RECOMBINATION PROCESSES DURING HUMAN B-CELL DIFFERENTIATION

Recombinatie processen tijdens humane
B-cel differentiatie

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GENERAL ASPECTS OF RECOMBINATION PROCESSES DURING B-CELL DIFFERENTIATION

1.1 Antigen-independent B-cell differentiation in the bone marrow.

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During normal human B-cell differentiation, B cells undergo several immunogenotypic and immunophenotypic changes, finally resulting in the formation of antigen-specific B cells. B-cell differentiation starts in the bone marrow (BM), where immunoglobulin (Ig) genes undergo V(D)J recombination in order to express a unique Ig molecule, which is composed of two identical Ig heavy chains (IgH) and two identical light chains that can be either Ig kappa (Igκ) or Ig lambda (Igλ) (Figure 1). This part of B-cell differentiation is independent of antigens. B cells that express a surface membrane bound Ig molecule (SmIg), which is not autoreactive, leave the BM compartment and migrate to the periphery where they can encounter antigen. Ig genes of antigen-activated B cells are further diversified by somatic hypermutation (SHM), which results in affinity maturation. This process occurs in the germinal centers of peripheral lymphoid organs and is followed by further differentiation into Ig secreting plasma cells. During the germinal center reaction the constant domain of the IgH chain, through which the Ig molecule can exert its effector function, can be changed via class switch recombination (CSR).

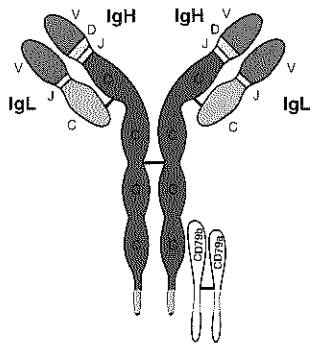


Figure 1 . Schematic representation of an Ig molecule, which is composed of two identical Ig heavy chains and two Ig light chains that could be either Igκ or Igλ. Both Ig heavy as well as Igκ and Igλ light chains are composed of a constant domain and a variable domain, which can be further devided in a variable, diversity, and joining part in case of the Ig heavy chains and in a variable and joining part in case of Ig light chains.

In this General Introduction, several aspects of B-cell differentiation are summarized, with a focus on normal and malignant recombination events. B-cell differentiation can be divided in two parts, i.e. antigen-independent differentiation in the BM (Section 1.1) and antigen-dependent differentiation in the periphery (Section 1.2). The three molecular processes, which play an important role in antigen-independent and antigen-dependent phases of B-cell differentiation are V(D)J recombination, SHM, and CSR. These three molecular processes are discussed.

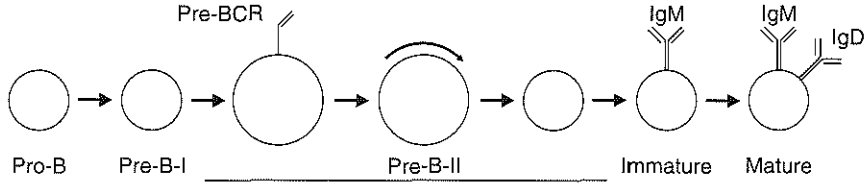
During differentiation, B cells can undergo malignant transformation. B-cell malignancies are clonal proliferations of B cells at various stages of differentiation, ranging from precursor B cells, which can transform in precursor-B acute lymphoblastic leukemia (precursor-B-ALL), to mature plasma cells that give rise to multiple myeloma (MM). B-cell

malignancies can be classified according to the corresponding normal B-cel differentiation stage. Several aspects of classification of B-cell malignancies are discussed in Section 1.3. Oncogenic events such as chromosomal translocations play an important role in malignant transformation. Chromosomal translocations in B-cell malignancies can roughly be divided in two categories (Section 1.4). The first category concerns translocations involving one of the Ig loci that result from aberrant V(D)J recombination, SHM or CSR. This type of translocations is most frequently found in mature B-cell malignancies. The second category includes translocations of two genes (not Ig genes) which result in the formation of a fusion gene that codes for a fusion protein with oncogenic potential. This type of translocations is primarily found in precursor-B-ALL. In the last part of the General Introduction (Section 1.5) the outline of this thesis is discussed.

1.1 ANTIGEN-INDEPENDENT B-CELL DIFFERENTIATION IN THE BONE MARROW

B-cell differentiation starts in the BM, where several B-cell differentiation stages (or subpopulations) have been defined based on differences in immunophenotype and immunogenotype. CD22 and CD34 positive pro-B cells are the first B-lineage cells that express components of the B-cell receptor, i.e. CD79a and CD79b (also known as Igα and Igβ) (Figure 2).[1,2] Mice pro-B cells express a precursor form of the B-cell receptor, which is composed of Igα, Igβ and calnexin. Crosslinking of Igβ elicits differentiation signals.[3]

The next differentiation stage is the pre-B-I cell that is characterized by expression of CD10 and CD19 together with components of the surrogate light chain in the cytoplasm that



Surface expression	CD10⁻ CD19⁻	CD10⁺ CD19⁻	CD10⁺ CD19⁺	CD10⁺ CD19⁺	CD10⁺ CD19⁻	CD10⁻ CD19⁺
Intracellular expression	(TdT)	TdT RAG CyVpreB Cyλ14.1	CyVpreB Cyλ14.1 CyIgμ	RAG CyVpreB Cyλ14.1 CyIgμ		
IGH	–	DJ→VDJ	VDJ	VDJ	VDJ	VDJ
IGK / *IGL*	–	–	–	VJ	VJ	VJ

