ohnishi *et al*. [27] showed homozygous *CDKN2* deletions in *MLL* rearranged childhood AML/ALL and adult AML. None of our three cases with t(1;19)(q23;p13.3) showed *CDKN2* deletions, confirming the finding of Maloney *et al*. [28] that *CDKN2* deletions and t(1;19)(q23;p13.3) might be mutually exclusive. Both t(9;22)(q34;q11) positive cases did have *CDKN2* deletions (1 homozygous, 1 mixed), as well as 7/25 t(12;21)(p13;q22) positive (4 hemizygous, 3 homozygous) and 12/41 hyperdiploid (> 50 chromosomes) cases, indicating that t(9;22)(q34;q11), t(12;21)(p13;q22) or hyperdiploidy and *CDKN2* deletions are not mutually exclusive. Our results indicated that *CDKN2* deletions occur more often in hyperdiploid cases than reported by Moreno *et al.* [29], who observed homozygous *CDKN2* deletions in only 2/21 (10%) cases with hyperdiploidy.

Diagnose-relapse couples

For 17 c/pre-B-ALL and 3 T-ALL patients, the *CDKN2* status could be analysed both at diagnosis and relapse (Table 5). In addition, one c/pre-B-ALL case developed secondary AML that could be analysed as well. In three cases, the *CDKN2* status differed between diagnostic and 1st relapse samples: two cases showed no *CDKN2* deletions at diagnosis, whereas the relapse samples did. The 3rd case showed a hemizygous deletion at diagnosis, and a homozygous deletion at relapse. In another case for which bone marrow at diagnosis, 1st relapse and 2nd relapse was available, no deletion was observed in diagnostic and 1st relapse samples, but in the 2nd relapse sample a homozygous *CDKN2* deletion was observed (Table 5).

Additionally, the case with secondary AML did not have *CDKN2* deletions at diagnosis, but showed a mixed type deletion when t-AML was diagnosed. Maloney *et al.* [30] and Carter *et al.* [31] observed increasing *CDKN2* loss in 5/18 and 8/22 cases respectively. Accordingly, they suggested that *CDKN2* deletions might be important in disease progression. In 13/17 c/pre-B-ALL, as well as 2/3 T-ALL couples, we analysed however, no differences were observed between diagnosis and relapse. Thus, in most cases *CDKN2* deletions did not represent an additional hit for disease progression.

Table 5: *CDKN2* status at diagnosis and relapse/secondary AML in 18 c/pre-B-ALL cases

CDKN2 status at diagnosis	*CDKN2* status at relapse	Number of cases	Remarks
No deletion	No deletion	11	Second relapse sample was additionally available in 1 case and this 2nd relapse sample showed a homozygous deletion
No deletion	Hemizygous	2	
No deletion	Mixed	1	Secondary AML
Hemizygous	Homozygous	1	
Homozygous	Homozygous	2	
Mixed	Mixed	1	

Prognostic relevance

Only the 109 c/pre-B-ALL cases successfully analysed for *CDKN2* deletions were included for prognostic evaluation of *CDKN2* deletions. The patient characteristics of this group are summarized in Table 2. Before starting survival analyses, correlations between any type of *CDKN2* deletion and sex, age, WBC, the presence of prognostic important chromosome abnormalities and treatment protocol were evaluated. Using Fisher's Exact test, we did not observe any significant p-values for the different variables analysed (data not shown). The known poor prognostic factors (male gender, age ≥ 10 years old, t(9;22) and 11q23 rearrangements) did indeed correlate with worse clinical outcome ($p \leq 0.05$ for each factor). No difference in clinical outcome was observed between the cases treated according to the ALL-8 and ALL-9 protocols.

Next, univariate Cox regression survival analysis was performed with stratification for treatment protocol. Median follow-up time for patients at risk for DFS was 4.8 years, and 5.1 years for OS. Univariate analysis showed that the probability of disease free survival (pDFS) of cases with *CDKN2* deletions did not differ significantly from those without deletions (Hazard ratio = 1.293, 95% Confidence Interval (CI) = 0.574-2.913, p=0.535). The 4-year pDFS was 74.2 ± 6.5% for cases without deletions and 73.1 ± 8.9% for cases with *CDKN2* deletions (Figure 2). The 4-year pOS was not significantly different either: 86.8 ± 4.9% for cases without deletions versus 79.8 ± 8.0% for cases with *CDKN2* deletions (Hazard ratio = 1.489, 95% CI = 0.574-3.866, p=0.413). Using multivariate Cox regression to correct for other prognostic factors, loss of *CDKN2* was not an independent prognostic factor in either DFS (Hazard ratio = 1.251, 95% CI = 0.532-2.941, p=0.608) or OS (Hazard ratio = 1.254, 95% CI = 0.469-3.353, p=0.652). Our results were in agreement with several reports [15-18], whereas others have reported a significantly worse outcome for patients with *CDKN2* deletions [8-14, 32]. In most of these studies however, no difference was made between childhood ALL cases with B- and T-cell phenotype, whereas we investigated a group of 109 childhood c/pre-B-ALL cases only. The reported poor prognostic value of *CDKN2* deletions in those studies may result from differences in patient groups, as T-ALL in general has a worse outcome and a high incidence of *CDKN2* deletions [7, 12].

In most of the studies mentioned above, the presence of *CDKN2* deletions was investigated using Southern Blot or loss of heterozygosity (LOH) analysis. Since FISH techniques analyse individual cells, it is possible to identify both homozygous and hemizygous deletions, and even to observe the presence of both deletions coexisting, providing insight in heterogeneity of *CDKN2* loss within a sample. Because Southern Blot analysis uses total DNA, it is hard to identify hemizygous deletions, as both the abnormal clone with and without *CDKN2* deletions or normal cells are present in the sample analysed. Consequently, quantification of DNA or mRNA can be difficult, and cases belonging to the mixed deletion group cannot be identified as such.

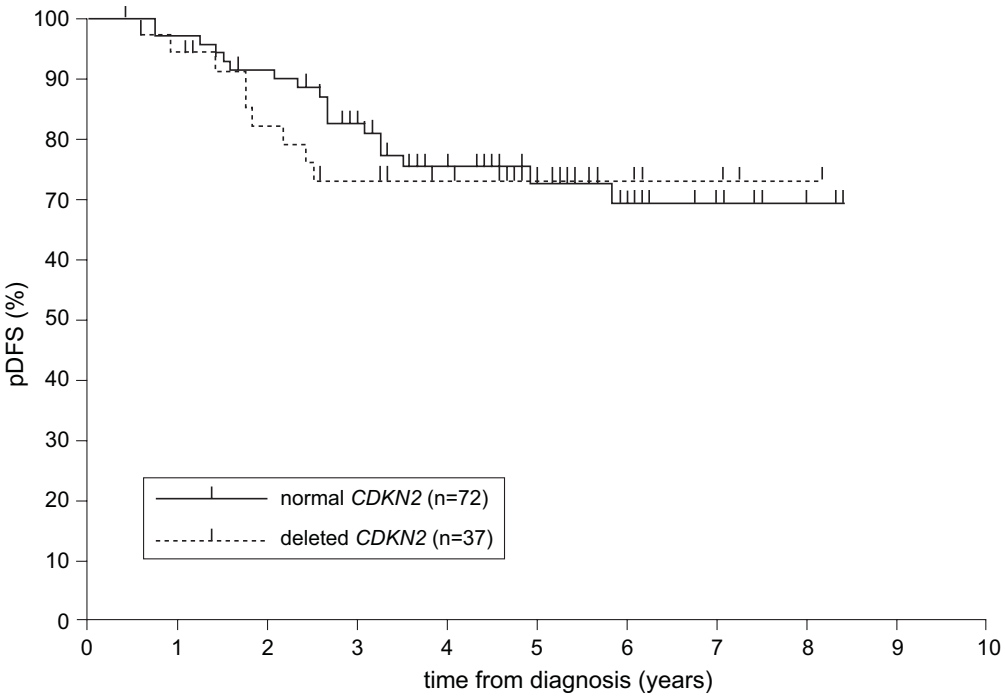

Figure 2: Disease free survival (DFS) in 109 c/pre-B-ALL cases with and without *CDKN2* deletions. DFS survival curves of 72 cases without *CDKN2* deletions and 37 cases with *CDKN2* deletions. On the X-axis time from initial diagnosis (in years) is depicted, and on the Y-axis the probability of DFS.

Furthermore, small aberrant, *CDKN2* deleted populations are missed more easily, due to the limited sensitivity of Southern Blotting. Using FISH, the problems with detecting hemizygous deletions are circumvented. However, very small deletions that might be present in some of the cases cannot be identified as the FISH or Southern blot probes used might be too large.

We did not investigate the other frequent mechanism through which *CDKN2* can be inactivated, promoter hypermethylation, as no DNA was available in most cases. Three studies, investigating hypermethylation of *p15*, showed unfavourable outcome for adult ALL or both adult and childhood ALL patients [32-34], whereas no correlation was observed in three other studies [19, 35, 36]. In five of these six studies however, no difference between B and T-ALL phenotype was made either.

We investigated the occurrence of homozygous and hemizygous deletions, but we did not examine the prognostic value of these types of deletions separately, because we considered the groups for the different types of deletions too small to reach statistical significance. Heyman *et al.* [20], however, showed, in a small group (n=24 for homozygous deletions, n=12 for hemizygous deletions), a difference in prognostic value between homozygous and hemizygous deletions using Southern Blot combined with microsatellite analysis [20]. When

we performed preliminary survival analysis with distinguishing deletion types in our group of c/pre-B-ALL patients (15 homozygous deletions, 7 hemizygous deletions and 15 mixed deletions), no differences were observed. Future investigations on larger patient groups will have to provide more insight into the prognostic value of each type of deletion.
In conclusion, we observed three types of *CDKN2* deletions, homozygous, hemizygous and mixed hemi/homozygous in 34% of 109 c/pre-B-ALL cases. The presence of *CDKN2* deletions had no prognostic value in 109 childhood c/pre-B-ALL cases analysed in this single-centre study. No difference in *CDKN2* status was observed between diagnosis and relapse in 76% of diagnosis-relapse couples. However, the incidence of *CDKN2* deletions in c/pre-B-ALL (34%) indicates that the genes harboured at the *CDKN2* locus might play an important role in leukaemogenesis not only in T-ALL but also in precursor B-ALL.

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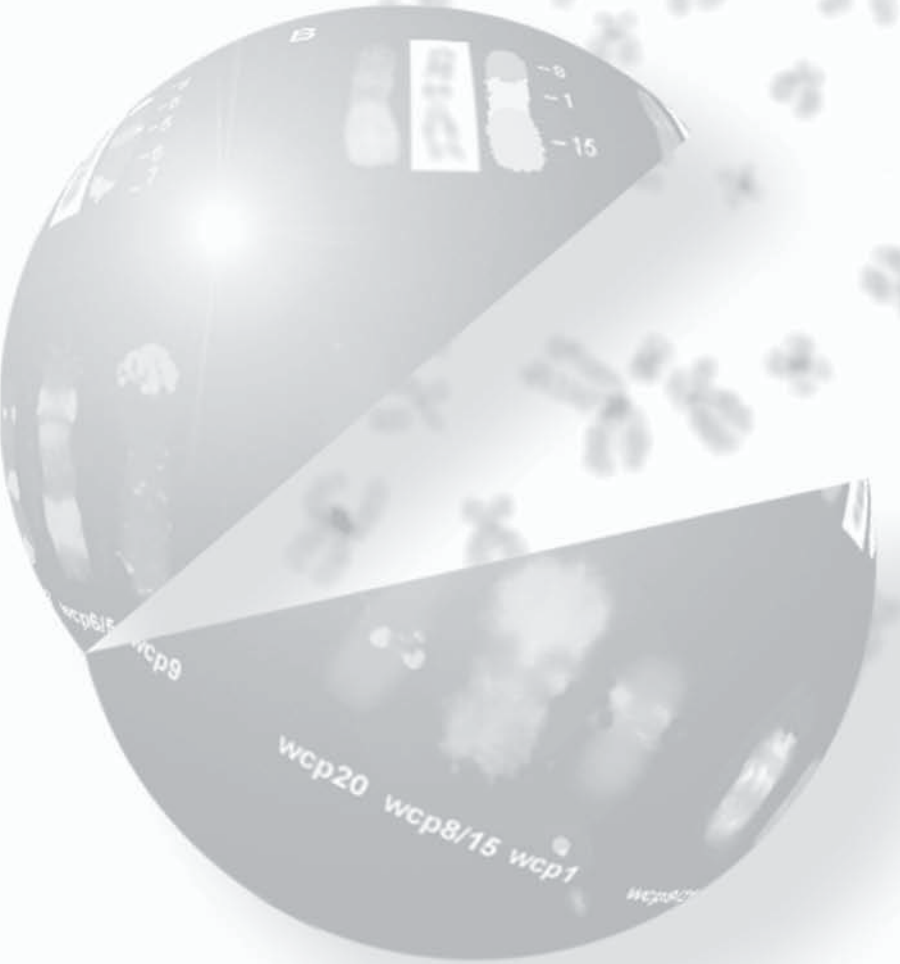
Chapter 7

General discussion



Chapter 7

General discussion

Chromosome abnormalities, which are frequently observed in haematological malignancies, constitute a prognostic factor for the outcome of disease and have therapeutic consequences. However, in approximately 50% of all new acute leukaemia cases, no or only non-specific clonal abnormalities are present. This absence of specific chromosomal abnormalities might be explained amongst others by the presence of small, cryptic rearrangements, which cannot be detected using conventional cytogenetic banding techniques. Therefore, specific aberrations may exist that have not been detected yet. The aim of this thesis was to identify new cryptic chromosome rearrangements, which could be important for diagnosis, prognosis and treatment stratification, and further investigate recently described aberrations using molecular cytogenetic techniques. In general, research on chromosome abnormalities in leukaemia can be divided into three main phases. In the first phase new chromosomal abnormalities are identified. This is followed by the characterisation of newly identified chromosome aberrations (second phase), after which the clinical relevance of the chromosome aberrations can be evaluated (third phase). The research described in this thesis included subjects of each of these 3 phases.

Identification of new chromosome abnormalities

For the identification of new chromosome abnormalities, conventional karyotypes can be investigated, but cryptic chromosome aberrations will be missed easily or only partly detected. Therefore, complementing the conventional karyotypes with molecular cytogenetic techniques, such as FISH, SKY and CGH, is helpful. FISH is a rapid technique, with high sensitivity and specificity, which can be used on both non-dividing cells and metaphases. A major limitation, however, is that only a few abnormalities can be investigated simultaneously, and data are obtained only for the loci targeted by the probes used [1]. Thus, FISH for the detection of aberrations requires a priori knowledge of the possible abnormality, which is usually available if the sample to be analysed has relapsed or is being followed in minimal residual disease (MRD) studies. For diagnostic samples this information mostly is not available. Depending on the leukaemia subtype, aberrations most likely to occur in this specific phenotype can be investigated, but this results in non-complete cytogenetic data, excluding the possibility of identifying new specific chromosome abnormalities. This problem is largely reduced by combining conventional cytogenetics with genome-wide multicolour FISH-based techniques, such as spectral karyotyping (SKY) [2, 3] or multicolour FISH (M-FISH) [4], which allow 24-colour karyotyping of chromosomes. Having identified chromosome rearrangements using these techniques, classical FISH is a well-suited technique to confirm the aberrations

observed and to investigate them in more detail. Additionally, FISH can be used to screen large cell populations and patient series very fast. In Chapter 2, we used SKY to identify new cryptic chromosome rearrangements in 25 AML, 17 MDS and 8 ALL patients with mostly complex karyotypes that had been karyotyped for routine diagnostic purposes. Chromosomes 2, 5, 7, 12, 17, 21 and 22 were more frequently involved in structural chromosome aberrations than could be seen from conventional karyotyping only, and SKY elucidated the composition of 88 marker chromosomes. Additionally, in 5 cases we observed 6 rearrangements of apparently normal chromosomes: a der(2)t(2;3)(q3?;q?) and a der(7)t(7;17)(q?;p?) in AML, a der(10)t(10;20)(p1?;?) and a der(16)t(11;16)(?;?q) in MDS and a der(1)t(1;22)(p3?2;q1?) and a der(21)t(5;21)(?;p?) in ALL. These aberrations were, however, only identified in one case each. Additional cases with identical cryptic abnormalities need to be identified, after which the aberration can be studied in more detail. This might result in the discovery of novel recurrent cryptic, but specific translocations, which could be important in diagnosis and could have prognostic value. Having identified possibly present cryptic translocations, new fusion genes or genes deregulated as a consequence of the translocation might be discovered, which could uncover new pathways playing a role in leukaemogenesis.

Several other groups also have shown that SKY and M-FISH are useful for refining conventional karyotypes and elucidating composition of marker chromosomes or incompletely identified abnormalities [5-7]. However, there are some intrinsic limitations with these techniques, as fluorescence interference at the border of 2 combinatorial-labelled probes can lead to classification problems, especially apparent for the detection of small translocations or insertions [8]. Azofeifa *et al.* [9] showed that this problem can be partly overcome by the introduction of more fluorescent labels. The sensitivity is dependent on the degree of condensation of the obtained chromosomes. However, intrachromosomal rearrangements and small deletions or amplifications will still be missed. Multicolour banding techniques can help identifying intrachromosomal rearrangements [10-12], but for detection of deletions and amplifications, CGH is a better technique [3, 13-16].

Using CGH, we additionally analysed 40 cases, in particular with involvement of chromosomes 2, 5, 7, 12, 17, 21 and 22 as observed in the conventional and/or SKY karyotypes. Regions on chromosomes 3 (p21-p14.1), 5 (q31.2-q32), 7 (q11.1-q11.23 and q33-q35), 12 (p12.3-p12.1) and 17 (p13-p12) were frequently lost, whereas regions on chromosomes 8 (q23-q24.1), 21 (q22.1-q22.3) and 22 (q13.2-q13.3) were frequently gained (Chapter 3). Our findings confirmed earlier publications [6, 17-20], suggesting that genes present in these minimally lost or gained regions are important for leukaemogenesis. Additional investigations concerning these regions, using FISH or array-CGH, might narrow down these regions to single genes. Those genes might represent tumour suppressor genes or oncogenes and lead to a better understanding of mechanisms contributing to leukaemogenesis. However, this might not be as simple as posed above, since for several frequently deleted chromosome regions, e.g. del(5q), del(7q) and del(20q), no genes have been identified yet. A favourable circumstance is that the sequence of the entire human genome is now available, which might help identifying candidate genes. Also, the use of expression profiles might facilitate the finding of likely genes implicated in the process of oncogenesis.

It should be noted that balanced chromosome rearrangements cannot be detected using CGH and the obtained data represent the genomic status of the major proportion of cells in the sample. Thus, heterogeneity within tumour samples, which is a well-known phenomenon also in leukaemia, may remain undetected [21]. In our experiments, we often observed gains or losses that did not reach the threshold value. These could be divided into two categories: those explained by the presence of unbalanced abnormalities in smaller cytogenetic subclones (percentage of leukaemic cells in samples of patients falling within this category: 8.5-70%), and those that remained unexplained when comparing them to conventional and SKY karyotypes (percentage of leukaemic cells in samples of patients falling within this category: 14-40%). Using FISH, we showed that the gains/losses in the first category represented true gains/losses, whereas those in the latter category could not be confirmed. Thus, heterogeneity in the sample, due to low numbers of leukaemic cells in the sample or the presence of subclones, can be observed as deviations in CGH profiles below threshold value. However, before conclusions can be drawn from this, additional experiments using different molecular cytogenetic techniques should be performed if possible.

Characterisation of newly identified chromosome abnormalities

As soon as more cases with identical chromosome abnormalities are observed, an aberration can be called recurrent, and the incidence can be investigated by screening larger patients series to identify new cases. Exact cytogenetic breakpoints can be assigned through FISH studies using locus specific probes. The whole human genome has now been sequenced, and therefore the human sequences harboured in BAC clones, which, amongst others, are frequently used as FISH probes, are known. Therefore, the sequence of the breakpoint-spanning clone can be used to determine the genes involved and investigate the breakpoints on a molecular level. Expression array studies can elucidate deregulated pathways in cases with identical chromosome aberrations, resulting in better understanding of leukaemogenesis and providing entries for therapy development. Additionally, detection methods for diagnostic purposes can be developed, such as fusion or split signal FISH. In fusion signal FISH, locus-specific probes for the two genes fused by the translocation are differently labelled with green or red fluorescent labels. If the translocation is absent, four separate signals will be visible, two of each colour, whereas one fusion signal, one green and one red signal will be observed in the case of a translocation. This type of probe has a relatively high number of false-positive fusion signals, due to close accidental proximity and due to 2D visualisation of target chromosomes in interphase nuclei. Extra-signal (ES) probes constitute a variant of fusion signal FISH, where part of probe for one gene remains at its original site. This results in an extra signal in a single colour if a translocation is present, thus reducing the number of false-positive fusion signals. Split signal FISH is particularly attractive for genes that can be fused to many different partner genes, e.g. the *MLL* gene, which has more than 60 translocation partners. Using this technique, probes flanking either side of the breakpoint region are differently labelled, resulting in two fused signals when no translocation occurred. If a translocation is present, one of the fused signals will split, resulting in one fused signal, one separate green and one separate red signal [1].