Figure 2. Human B-cell differentiation scheme, which is mainly based on classification of Ghia *et al.* and Noordzij *et al.*[2,4]

consists of λ14.1 (λ5 in mice) and VpreB proteins.[2,4,5] In this subpopulation the recombination activating genes (*RAG1* and *RAG2*) are expressed and the recombination process is initiated at the *IGH* locus with D_H-J_H gene rearrangements.[1,4] In addition, the enzyme terminal deoxynucleotidyl transferase (TdT) is expressed, which is involved in the Ig gene rearrangement process by randomly adding nucleotides at the junctions of V (D) and J segments. After D_H-J_H rearrangements, the V_H gene segments become accessible and subsequent V_H to D_HJ_H joining takes place.[6,7] As soon as a complete in-frame V_H-D_H-J_H rearrangement is formed, an Igμ heavy chain is expressed in the cytoplasm (CyIgμ). It is assumed that the CyIgμ chain becomes first associated with the chaperone protein BiP in the endoplasmatic reticulum, which is replaced by the surrogate light chain forming a pre B-cell receptor (preBCR), that is subsequently expressed on the cell surface (pre-B-II cell).[8,9] Approximately half of the Igμ chains are unable to pair with the surrogate light chain. These cells that do not express a preBCR are negatively selected as has also been demonstrated in human B cells.[10]

Upon pre-BCR expression and activation, pre-B-II cells start proliferating. These proliferating precursor-B cells are called large preBCR$^+$ pre-B-II cells. It has been suggested that pre-BCR cross-linking with as yet unknown BM stromal cell ligands is required for induction of pre-B-II cell proliferation.[11] However, other studies suggest that BM ligands are not required for driving pre-B-II cell proliferation.[9,12] At this time point, RAG enzymes as well as TdT are down regulated, thereby preventing ongoing *IGH* gene rearrangements at the second allele, a process which is called allelic exclusion.[13] In addition to downregulation of the *RAG* genes, the V_H gene segments, but not the J_H gene segments, become less accessible.[7]

The clonal expansion phase is followed by G_1 arrest, during which the surrogate light chain is down regulated and the cells lose preBCR expression (small preBCR-negative pre-B-II cells). The *RAG* genes are re-expressed and RAG proteins are stable during G_1 phase allowing further V(D)J recombination.[14] At the transition from large preBCR$^+$ pre-B-II cells to small preBCR$^-$ pre-B-II cells, Ig light chain gene (*IGK* and *IGL*) recombination is initiated. Two models have been proposed for explaining the regulation of Ig light chain gene recombination: the ordered model and the stochastic model.[15-18] The ordered model proposes that *IGK* genes rearrange prior to *IGL*, while the stochastic model postulates that, in principal, the two types of Ig light chain genes rearrange totally independent, but that other factors render *IGL* gene rearrangements more difficult.[19,20] The latter would imply that *IGL* gene rearrangements can occur in the absence of *IGK* gene rearrangements and vice-versa. Chapter 3 of this thesis deals with the regulation of Ig light chain gene recombination. Multiple rearrangements might take place before a functional Ig light chain rearrangement results in Ig light chain expression (Igκ or Igλ). If the Ig light chain is able to pair with the pre-existing Igμ chain, the cell further differentiates into a surface IgM (sIgM) positive immature B cell. Autoreactive immature B cells are negatively selected. They are either deleted by apoptosis, become anergic, or they can be rescued via receptor editing, which is defined as auto-antigen induced secondary Ig gene rearrangement.[21-23] Immature B cells that are not autoreactive leave the BM and migrate to the periphery, where further differentiation takes place.

Transcription factors involved in B-cell differentiation

The first step in B-cell differentiation is B-cell commitment of a hematopoietic stem cell, which takes place in the BM.[24,25] Several transcription factors are involved in the process of B-cell commitment and early B-cell differentiation such as PU.1, Ikaros, E2A, EBF, and Pax5 (BSAP).[24,26-30] PU.1 and Ikaros are involved in both survival and competence of lymphoid progenitor cells and the regulation of genes, which are important during B-cell differentiation. [31-34] E2A and EBF act synergistically to initiate B-cell specific regulatory factors.[35,36] Pax5 is important for B-cell commitment, but also for maintaining the identity of B cells.[37] Early B-lineage transcription factors function in transcriptional hierarchy, from E2A to induction of EBF followed by Pax5.[38] In Figure 3, a scheme of B-cell differentiation is depicted in which developmental blocks are indicated that result from disruption of genes encoding transcription factors. These findings are primarily based on studies in knock-out mice, but might operate as well for human B-cell differentiation.

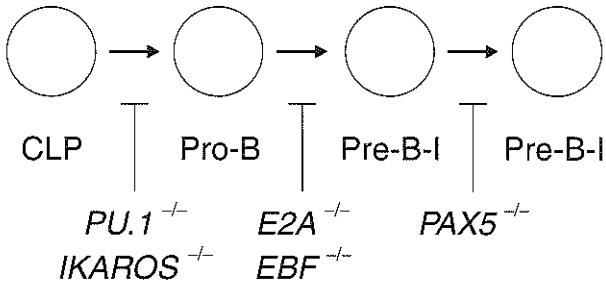


Figure 3. Simplified B-cell differentiation scheme. The positions of the differentiation blocks of the *PU.1, IKAROS, E2A, EBF* and *Pax5* knock-out mice are indicated.

E2A and EBF

The *E2A* gene encodes two proteins, E12 and E47, which arise through alternative splicing.[39] Both E12 and E47 have a helix loop helix (HLH) dimerization domain and a basic DNA binding domain.[40] E2A is widely expressed, but tissue specificity is, in part, determined by the dimerization partner. E2A exists only as homodimer in B cells. Deletion of the *E2A* gene results in a block in B-cell differentiation before the onset of Ig gene recombination.[36,41] Heterozygous *E2A* deletion leads to a two-fold decrease in the number of B cells. Activity of E2A and other class I bHLH proteins can be antagonized by inhibitors of differentiation (Id) proteins.[42] Of the four Id proteins (Id1-Id4), only Id2 and Id3 have been detected in lymphoid cells.

Expression of EBF (early B-cell factor) is restricted to B cells and some other non-lymphoid cells. EBF binds DNA as homodimer with a DNA-binding domain (zinc coordination).[43,44] Its dimerization domain is related to the HLH motif. Like *E2A*, disruption of the *EBF* gene results in a block in differentiation before D_H-J_H recombination and heterozygous

EBF deletions show decreased numbers of pro-B cells, indicating that normal B-cell development requires two *EBF* alleles.[45]

Analysis of *E2A/EBF* double heterozygous mice showed a more pronounced reduction of pro-B cells, indicating that EBF and E2A co-operatively regulate early B-cell differentiation.[35] In these mice reduced expression of *PAX5, RAG1, RAG2*, and *Igα* was observed. Therefore, co-ordinate regulation by the two transcription factors E2A and EBF seems to be important for establishing temporal patterns of gene activation during B-cell differentiation.[30,35] Functional co-operativity of E2A and EBF might be at the level of DNA binding, as has been shown by formation of a ternary complex on sequences in the λ5 enhancer/promoter. However, the transcription factors can also exert their co-operative function without direct interaction. EBF might for example bind to its target in the nuclear chromatin, thereby making the locus accessible for E2A.[35]

E2A and EBF also play an essential role in regulation of Ig gene rearrangements by making the Ig loci accessible for recombination (see also later in this section *Regulation of V(D)J recombination*).[46] E2A promotes germline transcription through the presence of E-box motifs in the Ig promoter and enhancer regions.[30,36,39] Moreover, E2A is able to interact with a number of histone acetyltransferases, suggesting that E2A promotes germline transcription and recombination by modifying the chromatin structure.[47] Also late in B-cell differentiation, E2A has been shown to be crucial, i.e. in the regulation of CSR.[48]

Pax5

Pax5 has functions throughout B-cell differentiation and is expressed throughout all stages except terminally differentiated plasma cells (reviewed by Nutt *et al.*).[49] *Pax5*-deficient mice have a block in early B-cell differentiation at the pre-B-I-cell stage in the adult BM.[29] In fetal liver of *Pax5*-deficient mice the differentiation block is already before the pro-B-cell stage.[29,49] These pro-B cells fail to complete the V_H to D_HJ_H joining, although RAG1 and RAG2 expression are not affected. It might be that the V_H gene do not become accessible in *Pax5*-deficient mice. Expression of B-cell specific genes, such as *VpreB, λ5, Oct-1, Oct-2, EBF* and *E2A*, did not differ between wild type and *Pax5*-deficient pre-B-I cells. Only CD19, Igα and N-myc were down-regulated in the absence of Pax5; expression of Igα and N-myc was reduced, whereas CD19 was completely absent.

Transgenic expression of Igμ or Igμ-Igβ in *Pax5*-deficient mice is not sufficient to advance B-cell differentiation to the pre-B-II cell stage in contrast to *RAG2*-deficient mice. This demonstrates that the observed differentiation block does not solely result from the inability to generate a complete $V_HD_HJ_H$ gene rearrangement.[26,28] Both *in vivo* and *in vitro* studies have demonstrated that *Pax5*-deficient pro-B-cells are unable to mature. Moreover, these pro-B cells have been shown not to be committed to the B-lymphoid lineage. Depending on lineage-specific *in vitro* culture conditions, *Pax5*-deficient pro-B cells can differentiate into mature macrophages, dendritic cells, osteoclasts, granulocytes, NK cells and T cells, which was for the latter also demonstrated *in vivo*.[26,27] So, *Pax5*-deficient pro-B cells can be propagated *in vitro* as uncommitted hematopoietic progenitors. Therefore, B-cell com-

mitment is not determined by D_H-J_H recombination nor by expression of E2A, EBF, or components of the pre-BCR (VpreB, λ5, Igα, Igβ), but by Pax5.

Pax5 exerts its function in B-cell lineage commitment via repression of lineage-promiscuous transcription of non-B-lymphoid genes, thereby preventing differentiation into other (non-B) lineages. Once B-lineage commitment has occurred, B-cells become dependent on the function of EBF and E2A in addition to Pax5.[24,30]

V(D)J recombination mechanism

Diversity of Ig molecules

The most important goal of B-cell differentiation is the generation of an antigen-specific Ig molecule. As the number of possible antigens or antigenic epitopes is innumerable, the B-cell repertoire should have an enormous diversity in Ig molecules. This diversity is achieved at three different levels. The first level is determined by combinatorial diversity. The Ig genes are composed of multiple V, (D,) and J segments, which are coupled during V(D)J recombination. In this way a diversity of 10^6 combinations is reached. During the process of V(D)J recombination random deletion and insertion of nucleotides takes place, resulting in additional junctional diversity potentially leading to $>10^{12}$ different specificities. Finally, affinity maturation through SHM in the V regions creates additional diversity.