The development and application of such detection methods, i.e. split signal FISH assays, is described in Chapters 4 and 5. In Chapter 4, two assays for detection of the t(5;14)(q35;q32) variants that occur in T-ALL were validated on T-ALL cell lines known to bear the aberrations, and on T-ALL patients. We identified five new t(5;14)(q35;q32) cases involving *HOX11L2* out of 32 T-ALL cases. Thus, the assays are very helpful in routine diagnostics for the identification of t(5;14)(q35;32) positive cases. *HOX11L2* expression is suggested to occur only as a result of 5q35 rearrangements [22, 23]. All our t(5;14)(q35;q32) positive cases showed *HOX11L2* expression; however one case without t(5;14)(q35;q32), as confirmed by FISH for *HOX11L2* and *CSX*, did as well. Therefore, analysing *HOX11L2* expression only is not a good diagnostic test for identification of t(5;14)(q35;q32) in new T-ALL patients. Several groups have shown that *HOX11L2* expression in T-ALL patients is associated with a poor prognosis [24, 25], but recently another study, based on a larger patient series, showed no difference in clinical outcome between patients with and without *HOX11L2* expression [26]. The mechanism of *HOX11L2* upregulation through the t(5;14)(q35;q32) is currently not known. One possible explanation posits ectopic activation of *HOX11L2* following juxtaposition with regulatory elements present on chromosome 14 in the far downstream region of *BCL11B*. Further research might provide better insight into the mechanism of *HOX11L2* upregulation, and into the leukaemogenic process occurring in cases with t(5;14)(q35;q32). This might also create entries for the development of new therapeutic strategies.
In Chapter 5, we screened 84 leukaemia cases using a *NUP98* specific split signal FISH probe combination. Our results show that the probe combination used is useful for detection of *NUP98* rearrangements, both on interphase nuclei and metaphases. We identified an unbalanced t(11;21)(p15;p13) involving *NUP98* and *NCL* in one case, and a t(4;11)(q1?3;p15) with expression of a *NUP98-RAP1GDS1* fusion product in another case. The unbalanced t(11;21)(p15;p13) represents a new rearrangement involving *NUP98*. Both our *NUP98* rearranged cases showed 11p abnormalities at diagnosis, indicating that *NUP98* rearrangements occur with a certain frequency in cases with 11p abnormalities (in our series 6%) , but are rare in our relatively small and selected group of leukaemia cases (2%). Excluding the 2 cases described in Chapter 5, thus far only 55 *NUP98* rearranged AML/MDS cases have been reported worldwide [27], demonstrating the rare nature of *NUP98* rearrangements in general. Large-scale screening will provide better estimates on the *NUP98* frequency and new rearranged cases will be detected. This will allow determination of the prognostic value, although this might be complicated by the seemingly rare nature of *NUP98* rearrangements. Moreover, differences in clinical outcome within the small group of *NUP98* rearranged cases might be observed resulting from heterogeneity in partner genes.

Evaluation of clinical relevance

Having identified a new recurrent abnormality, the prognostic value of the chromosome aberration can be evaluated. One very well characterised type of chromosome abnormality in acute leukaemia is the occurrence of *CDKN2* deletions. However, the publications on the prognostic value of *CDKN2* inactivation in acute leukaemia are inconsistent, probably due to

differences in methods used and disease heterogeneity in the studied patient groups [28-42]. Using Southern Blotting, RT-PCR or loss of heterozygosity (LOH), techniques used in several studies, *CDKN2* deletions might be missed, as tumour heterogeneity can only partly be detected and small aberrant, homozygous deleted populations can be missed easily. With both Southern blotting and FISH, very small deletions might be missed depending on the size of probes used, but unlike Southern blotting, FISH can detect tumour heterogeneity of small subclones. These technical issues and differences in study groups could result in the observed differences in prognosis between several studies. For these reasons, FISH is the most suitable technique for detecting *CDKN2* deletions at this moment. Additionally, most previous studies did not discriminate different ALL phenotypes, but studied them as one group. In our single centre study, using FISH, investigating 109 common/preB-ALL cases, we did not observe a significant different clinical outcome between patients with and without *CDKN2* deletions (Chapter 6). *CDKN2* deletions, mainly of *p16*, result in inactivation of genes at this locus. The alternative mechanism of *CDKN2* inactivation, *p15* promoter hypermethylation, has been observed frequently in AML and to a lesser extent in ALL and MDS. This suggests that inactivation of genes at this locus contribute to leukaemogenesis in acute leukaemia. Future investigations, distinguishing different leukaemia subtypes and combining different techniques for the detection of *CDKN2* deletions and promoter hypermethylation, might result in more consistent views regarding the clinical outcome of patients with *CDKN2* inactivation.

Besides evaluating the prognostic value of chromosome abnormalities, new therapies can be designed based on insights in leukaemogenesis obtained from the characterisation of specific chromosome abnormalities. Additionally, the in-vitro-sensitivity for generally used therapeutic drugs, which can be correlated to the presence of certain chromosome abnormalities, can be investigated. This will lead to better understanding of the clinical relevance of chromosome aberrations.

Research versus diagnostics

Interests of researchers mostly differ from priorities of scientists involved in diagnostics. Researchers are interested in identifying new aberrations, elucidating molecular abnormalities underlying chromosome rearrangements, elucidating genes and pathways involved in leukaemogenesis and development of therapeutics, and to a lesser extent in development of new tools for diagnosis. Since cryptic chromosome aberrations, that have not yet been identified, might be present in approximately 50% of new leukaemia cases, research is conducted to achieve a complete genetic picture of each case studied. Combination of conventional, molecular cytogenetic and molecular biological techniques results in the most detailed information for each case. By comparing cases, new cryptic specific aberrations can then be identified, which might result in the identification of new fusion genes or deregulation of so far unknown tumour suppressor and/or oncogenes. This will provide more insight into mechanisms of tumourigenesis, leading to new entries for therapy development. Based on researchers' findings, new diagnostic tests can be developed for easy, fast or cost-effective

detection of these newly identified aberrations in new acute leukaemia cases. Consequently, diagnostics will greatly benefit from this research.

At diagnosis, different prognostic factors next to age and gender, such as cytogenetics, leukocyte (WBC) count, age, gender, in vitro drug sensitivity and immunophenotype, need to be investigated quickly and cost-effectively. Regarding chromosome aberrations, the best way to establish the presence of each prognostically important abnormality may be different. For instance, cytogenetic observation of the t(9;22)(q34;q11), detection of this translocation with FISH or quantitative analysis of the *BCR-ABL* fusion transcript are all different ways to determine whether this abnormality is present. However, for conventional karyotyping, bone marrow cells need to be cultured (1-2 days) and harvested to obtain metaphases. After this, time must be taken into account to analyse metaphases, as heterogeneity is possible in the leukaemic sample and a single metaphase is not sufficient to draw conclusions. For FISH analysis, uncultured cells can be used as well, which saves time, but the technique requires at least one night of hybridisation for optimal results before analysis can start. RNA isolation and cDNA preparation are relatively short procedures. Thus, cDNA can be generated for quantitative RT-PCR in a short time period. However, the rapid method of isolating RNA, preparing cDNA and RT-PCR of one sample is not applicable on a routine basis, where several samples are collected before the procedure starts. Thus, in routine practice, diagnostics using these methods requires several days as well. On the other hand, for other abnormalities, such as deletions, or in cases as described below, RT-PCR may not be the most optimal way of detection.

Detection of rearrangements involving genes with many different partner genes, such as *MLL*, requires different considerations. *MLL* rearrangements, easily missed using conventional cytogenetics, are correlated with poor prognosis in most studies. Detection of these aberrations is thus important for correct therapeutic decisions. However, *MLL* fused to at least 37 different partner genes, which makes it impossible to perform RT-PCR reactions. FISH using a split signal probe combination on the other hand can detect all *MLL* translocations in a single experiment, which makes this a more suitable technique than RT-PCR for detection of this type of aberrations. As soon as the partner chromosome is known, the partner gene can be identified and the fusion product can be detected quickly in relapse samples or for following MRD. *MLL* partial tandem duplications (see below) cannot be detected with FISH, but can be detected using Southern Blot or RT-PCR.

Some specific chromosome aberrations can also be discriminated based on expression patterns elucidated by microarray analysis. In AML, it has been shown that the t(8;21)(q22;q22), inv(16)(p13q22) or t(15;17)(q22;q21) can be discriminated by expression of 1-3 genes [43], whereas t(12;21)(p13;q22), 11q23 rearrangements and t(9;22)(q34;q11) in ALL have been shown to have more heterogeneous expression patterns with many genes needed to discriminate these aberrations [44]. Microarray analysis in diagnostics is, similar to cytogenetics, both time-consuming and expensive. Information obtained from these experiments in a research setting may be used to select genes whose expression discriminates a specific translocation, or indicates drug resistance and treatment outcome [45]. If a translocation is discriminated by expression of a single or just a few genes, this can be quickly analysed with RT-PCR. However,

if expression of many genes discriminates a subset of patients, other techniques for diagnosing this patient group are more appropriate. Expression array analysis additionally has shown that samples of AML patients with normal cytogenetics cluster in groups not characterised by a certain chromosome abnormality [43], or show expression profiles more closely resembling those of AML samples with translocations [46].

In the majority of leukaemia patients with normal karyotypes, molecular cytogenetic techniques have shown that indeed no microscopically detectable abnormalities are present (Chapter 2). Patients with normal karyotypes are usually stratified in the intermediate risk group regarding therapy and prognosis. However, as also suggested by the expression array results, these patients are heterogeneous at the molecular level [43, 46]. It has been shown that clinical outcome in this group can be predicted by the presence or absence of mutations or changes in expression of specific genes, usually not associated with recurrent chromosome aberrations [47]. Examples of these mutations or changes involve *FLT3* (13q12), *MLL* (11q23), *C/EBP*α (19q13.1), *BAALC* (8q22.3) and *NPM* (5q35).