V(D)J recombination

Many enzymes and factors are involved in the V(D)J recombination process. During this process V, (D,) and J segments are joined to form a V(D)J exon. The recombination process can be divided in several stages:

1. RAG binding to recombination signal sequences (RSS). V(D)J recombination is initiated by binding of the recombination activating gene proteins (RAG1 and RAG2) to the RSS. Every V_H, D_H, and J_H gene segment is flanked by an RSS, which is composed of a conserved heptamer motif (CACAGTG) and a conserved nonamer sequence (ACAAAAACC) separated by a spacer of 12 or 23 non-conserved nucleotides. Recombination mainly occurs between RSS with a 12 and a 23 spacer, ensuring that DNA rearrangements are limited to the appropriate coding segments (V to D to J) (see Figure 4).[50] RAG binding to the RSS results in the formation of a pre-cleavage complex, also called a synapsis. The DNA-bending high mobility group proteins (HMG1 and HMG2) stimulate RAG binding especially at the RSS

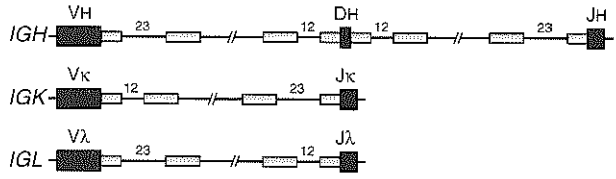


Figure 4. Schematic representation of the V, (D,) and J segments with the recombination signal sequences (RSS). The size of the spacer (12 or 23 nucleotides) are indicated as well.

with a 23bp spacer, which is suggested to stimulate the formation of the synaptic complex.[50,51]

2. Introduction of double stranded breaks (DSB). In the next step, RAG proteins introduce a single stranded nick just between the RSS and the gene segment.[52] The generated 3'OH group of the nick attacks the phosphodiester bond of the complementary DNA strand in a direct transesterification reaction, resulting in a DSB with a covalently sealed hairpinned coding end and a 5'phosphorylated signal end.[53,54] The generated DNA-ends are retained in a RAG post-cleavage complex.[50,52]

3. Processing of coding ends. The two signal ends of both RSS can ligate directly, resulting in the formation of a signal joint. Coding ends need to be processed before the formation of a coding joint can take place. Hairpinned coding ends can be nicked 5' of the tip, at the tip, or 3' of the tip for opening. It is not exactly known which factors are involved in hairpin opening. The RAG1/RAG2 complex might play a role in the processing of coding ends as is shown by *in vitro* studies.[55] *In vivo* analysis, however, shows that hairpin opening also requires active DNA-PK_{cs} and XRCC4.[56,57] Another candidate for hairpin opening is the Nijmegen breakage syndrome gene product (Nbs1), which has been shown in a complex with Mre11 and Rad50 to be able to unwind and cleave hairpins in the presence of ATP.[58] The recently identified protein Artemis has been shown to be required for V(D)J recombination.[59] Its function is not yet completely understood, but it might be involved in opening of the hairpins as well. Dependent on the site of hairpin opening, 5' or 3' overhanging palindromic (P) nucleotides are generated, or blunt ends. Nucleotides can be removed by exonuclease activity; the DNA ends are further processed by DNA polymerases that fill-in 5' overhanging ends resulting in a P-region, and by addition of nontemplated (N) nucleotides by TdT to the free 3'OH ends.[60-62]

4. Ligation of coding ends. The final step of V(D)J recombination is the ligation of the coding ends which is mediated by the components of the non-homologous end joining (NHEJ).[60] Ligation is supposed to be catalyzed by DNA ligase IV in complex with XRCC4.[63,64] The DNA binding proteins Ku70 and Ku80 bind as a complex to DNA ends.[65,66] The Ku proteins can associate with DNA-PK_{cs} forming the DNA-PK complex. Ku is thought to recruit DNA-PK_{cs}.[67]

Structure of *IGH, IGK,* and *IGL* loci

IGH locus

The human Ig heavy chain (*IGH*) gene locus at 14q32.33 spans a region of about 1.2 Mb. It contains 123 to 129 V_H segments, 27 D_H segments and six J_H gene segments.[68-70] (http://imgt.cnusc.fr:8104) The potential genomic *IGH* repertoire per haploid genome consists of 38 to 46 functional V_H genes belonging to seven gene families, 27 D_H gene segments belonging to seven families and six J_H gene segments (Table 1).[68-70] The V_H, D_H, and J_H gene segments precede nine functional and two pseudo constant (C) gene segments (Figure 5). Every functional C gene segment is preceded by a switch region, except for the Cδ gene seg-

Table 1. Number of gene segments (per haploid allele) which are present in the three Ig loci and the number of gene segments with functional RSS and the number of functional gene segments.[187,188]

	IGH			*IGK*		*IGL*	
	V	D	J	V	J	V	J
No. of gene segments[a]	123-129	27	9	76	5	73-74	7
No. of gene segments with functional RSS	~70	27[b]	6	~60	5	~40	5
No. of functional gene segments	38-46	27[b]	6	31-35	5	29-33	4

a. Orphan gene segments (gene segments outside the Ig locus) are not included.
b. Four D_H-segments have minor modification in their heptamer sequences and therefore might not be functional.

ment. During CSR, two switch regions recombine resulting in replacement of the Cμ region with another constant region while retaining the preceding VDJ exon.