The FLT3 protein is involved in regulation of proliferation, differentiation and apoptosis of haematopoietic cell progenitors. The most common mutation occurring in normal karyotype AML is the *FLT3* internal tandem duplication (ITD) [48], but also mutations at the Asp835 in the activation loop of the kinase domain (TKD) are possible [49]. The mutations are mutually exclusive and both result in constitutive activation of the protein [50]. Both ITD and TKD are associated with poor prognosis [51]. Besides *FTL3* ITD and TKD in AML patients with normal karyotypes (13-34% and 6-7% respectively), *FTL3* mutations are also frequently observed in other AML patients e.g. with t(15;17) or *MLL* abnormalities (30-39%) [50].

In approximately 10% of AML patients with normal cytogenetics, partial tandem duplications (PTD) in the *MLL* gene, associated with poor prognosis, are observed [47]. In expression microarray analysis, these aberrations cluster differently from translocations involving *MLL* and thus leukaemic transformation is thought to occur in a different way than in the case of translocations [52].

C/EBPα belongs to the CCAAT/enhancer binding protein family, which is involved in the balance between cell proliferation and terminal differentiation. C/EBPα itself is involved in granulocytic differentiation of common myeloid progenitors [53]. The protein occurs in two isoforms, a 42 kD protein, which is the functional one, and a 30 kD protein with unknown function [53]. Missense or nonsense mutations in AML produce a truncated 42 kD protein. The 30 kD protein, normally made in low amounts compared to the 42 kD form, has been shown to have a dominant-negative effect when expressed at levels equal to or greater than the 42 kD form, as happening in AML patients [54, 55]. *C/EBP*α mutations seem to be correlated with a better relapse-free or overall survival [56].

Aberrant expression of *BAALC* is a recurrent phenomenon in the group of patients with normal cytogenetics. Normally in CD34+ cells, *BAALC* is expressed at levels comparable to expression levels in normal brain. In haematological malignancies, strong overexpression has been observed, both in patients with and without cytogenetic abnormalities [57]. *BAALC* overexpression has been shown to be associated with a poor prognosis [58]. The mechanism underlying this abnormal expression is currently unknown, as no intragenic mutations in have been found [57]

Recently, aberrations of *NPM* have been observed to be involved in AML patients with normal karyotypes. NPM is a protein that shuttles between the nucleus and cytoplasm, but it is most prominently present in the nucleoli. In patients with normal karyotypes, cytoplasmic dislocation of the protein has been observed [59]. This dislocation is caused by mutations in exon 12, resulting in a frame-shift in the region encoding the C-terminal part of the protein. Patients with these mutations appear to show a good response to induction chemotherapy [59].
Besides these examples, other mutations or changes in expression level may exist in the group of leukaemia patients with normal cytogenetics that remain to be identified. This implies that in this group of patients the percentage with cryptic chromosomal aberrations will probably be lower than the approximately 50% stated earlier in this discussion. However, as several cryptic aberrations have been identified during the last 10 years, e.g. t(12;21)(p13;q22) [60-62], t(5;14)(q35;q32) [63, 64], and the very recent discovery of the episomal *NUP214-ABL1* fusion [65], it is likely that new abnormalities of this kind will be identified next to the mutations and expression level changes described above.
For diagnostic samples, at least the (cyto)genetic factors with known prognostic significance correlated to a leukaemia subtype should be investigated. As discussed above, different technical possibilities exist for most chromosome aberrations, the most suitable (combination) of which should be chosen. Different disciplines with their own technical specialities, cytogenetics, morphology, immunophenotyping and haematology, are involved in diagnosis of leukaemia, and they join forces to perform the different tests needed for the most optimal diagnosis. Together, the different disciplines can make an accurate diagnosis within 3-7 days, which is a reasonable time before clinicians have to start differential therapy. For relapsed cases, that showed a specific chromosome aberration at diagnosis, and for monitoring of MRD, (quantitative) expression analysis of the known aberrations, next to flowcytometric analysis [66], is very useful. It yields quick results and needs to be done only by the discipline specialized in molecular techniques. However, it is not a genome wide screening technique: no data will be available about other aberrations, and disease progression marked by additional chromosome abnormalities will not be visible.
One could argue that a combination of FISH and molecular techniques can replace the use of conventional cytogenetics at diagnosis completely. However, the presence of additional chromosome aberrations besides specific chromosome abnormalities with known prognostic significance might influence this prognostic value. Analysing expression of genes known to be associated with a better or poorer prognosis might be a fast way for treatment stratification and it will be a good tool to identify aberrant expression patterns with clinical consequences in leukaemia patients. However, if the mechanism underlying this abnormal expression is not known, one would want to identify the underlying mechanism causing the changes in expression levels, as this knowledge may contribute to the development of therapies specific for certain groups of patients. A combination of molecular genetic and (molecular) cytogenetic techniques provides a good basis for identifying both mutations at the molecular level and cryptic chromosome aberrations in patients with normal karyotypes. Furthermore, conventional cytogenetics at diagnosis, unlike FISH and molecular techniques, is a genome wide screening tool, providing information about heterogeneity within the tumour sample, which might be linked

to disease progression. Additionally, it provides a starting point for research. As described in this thesis, new chromosome aberrations and (complex) marker chromosomes will be observed, which can be investigated in more detail using standard and multicolour FISH techniques. This will lead to identification of new specific chromosome abnormalities, which might turn out to be new prognostic factors, especially in leukaemia subtypes that are not very well characterised yet. Therefore, besides expression analysis, conventional cytogenetics, complemented with FISH, SKY and array-CGH for optimal detection of balanced translocations, inversions and chromosomal imbalances, will continue to be an important tool in diagnosis of leukaemia in future diagnostics.

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Summary

Specific chromosome aberrations are observed in 50% of all new acute leukaemia patients. As a result of chromosome aberrations, genes located at the breakpoints can be disrupted and fusion genes can be formed. In addition, genes can be lost or amplified. An increasing number of these rearrangements are specific and can be correlated with diagnosis, prognosis and response to therapy. In the other 50% of new leukaemia patients no or non-specific abnormalities are found. It is thought that also in this group specific chromosome abnormalities are present, but that these are cryptic and not detectable using conventional karyotyping. The aim of this thesis was to identify these cryptic chromosome aberrations.

In Chapter 2, we used spectral karyotyping (SKY) to identify new cryptic chromosome rearrangements in 25 acute myeloid leukaemia (AML), 17 myelodysplastic syndrome (MDS) and 8 acute lymphoblastic leukaemia (ALL) patients. SKY showed that in AML chromosomes 5 and 17, chromosomes 2, 5, 12, 17 and 22 in MDS and chromosomes 7, 12 and 21 in ALL were involved in structural rearrangements more frequently than observed with conventional karyotyping. In 5 cases we observed 6 rearrangements of apparently normal chromosomes: a der(2)t(2;3)(q3?;?) and a der(17)t(7;17)(q?;p?) in AML, a der(10)t(10;20)(p1?;?) and a der(16)t(11;16)(?;?q) in MDS and a der(1)t(1;22)(p3?2;q1?) and a der(21)t(5;21)(?;p?) in ALL. Future investigations elucidating the exact breakpoints of these translocations will show whether these abnormalities are recurrent and identify the possible genes involved.

The cases with involvement of chromosomes 2, 5, 7, 12, 17, 21 and 22 were subsequently analysed with comparative genomic hybridisation (CGH) to determine whether the rearrangements led to gains and losses and to delineate the regions involved (Chapter 3). Besides significant gains and losses, we observed still relevant gains and losses that did not reach threshold values, in part due to a high proportion of normal cells in the tumour sample. Fluorescent in situ hybridisation (FISH) analysis showed that some of those gains/losses below threshold could be explained by unbalanced rearrangements present in minor cytogenetic subclones as observed in conventional and SKY karyotypes. High-level amplifications of 3q, 8q, 10q and 11q were observed. Comparison of different cases in the overall group showed that regions 8q, 21q and 22q were frequently gained. Additionally, regions 3p, 5q, 7q, 12p and 17p were frequently lost. In 18 AML cases, minimal lost regions were 3p24.2-p21.3, 3p21.1-p13, 5q31.2-q32, 7p22-q11.23, 7q22-q35, 12p13.3-p12.1, 16q22-q24, 17p13-p12 and 17q21.2-q21.3, and minimal gains were observed for 8q21.3-q24.3 and chromosome 22. In 15 MDS cases 3p26-p14.3, 5q31.2-q32, 7q31.1-q36 and 17p13-p12 were the minimally lost regions, whereas 8q23-q24.1 and 21q22.1-q22.3 were gained. In the ALL group, only gains of chromosomes 10, 12 and 21 were observed in more than one case. Most of these gains and losses have been observed previously as well, suggesting that genes present in these minimally lost or gained regions might be important for leukaemogenesis.

In Chapter 4, we developed split signal FISH assays for both *HOX11L2* and *CSX*, for metaphase and interphase detection of t(5;14)(q35;q32) possibly present in T-ALL patients. The assays were validated using T-ALL cell lines and patients. We did not identify cases with a t(5;14)(q35;q32) involving *CSX*, but we did identify five cases with t(5;14)(q35;q32) involving

HOX11L2 out of 32 T-ALL cases. All five positive cases showed *HOX11L2* expression, as well as one case without t(5;14)(q35;q32). Cases with t(5;14)(q35;q32) involving *HOX11L2* did not show *TAL1* abnormalities, whereas five *HOX11L2* negative cases did, suggesting that *TAL1* and *HOX11L2* expression, or *TAL1* aberrations and the t(5;14)(q35;q32) involving *HOX11L2* are mutually exclusive.

In Chapter 5, we used split signal FISH for detection of *NUP98* rearrangements. To date 16 translocations and one inversion involving *NUP98* have been described in haematological malignancies, and in all cases fusion genes are formed. To investigate whether other rearrangements exist that have not been identified yet, we analysed 84 leukaemia cases cases with AML, MDS or ALL with normal karyotypes or 11p abnormalities. In two cases, separated FISH signals were observed: we identified an unbalanced t(11;21)(p15;p13) with expression of a potential *NUP98-NCL* fusion products in a 1-year old boy with AML-M7, and a t(4;11)(q1?3;p15) with expression of the *NUP98-RAP1GDS1* fusion product in a 60-year old woman with AML-M0. Our results suggest that *NUP98* rearrangements are very rare. In addition, subclonal, cryptic *NUP98* deletions were observed in 5 patients with normal karyotypes.

As *CDKN2* deletions have been found with high frequencies in ALL, inactivation of this locus is thought to be important in leukaemogenesis and could have important clinical significance. However, the publications on the prognosis of *CDKN2* inactivation in acute leukaemia are inconsistent. Chapter 6 describes a single centre study using FISH, evaluating the prognostic significance of *CDKN2* deletions in 109 childhood common/precursor-B (c/preB) ALL patients. *CDKN2* deletions were observed in 34% of the cases. We did not observe a significant difference in disease free and overall survival between cases with and without *CDKN2* deletions using univariate and multivariate Cox regression analyses. No difference in *CDKN2* status was observed between diagnosis and relapse in 76% of diagnosis-relapse couples. However, we did observe deletions in a considerable number of cases, indicating that the genes harboured at the *CDKN2* locus contribute to leukaemogenesis.

In conclusion, the different chapters demonstrate examples of the stages distinguishable in research concerning chromosome aberrations in acute leukaemia. In the first phase, we identified five new chromosome abnormalities, each present in a single case until now. More cases need to be identified before these aberrations can be labelled as recurrent and additional research should be performed to obtain more insight into the breakpoints, incidence and clinical relevance. Additionally, we identified frequently lost and gained chromosome regions, which may be narrowed down to single genes involved in leukaemogenesis. In the second phase, we developed diagnostic split-signal FISH assays for easy diagnostic detection of the t(5;14)(q35;q32). Using these probe sets, we identified five new cases involving *HOX11L2* in our lab. For detection of *NUP98* rearrangements we used split signal FISH as well, and we showed *NUP98* aberrations are very rare, being present in 2/84 cases studied. As an example of the last phase in research, we showed that *CDKN2* deletions do not constitute a prognostic factor in childhood c/preB-ALL.

Samenvatting

Het lichaam van de mens is opgebouwd uit miljarden cellen. De verschillende typen cellen vormen de weefsels en organen en hebben daarin een eigen functie. De functie van de cellen is vastgelegd in genen op het erfelijke materiaal van de cel (DNA), dat verpakt is in chromosomen. Iedere menselijke cel heeft 46 chromosomen, 22 paren die zijn genummerd van 1 t/m 22 (autosomen) en 1 paar geslachtschromosomen (XX bij meisjes en XY bij jongens). Een chromosoom van elk paar is afkomstig van de vader, het andere van de moeder. De studie die zich met chromosomen bezig houdt, is de cytogenetica. Deze studie zoekt de 23 paren chromosomen bij elkaar en rangschikt de paren op basis van grootte en bandenpatronen. Deze rangschikking wordt beschreven in het karyotype. Door invloeden van buitenaf, zoals bijvoorbeeld UV-licht en radioactieve straling, maar ook stoffen in het voedsel, kan het DNA in de chromosomen beschadigd raken, waardoor genen niet goed meer werken, wat kan leiden tot het ontstaan van kanker. In verschillende soorten kanker worden vaak afwijkingen aan de chromosomen gezien. Ook bij leukemie is dit het geval. Leukemie is een vorm van kanker, die zichtbaar is in het beenmerg en in het bloed. De voorlopercellen, die zich normaliter tot verschillende soorten bloedcellen ontwikkelen, zijn aangedaan. Afhankelijk van het bloedceltype waartoe deze voorlopercel zich had moeten ontwikkelen, zijn verschillende vormen (subtypen) van leukemie te onderscheiden. Zoals bij andere vormen van kanker, worden in leukemie cellen vaak chromosomale afwijkingen waargenomen, die specifiek of niet-specifiek kunnen zijn. Specifieke afwijkingen zijn afwijkingen die kenmerkend zijn voor een bepaald subtype leukemie: de afwijking wordt opvallend vaak gevonden in leukemische cellen van patiënten met voornamelijk dat subtype leukemie. Niet-specifieke chromosomale afwijkingen zijn afwijkingen die in meerdere patiënten met verschillende subtypen leukemie worden gevonden. Ook worden niet-specifieke afwijkingen vaak in cellen van slechts één patiënt met een bepaalde vorm van leukemie waargenomen, terwijl de cellen van andere patiënten met dezelfde vorm van leukemie, heel andere chromosomale afwijkingen laten zien. Een veelvoorkomende soort chromosomale afwijking is de translocatie. Hierbij is materiaal van twee chromosomen uitgewisseld, waardoor twee afwijkende chromosomen ontstaan zijn, ieder afzonderlijk aangegeven met de afkorting der, gevolgd door het chromosoomnummer tussen haakjes. Wanneer bij deze uitwisseling geen chromosomaal materiaal verloren is gegaan, wordt gesproken over een gebalanceerde translocatie. Wanneer echter chromosomaal materiaal verloren is gegaan, bijvoorbeeld door verlies van één van beide afwijkende chromosomen, is er sprake van een ongebalanceerde translocatie. Ook de afwijkingen aan chromosomen kunnen in het karyotype beschreven worden. Bijvoorbeeld: een translocatie (t) waarbij materiaal van chromosomen 15 en 17 is uitgewisseld, wordt aangegeven als t(15;17). Hierachter kan, ook tussen haakjes, de precieze plaats aangegeven worden waar beide chromosomen zijn gebroken en verkeerd aan elkaar zijn geplakt.

Specifieke chromosomale afwijkingen worden waargenomen in ongeveer 50% van alle nieuwe patiënten met acute leukemie. Sommige specifieke afwijkingen, zoals bijvoorbeeld de t(15;17)(q22;q12) en t(8;21)(q22;q22) in AML, hangen samen met een gunstiger ziekteverloop (prognose) dan andere afwijkingen. Het behandelplan, dat voor een patiënt wordt gekozen,

is deels afhankelijk van de aanwezigheid van chromosomale afwijkingen met een betere of slechtere prognose. Ook kan de aanwezigheid van een bepaalde afwijking een indicatie geven of een patiënt slecht op de standaard chemotherapie zal reageren. Het is dus belangrijk om te weten welke chromosoomafwijkingen aanwezig zijn in de leukemische cellen.
In de overige 50% van de patiënten wordt een normaal karyotype waargenomen, of worden niet-specifieke afwijkingen gezien. Waarschijnlijk zijn in deze groep patiënten specifieke chromosomale afwijkingen aanwezig, die echter verborgen (cryptisch) zijn, waardoor ze niet m.b.v. normaal gebruikte cytogenetische technieken kunnen worden waargenomen. Het doel van dit promotieonderzoek was om dit soort cryptische chromosomale afwijkingen te identificeren, omdat deze afwijkingen van belang kunnen zijn bij het stellen van een juiste diagnose, het bepalen van de prognose en de classificatie van de leukemie. Dit werd onderzocht m.b.v. nieuwe moleculair cytogenetische en moleculair biologische technieken.
In het eerste deel van dit proefschrift (Hoofdstukken 2 en 3) worden twee van deze nieuwe moleculair cytogenetische technieken, SKY en CGH, gebruikt om nieuwe chromosomale afwijkingen te identificeren. Hiermee hebben wij vijf nieuwe cryptische afwijkingen gevonden, echter elk tot nu toe slechts in één patiënt. Ook hebben we chromosoomregio's gevonden, die in meerdere patiënten vaak verloren gegaan of juist extra aanwezig zijn. Door in de toekomst deze afwijkingen en regio's verder te bestuderen, kunnen waarschijnlijk nieuwe genen ontdekt worden die een rol spelen bij het ontstaan van de leukemie. Uiteindelijk kan dit leiden tot de ontwikkeling van nieuwe behandelingsmethoden. In Hoofdstukken 4 en 5 hebben we naar recente reeds beschreven afwijkingen gekeken. Dit deel van het proefschrift illustreert het soort onderzoek dat volgt wanneer een nieuwe specifieke chromosoomafwijking in meerdere patiënten geïdentificeerd is. Voor één van de afwijkingen die bestudeerd is, de cryptische en daarom moeilijk zichtbare t(5;14)(q35;q32), is een diagnostische test ontwikkeld om de translocatie beter en sneller te kunnen opsporen bij de diagnose van patiënten met leukemie. Met deze test hebben wij vijf nieuwe patiënten gevonden, die deze translocatie wel hadden, maar bij wie dit eerder niet was gezien met het traditionele chromosomenonderzoek bij diagnose. Voor een tweede groep afwijkingen, translocaties die het *NUP98* gen op het uiteinde van de korte arm van chromosoom 11 betreffen, hebben we gezocht naar nieuwe afwijkingen waarbij dit gen betrokken is. In totaal is materiaal van 84 patiënten onderzocht. Slechts twee van de 84 patiënten vertoonden afwijkingen in het *NUP98* gen, wat aangeeft dat deze afwijkingen zeldzaam zijn. Verder onderzoek is nodig om meer inzicht te verschaffen in de incidentie van deze afwijkingen en de mechanismen van het ontstaan van de leukemie, zodat ook voor deze twee soorten afwijkingen uiteindelijk betere behandelingsmethoden mogelijk zullen worden.
Hoofdstuk 6 is een voorbeeld van het volgende stadium in het wetenschappelijk onderzoek naar chromosomale afwijkingen in leukemie: wat betekent het hebben van chromosomale afwijkingen voor de behandelingsvooruitzichten van de patiënt? Wij hebben gekeken naar de prognostische waarde van verlies (deletie) van het *CDKN2* gen in kinderen met een bepaald subtype leukemie, voorloper B-ALL. Eerdere publicaties over de betekenis van deze deleties in diverse vormen van leukemie lieten tegenstrijdige resultaten zien. Bij de onderzochte groep kinderen met voorloper B-ALL uit het ErasmucMC - Sophia Kinderziekenhuis werd geen

verschil in prognostische waarde gevonden tussen patiënten met en zonder verlies van het *CDKN2* gen.