There are two enhancers in the *IGH* locus, an intronic enhancer (iEμ), which is located between the J and the Cμ gene segments, and a 3' enhancer (3'Eμ), which is located downstream of Cα2. Germline transcription of Cμ, initiation of V(D)J recombination, and opening of the switch-μ region for CSR, is mainly regulated by promoters and the iEμ enhancer.[71]. The 3'Eμ enhancer, containing the four lymphoid-specific transcriptional enhancers: *hs3a, hs1,2, hs3b,* and *hs4*, which are individually weak enhancers, but have a strong synergistic effect. Especially *hs3b* and *hs4* play an important role in germline transcription and CSR, and may affect Igμ expression in resting B cells.[72]

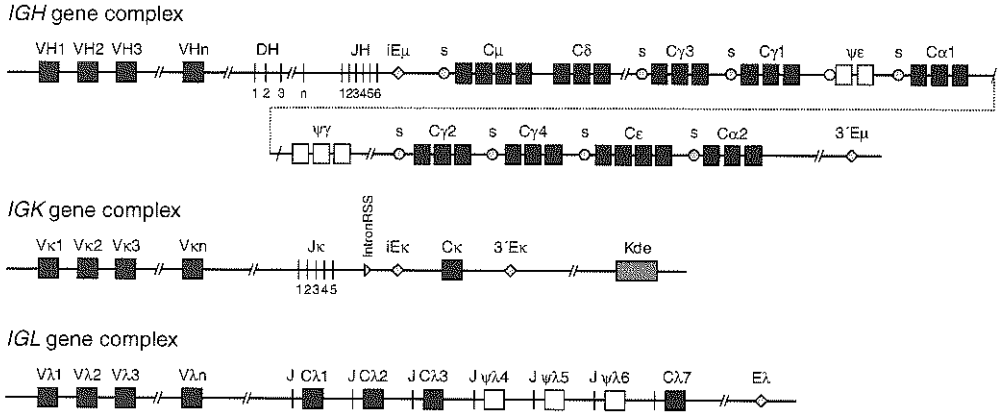


Figure 5. *IGH, IGK,* and *IGL* locus representations including the positioning of the enhancer elements.

IGK locus

The human *IGK* locus on chromosome 2 (2p11.2) consists of 76 Vκ gene segments belonging to seven gene families, five Jκ and a single Cκ gene segment (Figure 5).[70,73] (http://imgt.cnusc.fr:8104) The total region of the *IGK* locus is 1.8 Mb. The Vκ gene segments are organized in two clusters (proximal and distal) that are separated by 800kb. The proximal cluster contains 40 Vκ segments and is located closest to the Jκ gene segments. The distal cluster containing 36 Vκ gene segments probably originates from the proximal one by duplication and inversion (Table 1).[74,75] Recombination involving Vκ gene segments from the distal cluster occurs via inversional rearrangements.[76,77]

An isolated RSS heptamer is located between the Jκ and Cκ gene segments. This intronRSS can recombine with the kappa deleting element (Kde), which is located 24 kb downstream of the Cκ gene segment.[78,79] Upon an intronRSS-Kde rearrangement the Cκ gene segment is deleted thereby inactivating that *IGK* allele (Figure 6). Alternatively, inactivation of the *IGK* allele can occur via rearrangement of a Vκ gene segment upstream of a pre-existing Vκ-Jκ rearrangement to Kde, resulting in the deletion of the Vκ-Jκ rearrangement, the remaining Jκ segments, and the Cκ region.

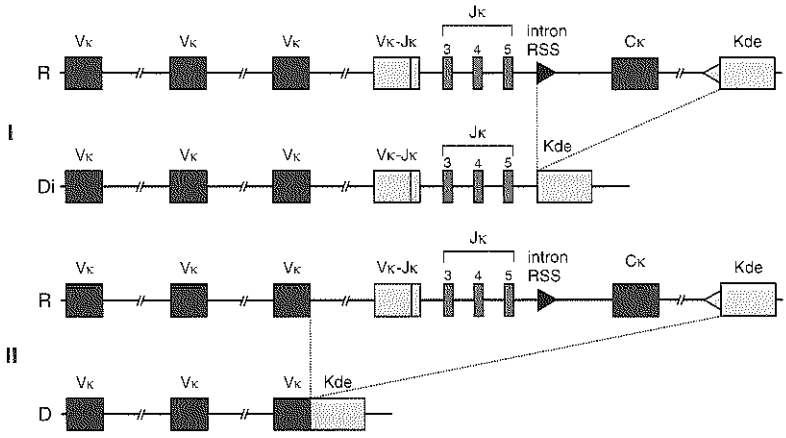


Figure 6. Two *IGK* deletion mechanisms. (I) Rearrangement of the intronRSS to the kappa deleting element (Kde), which results in deletion of the Cκ gene segment. (II) Rearrangement of a Vκ gene segment, which is located upstream of the Vκ-Jκ rearrangement, to Kde, resulting in deletion of the of the Vκ-Jκ rearrangement, the remaining Jκ segments, and the Cκ gene segment.

Similar to the *IGH* locus, two enhancers are located in the *IGK* locus; an intron enhancer (iEκ) located between intronRSS and Cκ, and an enhancer downstream of Cκ, but upstream of Kde (3'Eκ). The organization of the Jκ and Cκ gene segments and the enhancers are similar in human and mice. The functions of the two enhancers have been determined in mice. The iEκ becomes active during pre-B to immature B-cell transition and is involved in activation of *IGK* gene rearrangements and transcription. Mice with an homozygous Jκ-Cκ

or iEκ deletion have decreased *IGK* gene rearrangements as compared to *IGL* gene recombinations resulting in about half the number of B-cells and the production of 10 times more Igλ+ B-cells.[16,80-82] The 3'Eκ becomes active in mature B-cell stages.[83] This enhancer has been shown to play an important role in the transcription of rearranged *IGK* genes.[17,84,85]

IGL locus

The human immunoglobulin (*IGL*) lambda locus is located on chromosome band 22q11.2 and spans a region of about 1 Mb.[86] The *IGL* locus contains 73-74 Vλ gene segments, of which 29-33 can be expressed (Figure 5 and Table 1).[70,86-90] (http://imgt.cnusc.fr:8104) Seven J-Cλ gene regions are located downstream of the Vλ segments. Each Cλ gene segment is preceded by a Jλ gene segment. Only four J-Cλ gene regions are functional: i.e. J-Cλ1, J-Cλ2, J-Cλ3, and J-Cλ7; the regions J-Cλ4, J-Cλ5, and J-Cλ6 are non-functional (pseudo) gene regions. [91-93] One enhancer has been identified 8 kb downstream of the J-Cλ7 region, which consists of three DNaseI hypersensitive sites (HSS-1, -2, and -3).[94,95] The three HSS synergize in transcription activation.[96] Although it was initially assumed that the *IGL* enhancer was independent of the transcription factor NFκB, it has now been shown that the *IGL* enhancer, like the *IGK* and *IGH* intronic enhancers, contains four NFκB binding sites, that all have different effects on the enhancer activity.[97]

Regulation of V(D)J recombination

Several aspects of the V(D)J recombination mechanism itself are known in detail, but the tight lineage- and stage-specific regulation of V(D)J recombination is less well understood. Lineage- and stage-specific V(D)J recombination can be achieved via restricted expression of the *RAG* genes on one hand [98] and regulation of accessibility of the different Ig loci during the course of B-cell differentiation on the other hand. V(D)J recombination is initiated at the *IGH* locus and as soon as Igμ is expressed on the cell surface in the context of a pre-BCR, expression of *RAG1* and *RAG2* is downregulated to prevent further rearrangement that might potentially result in the generation of more than one functional allele. This process is called allelic exclusion and ensures generation of monospecific Ig B cells.[99] Allelic exclusion also operates for the Ig light chain loci. In addition to allelic exclusion, i.e. expression of one allele, isotypic exclusion applies to Ig light chain loci. Isotypic exclusion is defined by regulation of expression of either *IGK* or *IGL*.

From recent studies it became apparent that the initiation of *IGH* or *IGK* and *IGL* gene rearrangements do not occur randomly. The two chromosomes on which the *IGH* loci are located, have been shown to replicate asynchronously. Rearrangements start at the Ig loci, which are located on the chromosome that replicates first.[100] In addition, the nuclear localization of the six Ig alleles differs. Nuclear localization have been shown to correlate with expression and might also be involved in isotypic exclusion of Ig light chains.[101] This information contributes to the understanding of how accessibility is regulated and how monospecificity might be maintained.

Chromatin structures generally underlie the accessibility of genes. Like in all eukary-

otic cells, DNA is wrapped around nucleosomes, which consist of histone proteins (H2, H3, and H4), which form higher order chromatin structures. Modulation of the chromatin structure is important for transcription regulation, but also for gene-specific recombinase accessibility. In general, there are two main types of nucleosome remodeling. The first is acetylation of the N-termini of histones H3 and H4 by histone acetyltransferases resulting in gene activation. Inactive genes are associated with hypoacetylated histones.[102] Methylated CpG DNA motifs also correlate with inactive genes. Moreover, it has been shown that histone deacetylases can interact with methylcytosine-binding proteins.[103,104] A second type of chromatin modulation includes modification of the positions of the nucleosomes via SWI and SNF. SWI/SNF remodeling complexes alter the wrapping of the DNA around the histone proteins, resulting in nucleosome mobilization. Activity of SWI/SNF remodeling complexes is generally correlated with gene activation.[102]

Both remodeling via histone acetylation/CpG demethylation as well as SWI/SNF remodeling complexes are involved in V(D)J recombination.[105-108] Histone acetylation is thought to enhance the individual nucleosome accessibility for the V(D)J recombinase machinery, but might also influence unfolding of higher order chromatin structures.[107] SWI/SNF remodeling has been demonstrated to stimulate cleavage by RAG proteins at the RSS.[107] The combined effect of SWI/SNF chromatin remodeling and histone acetylation has a greater impact on V(D)J recombination than the individual effects.[107]

The next question is how chromatin remodeling at the Ig loci is controlled. Transcription factors might be responsible for targeting chromatin modulation complexes. In this respect E2A and EBF might be candidates for recruitment of histone acetyltransferases to Ig loci, because E2A binds to target sites and activates V(D)J recombination at Ig loci.[46,109] E2A and EBF have been shown to be together with RAG1 and RAG2 the only factors that are required for initiation of V(D)J recombination in a non-lymphoid cell line.[46] However, the two transcription factors promote different types of rearrangements. Both E2A and EBF can induce incomplete D_H-J_H rearrangement, but E2A, and not EBF, induces Vκ-Jκ rearrangements (mainly Vκ-Jκ), whereas EBF induces Vλ-Jλ rearrangements (mainly Vλ3-Jλ).