Samengevat: de verschillende hoofdstukken laten zien welke fasen van onderzoek nodig zijn om de betekenis van gevonden chromosomale afwijkingen te achterhalen. Wij hebben enkele nieuwe afwijkingen geïdentificeerd, ieder in slechts één patiënt, die nog helemaal aan het begin van dit traject staan. Er moeten nog meer patiënten worden gevonden die dezelfde afwijking in de leukemische cellen laten zien, voor het onderzoek kan verder gaan naar de volgende fase. In deze volgende fase, hebben wij bijgedragen aan de ontwikkeling van betere diagnostische methoden voor detectie van de t(5;14)(q35;q32) en aan een beter inzicht in deze translocatie en afwijkingen die het *NUP98* gen betreffen. Tenslotte hebben we ook aangetoond dat bij kinderen met voorloper B-ALL verlies van het *CDKN2* gen geen aantoonbare prognostische waarde heeft, een onderzoek dat in de laatste fase van het onderzoek naar chromosomale afwijkingen geplaatst kan worden.

Curriculum Vitae

Persoonlijke gegevens

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Opleidingen

1996-2000	Biologie, Katholieke Universiteit Nijmegen Doctoraal examen behaald in de moleculair/medisch biologische vakken
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Werkervaring en stages

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februari 1999 - juli 1999	Stage Moleculaire dierfysiologie, Subfaculteit Biologie, Katholieke Universiteit Nijmegen Biochemical alterations in the Zucker Diabetic Fatty rat, an animal model for diabetes mellitus type II

Publicaties

Laura J.C.M. van Zutven, Sandra C.J.M. Velthuizen, Ingrid L.M. Wolvers-Tettero, Jacques J.M. van Dongen, Tim S. Poulsen, Roderick A.F. MacLeod, H. Berna Beverloo, Anton W. Langerak
Two dual colour split signal FISH assays for detection of the t(5;14) involving *HOX11L2* or *CSX* in T-cell acute lymphoblastic leukaemia. *Haematologica; 89(6): 671-678*

Laura J.C.M. van Zutven, Ellen van Drunen, Moniek M. Wattel, Judith M. de Bont, Monique L. den Boer, Rob Pieters, Anne Hagemeijer, Rosalyn M. Slater, H. Berna Beverloo
CDKN2 deletions have no prognostic value in childhood precursor-B acute lymphoblastic leukaemia. *Leukemia 2005; 19: 1281-1284*

Laura J.C.M. van Zutven, Emine Önen, Sandra C.J.M. Velthuizen, Ellen van Drunen, Marry M. van den Heuvel, Angelo Veronese, Cristina Mecucci, Massimo Negrini, Georgine E. de Greeff, H. Berna Beverloo
Identification of *NUP98* abnormalities in acute leukaemia: *NCL* (2q37) as a potential new partner gene. (submitted for publication to *Genes Chromosomes & Cancer*)

Laura J.C.M. van Zutven, Ellen van Drunen, Sandra C.J.M. Velthuizen, H. Berna Beverloo
Identification of new cryptic chromosome aberrations in acute leukaemia using molecular cytogenetic techniques. (in preparation)

Laura J.C.M. van Zutven, Ellen van Drunen, Sandra C.J.M. Velthuizen, Rosalyn M. Slater, H. Berna Beverloo
CGH analysis of complex chromosome aberrations in acute leukaemia. (in preparation)

Olivier Hagens, Aline Dubos, Fatima Abidi, Gotthold Barbi, Laura Van Zutven, Maria Hoeltzenbein, Niels Tommerup, Claude Moraine, Jean-Pierre Fryns, Jamel Chelly, Hans van Bokhoven, Jozef Gécz, Hélène Dollfus, Hans-Hilger Ropers, Charles E. Schwartz, Rita de Cassia Stocco dos Santos, Vera Kalscheuer and André Hanauer
Disruptions of the novel *KIAA1202* gene are associated with X-linked mental retardation (submitted for publication to *Human Genetics*)

Appendix: SKY combinatorial labelling scheme

Label code	Label
A	Rhodamine
B	Texas-Red
C	Cy5
D	FITC
E	Cy5.5

Chromosome	Label
1	BCD
2	E
3	ACDE
4	CD
5	ABDE
6	BCDE
7	BC
8	D
9	ADE
10	CE
11	ACD
12	BE
13	AD
14	B
15	ABC
16	BD
17	C
18	ABD
19	AC
20	A
21	DE
22	ABCE
X	AE
Y	CDE

Dankwoord

Een balletje kan raar rollen….. Wie had er tijdens/na mijn eerste stage ooit verwacht dat ik nog eens in een lab zou gaan werken en een proefschrift zou schrijven? Ik niet in elk geval, en met mij denk ik verschillende anderen. Maar verplicht verschillende stages lopen (op verschillende afdelingen en vakgebieden) heeft ook voordelen: je krijgt een tweede kans om te ontdekken hoe leuk het onderzoek kan zijn. Die tweede stage heeft me dat laten zien, en ik heb er ook de afgelopen jaren nooit spijt van gehad dat ik aan het onderzoek, beschreven in dit boekje, begonnen ben. Niet alleen het onderzoek was erg interessant en leuk, maar vooral ook heb ik veel te danken aan de mensen die me hebben geholpen het werk gedaan te krijgen en die ervoor hebben gezorgd dat ik het altijd goed naar mijn zin heb gehad. Bedankt allemaal!

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Natuurlijk heb ik gedurende de afgelopen 5 jaar nog veel meer mensen om me heen gehad. Om te beginnen de mensen van lab 816 die hierboven nog niet genoemd zijn: Bep, Moniek, Cindy, Ina, Elsa, Lizette, Marjan, Tessa, Anne, Maureen, en ook Hannie en José voor jullie naar de 24e verhuisden, iedereen bedankt voor de interesse in mijn onderzoek, de hulp bij allerdaagse dingen (zoals het druppelen van glaasjes of meenemen van een glaasje in de FISH) en het enthousiasme om mij ook bij leuke, ingewikkelde en interessante patiënten te betrekken. Moniek, dank je voor je bijdrage aan het *CDKN2* stuk. Anne, zonder jouw hulp bij het RACE-en was het vast niet gelukt om dat stukje van het *NUP98* artikel op tijd af te krijgen, dank je wel. Verder horen bij het bovenstaande lijstje ook de vaste gasten van 816, An en de oogmelanomen-mensen die ook regelmatig even binnenliepen: Emine, Michael, Walter en Hennie: bedankt allemaal!
Naast het cytogenetisch lab heb ik ook behoorlijk wat tijd op het voormalige lab 734 (nu verdeeld over diverse labs en het hele land) doorgebracht. Alex, Iris, Linda, Ingrid, Mandy, Mauro, Magda, en de mensen die er in lab 659 zijn bijgekomen, bedankt voor jullie hulp op moleculair niveau en de prettige sfeer op het lab, die er voor zorgde dat ik me er goed thuis voelde ook al was ik er niet de hele tijd. Voor de inwijding in de wereld van CGH bedank ik Jacqueline en Bert. Ook wil ik Emine en Hannie bedanken voor hun tips en hulp bij de CGH als ik ergens tegen aan liep. Verder wil ik alle “nieuwe” collega’s van de postnatale cytogenetica bedanken voor hun interesse en het meeleven tijdens de laatste schrijfloodjes sinds mijn start bij jullie.

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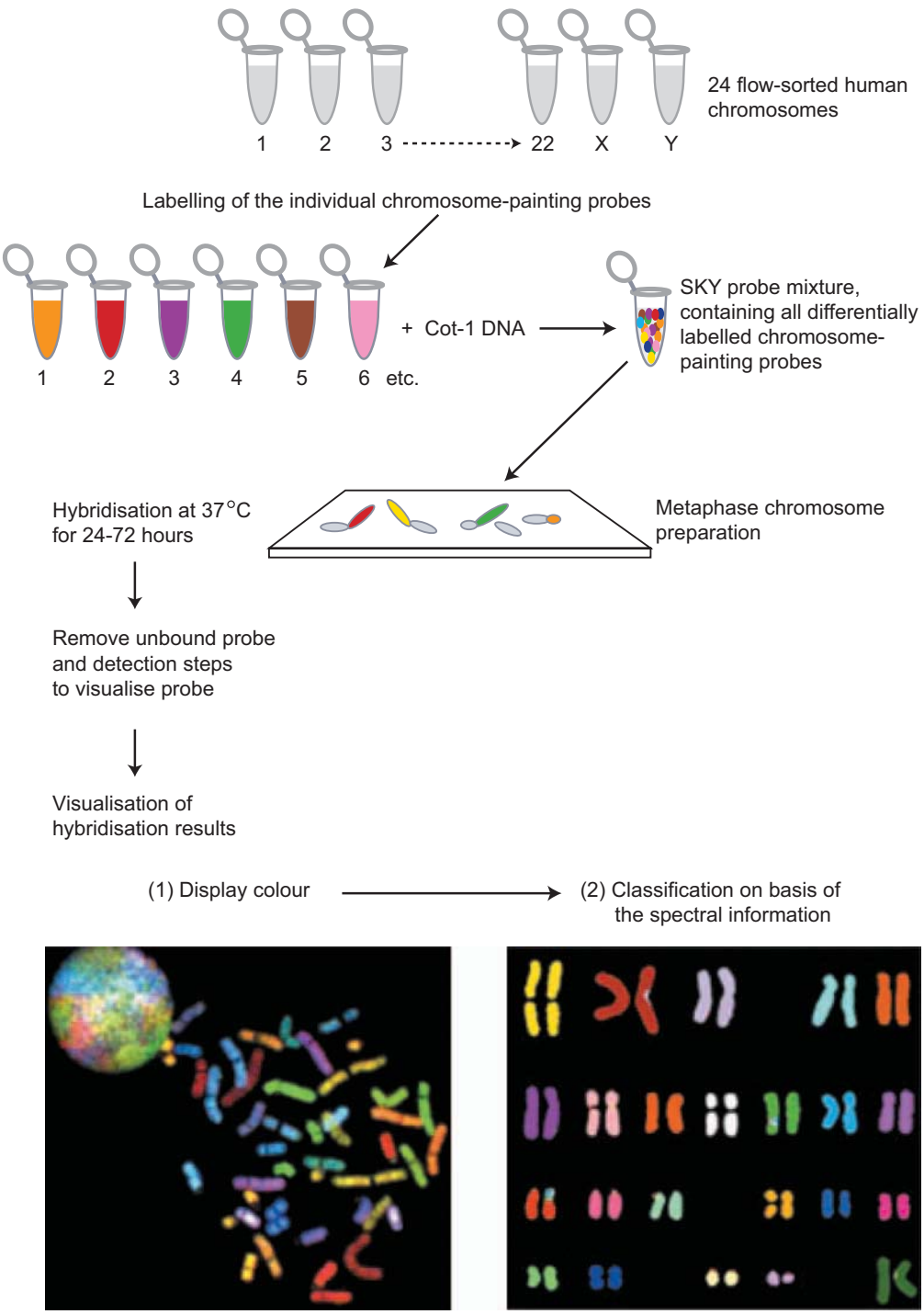
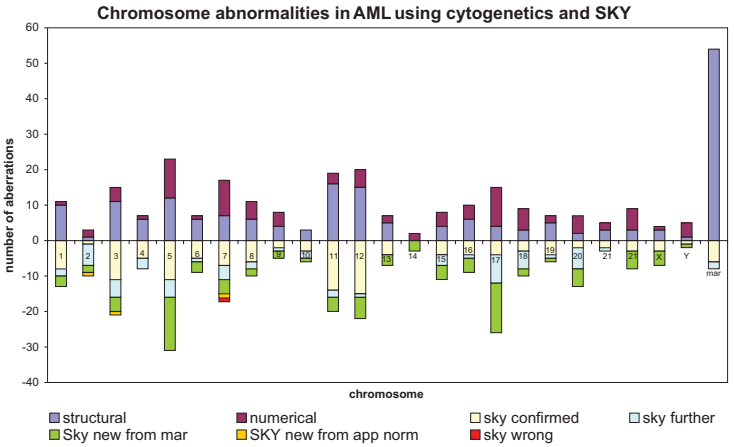
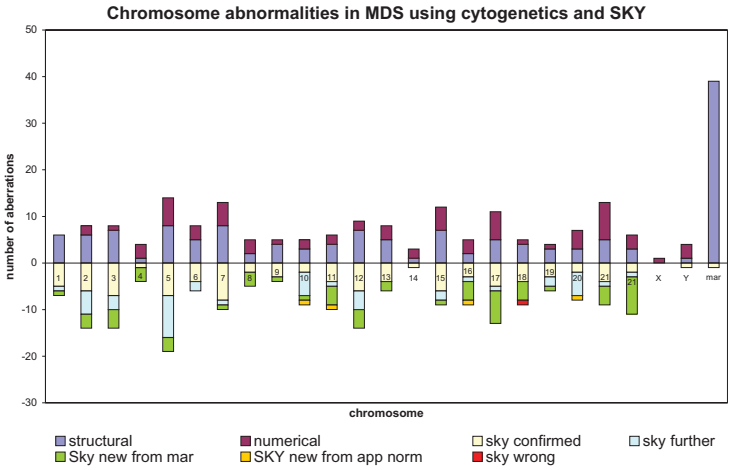
Tot slot, Maarten, hartstikke bedankt voor alle steun, interesse en geduld de afgelopen 2,5 jaar en vooral de laatste maanden! Ik kan hier alles wat je voor me gedaan hebt wel in detail gaan opschrijven, maar het belangrijkste is dat jij weet hoeveel dat voor me heeft betekend, en dat heb ik je al vele malen eerder verteld!