E2A has already been shown to interact with acetyl-transferase complexes.[47] The transcription factor NF-κB, which is a critical factor in B-cell differentiation, might be another candidate, as it has been shown that NF-κB recruits CBP/p300, which is a histone acetyltransferase.[110,111]

Histone acetylation, DNA demethylation, germinline transcription, and DNase I hypersensitivity are all associated with the possibility of V(D)J recombination. These phenomena have been determined during different B-cell differentiation stages in several studies.[7,97,112-114] The *IGH* locus becomes accessible at the pro/pre B-cell stage. Initially, only the region ranging from the D_H gene segments to a certain point between Cμ and Cδ are accessible allowing D_H-J_H rearrangements. DJ rearrangements are followed by accessibility of the V_H region. Activation of the V_H gene region is also differentially regulated. The proximal V_H gene segments (closest to D segments) become accessible at an earlier stage than the

distal V_H gene segments. This differential accessibility of proximal versus distal V gene segments has been shown to be dependent on IL7-sensitivity.[112] After a complete VDJ rearrangement, the V_H gene segments and not the J_H region become less accessible in parallel with an increased accessibility of the *IGK* locus.[7] Activation of the *IGK* locus for recombination has been shown to initially occur via demethylation of one allele. This differential accessibility of the two *IGK* alleles most probably plays an important role in allelic exclusion.[113] The *IGL* locus has been shown to be activated at a later stage than the *IGK* locus.[114]

1.2 ANTIGEN-DEPENDENT B-CELL DIFFERENTIATION IN THE PERIPHERY

Immature B cells leave the BM and enter the periphery as naive B cells. These B cells enter the lymphoid organs via blood vessels through high endothelial venules and leave via efferent lymphatic vessels.[115] Naive B cells can be activated upon crosslinking of the Ig receptor by native antigen.[116] At this point the activated B cell can either differentiate into an IgM secreting plasma cell or can undergo a so-called germinal center (GC) reaction. GC are specialized microenvironments in peripheral lymphoid organs (lymph nodes, spleen, and mucosa-associated lymphoid tissue (MALT)), where B cells undergo affinity maturation and CSR. Affinity maturation is achieved via the generation of SHM in the variable regions of the Ig molecule followed by selection of the best-fitting Ig molecule by follicular dendritic cells (FDC). Lindhout *et al.* described three antigen-specific checkpoints that ensure appropriate activation, selection, and differentiation of antigen-specific B cells (Figure 7).[117]

1. Antigen-specific initiation of the GC reaction via cognate B-T cell interaction.

The first checkpoint of the GC reaction actually occurs outside the GC. The antigen-specific B cell is activated upon crosslinking of the Ig receptor by native antigen. These B cells internalize and process the antigen and present the processed antigen in MHC class II. T cells are activated by processed antigen in the context of MHC class II presented by an antigen presenting cell (APC) and two co-stimulatory signals from the APC, i.e. CD40-CD40L and CD80-CD28 interaction.

In the T cell zone of the lymphoid organ, antigen-specific interaction of activated B and T cells takes place via processed antigen presented by B cells in MHC class II and the antigen-specific T-cell receptor (TCR) together with CD28-CD86 costimulation. The T cells up-regulate CD40L and the CD40-CD40L signaling is the second B-cell activation signal. This antigen-specific B-T interaction is required for initiation of the GC reaction and results in migration of the B and T cell into the dark zone of the GC.

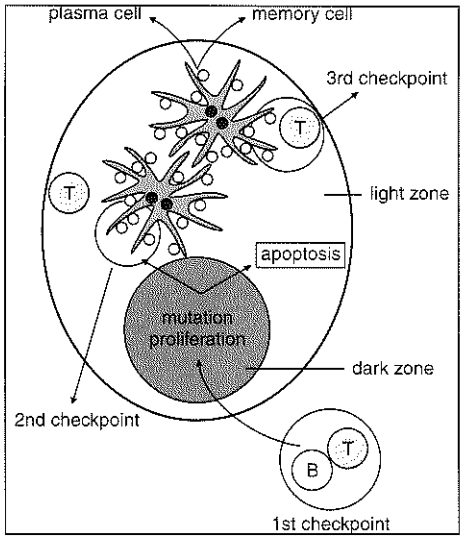


Figure 7. Ag-dependent B-cell differentiation: the germinal center reaction divided in three antigen-specific checkpoints that ensure appropriate activation, selection, and differentiation of antigen-specific B cells as described by Lindhout *et al.*[116]

2. SHM and Affinity maturation

During the GC reaction affinity maturation of the Ig molecule results in selection of the B cells with the highest affinity for the antigen. In the dark zone, B cells start proliferating and the variable domains of the Ig molecules are subjected to the SHM process. During this stage, the B cells are referred to as centroblasts and lose Ig expression. After the proliferation phase the centroblasts become centrocytes and migrate to the light zone of the GC, where centrocytes are selected based on antigen binding on the FDC.[117] Centrocytes will die by apoptosis unless they receive an anti-apoptosis signal from the FDC.[118,119] In this way, centrocytes with the highest affinity are selected.

3. CSR and maturation into plasma cell or memory B cell

The selected centrocytes migrate further in the light zone and present antigen taken up from the FDC to antigen-specific GC T cells, which subsequently upregulate CD40L. CD40-CD40L interaction initiates CSR and final differentiation into either plasma cells or memory B cells, which is dependent on the level of CD40-CD40L signaling.[120-122]

Somatic hypermutation

Although the complete mechanism of SHM has not yet been understood, based on a large number of studies several characteristics of SHM have emerged.[123]

The upstream boundary of the SHM is the promoter region with an increasing SHM

frequency along the leader and the V(D)J exon.[124,125] SHMs extend over about 1.5 kb with a decrease beyond the J region, but there is no fixed 3' boundary (Figure 8).[124] Based on available sequences an intrinsic decrease of the mutation frequency was calculated and showed an exponential decrease for both Ig heavy and Ig light chains.[126] The frequency of somatic mutations has been estimated to be 10^{-3} per cell generation.[127] Based on analysis of SHM in a large number of rearrangements, an RGYW (A/G,G,C/T,A/T) hotspot motif has been identified.[128,129]

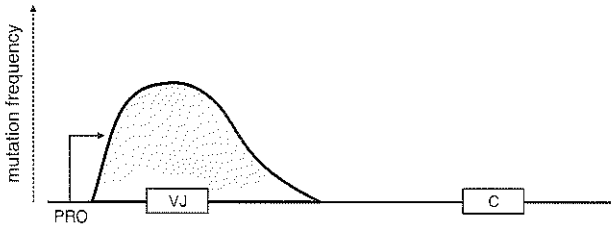


Figure 8. Schematic representation of the distribution of somatic mutations.