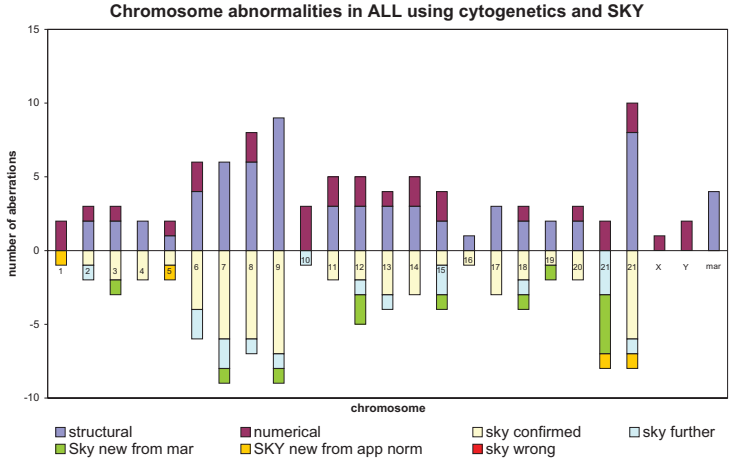
Chapter 1, figure 4



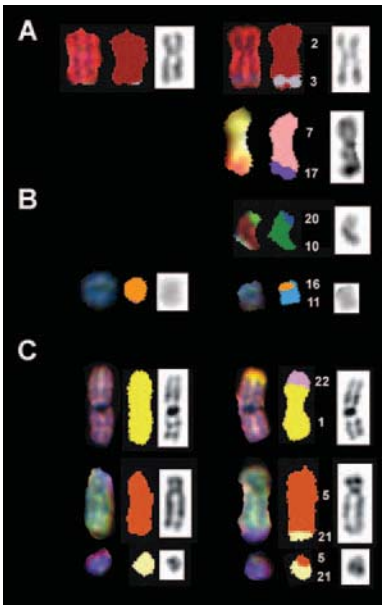
Chapter 2, figure 1



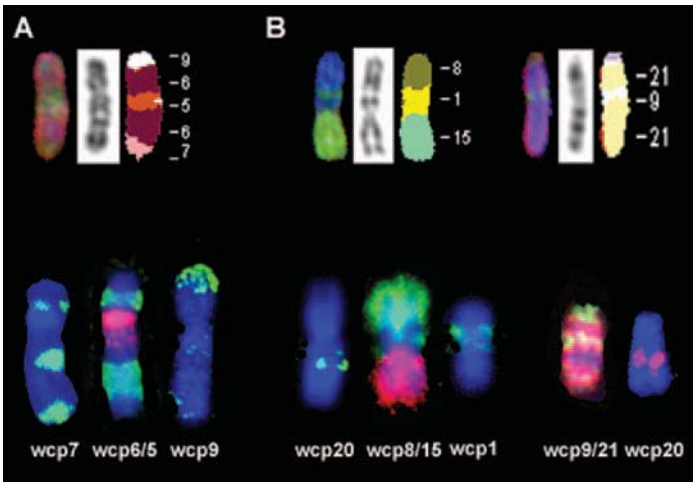




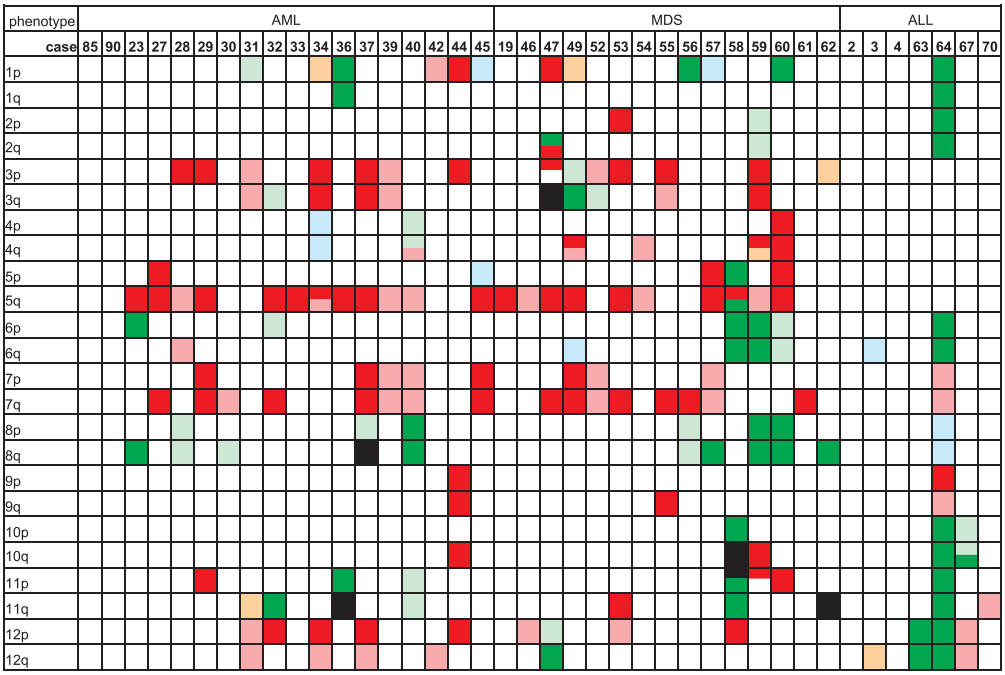
Chapter 2, figure 2

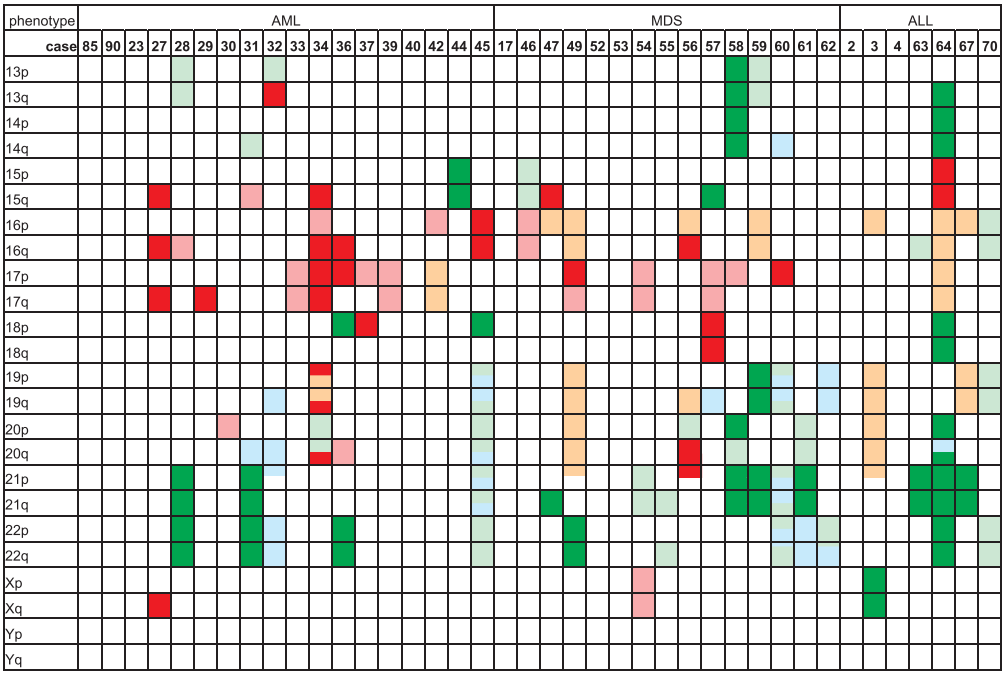


Chapter 2, figure 3

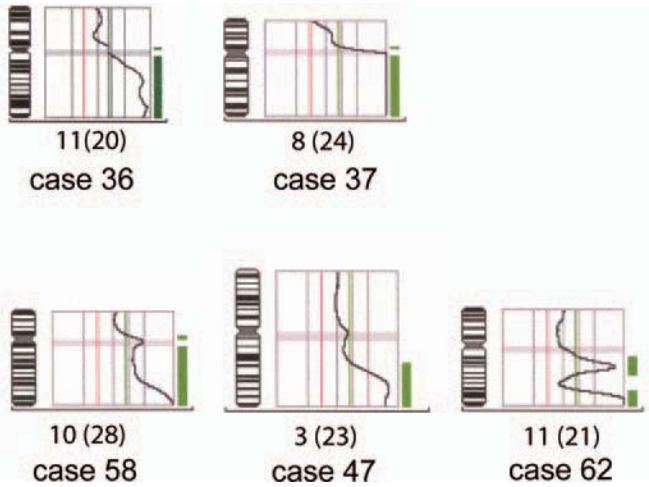


Chapter 3, table 1





Chapter 3, figure 1



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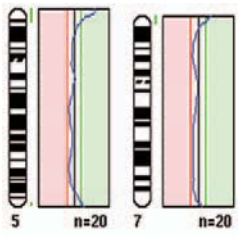
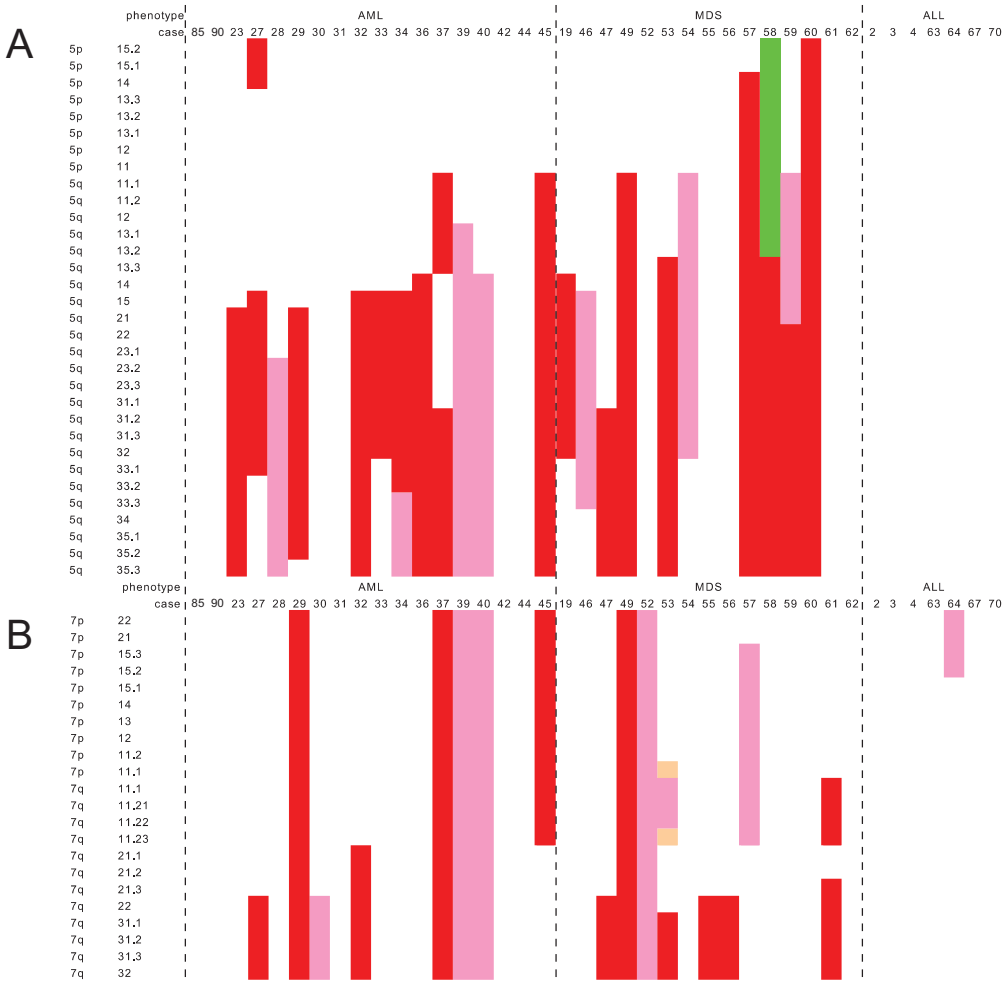
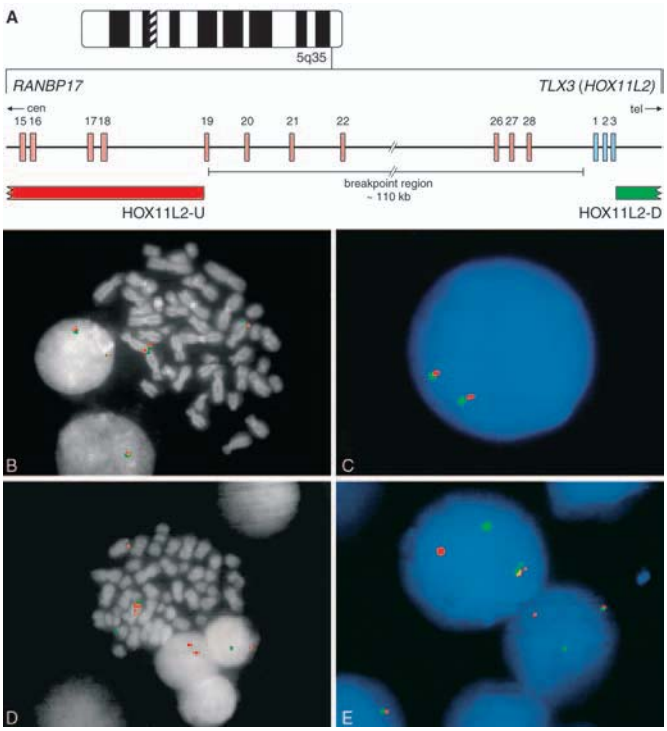


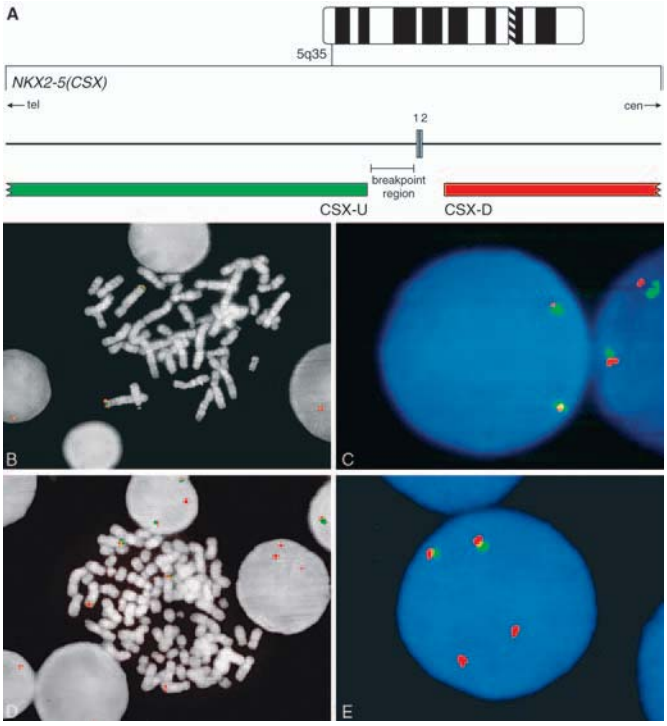
Chapter 3, figure 3



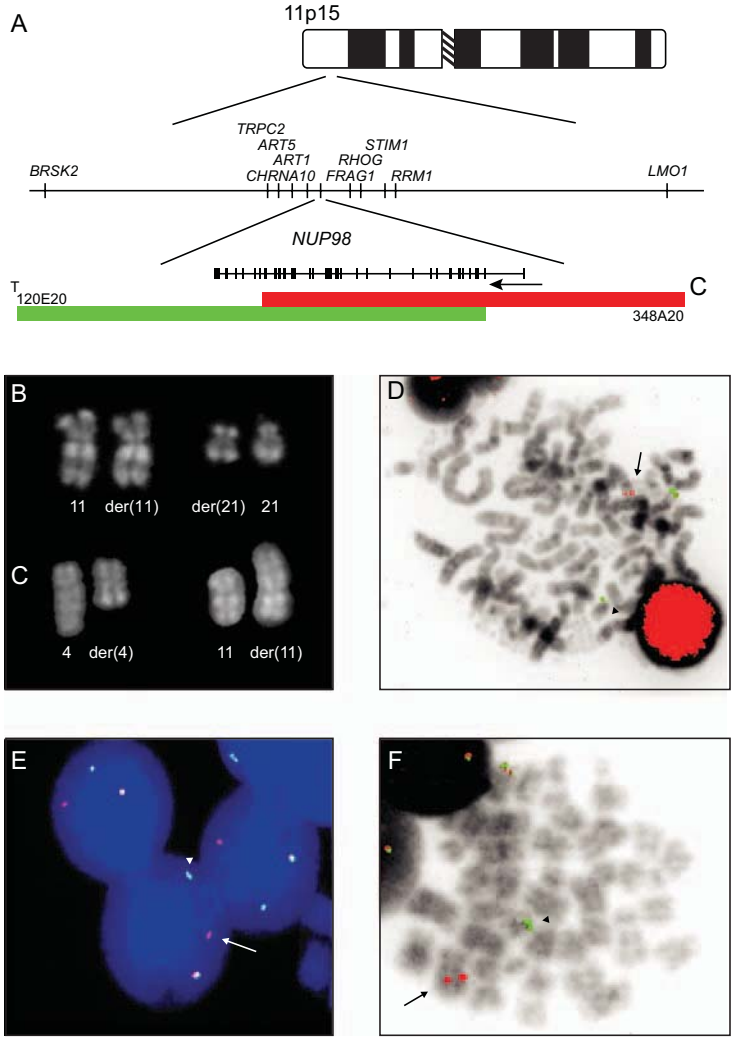
Chapter 4, figure 1



Chapter 4, figure 2



Chapter 5, figure 1



Chapter 5, figure 2