Promoters and Ig enhancers are critical elements for SHM, but not the V(D)J exon itself.[130-133] The presence of a promoter is essential for SHM rather than the specificity of the Ig promoter, because replacement of e.g. the Vκ promoter with another RNA polymerase II-dependent promoter (beta-globin) does not affect SHM.[131] These data indicate that the SHM process is linked to transcription.[125,134] It has also been demonstrated that the SHM frequency is dependent on the level of transcription.[135]

Several proteins that might be involved in SHM were studied by analyzing mice that are defective in various repair processes, including those that are linked to transcription.[136] It appeared that transcription-associated base and nucleotide excision repair is not involved in the SHM process.[136] The role of mismatch repair in SHM seems to be limited as shown by mutation analysis in knockout mice, although there are some conflicting data in literature.[137-140]

SHM has been shown to be mediated via DSBs.[141-143] The generation of DSBs is coupled to transcription, is enhancer-dependent, and seems to correlate with nearby mutations. Fifty to sixty percent of these DSBs occur preferentially at RGYW motifs; within this motif DSBs occur preferentially 5' of the G and R residues. There are generally two mechanisms for repair of DSBs, i.e. via NHEJ or via homologous recombination. The mechanism of repair is dependent on the phase of the cell cycle. NHEJ operates predominantly during the G_1/early S phase, whereas homologous recombination is active during late S/G_2 phase. DSBs have been shown to be much more abundant during the G_2 phase as compared to the G_1, suggesting a predominant role for homologous recombination in combination with an error-prone polymerase.[142,143] With this respect several error prone polymerases have been studied, such as polι, polη, polζ, and polμ, but it is yet unknown which is the major player.[144,145]

Recently, the potential RNA editing enzyme activation-induced cytidine deanimase (AID) has been shown to be not absolutely required for SHM, but clearly enhances the process.[146,147] The exact role of AID in the SHM process needs to be further analyzed.

Class switch recombination

During CSR the V(D)J exon is juxtaposed to a downstream C_H region resulting in the generation of different antibody isotypes (i.e. IgG, IgA, and IgE). CSR occurs between the switch (S) regions, which are composed of repetitive sequences.[148] The precise function of the S regions is yet unknown, but the isotype specificity of CSR is not determined by the exact nucleotide sequence of the S region nor by its orientation.[149] CSR to specific isotypes is rather determined by accessibility of the different C_H regions.

CSR is activated by specific cytokines that induce germline transcription. Germline transcription originates from a promoter upstream of the I exon, which precedes all switch and C regions, and directs the C region to which CSR occurs. Germline transcripts are further processed by RNA splicing that fuses the I exon to the C exons, thereby splicing out the S regions. The role of germline transcription might be opening of the S region to a specific, yet undefined, CSR recombinase. However, it has been demonstrated that not only germline transcription, but also the subsequent splicing of germline transcripts is required for CSR.[150] This suggests that either the splicing machinery or the processed transcripts are involved in CSR.[150] In the latter case, germline transcripts might play a role in direct initiation of CSR through the formation of a so-called R-loop in S-region via the formation of RNA-DNA hybrids. However, this has only been shown in *in vitro* studies. The transcriptional enhancer (iEμ) plays a role in efficiency of germline transcription,[151,152] but the control elements in the 3' part of the *IGH* locus play a major part in CSR. This 40 kb region downstream of the Cα region contains four enhancer elements (*hs3a, hs1,2, hs3b,* and *hs4*) that are conserved between mice and human. Several deletion and insertion studies showed that *hs4* might play a prominent role in CSR. The precise function of the individual elements remains to be elucidated.[72]

Unlike V(D)J recombination, the actual mechanism of CSR is not yet unraveled. It is generally assumed that DSBs are introduced in the S regions. It is also not yet known whether these DSBs are introduced by a CSR recombinase enzyme similar to RAG in V(D)J recombination or that a more general factor or process associated with S region transcription is involved in the generation of DSBs. The DSBs are joined forming a hybrid S region, and the intervening sequence is deleted as an excision circle.[153] NHEJ might play a role, as mutations in components of this system (Ku70, Ku80, DNA-PK_{CS}) have been shown to inhibit CSR.[154-156] The NHEJ-repair system is active during the G_1 phase of the cell cycle. V(D)J recombination also takes place during this phase. However, CSR takes place in proliferating cells most probably during the S phase. It might therefore be that enzymes involved in DNA synthesis are involved in CSR. A role for mismatch repair (MMR) pathways has been suggested based on the findings that mice deficient in MMR show reduced CSR.[157-159] The MMR pathway might play a role in the suggested R-loop cleavage.

Recently, activation-induced cytidine deanimase (AID), has been shown to be absolutely required for CSR and somatic hypermutation.[146,160] Mutations in AID result in an autosomal recessive form of the hyper-IgM syndrome (HIGM type 2), in which no CSR can take place.[161] AID is an RNA editing enzyme which is homologous to the RNA editing enzyme APOBEC-1, which removes an aminogroup from a cytidine making it a uridine in the intestinal apolipoprotein-B mRNA.[160] AID expression is restricted to germinal centers. The formation of GC in AID deficient mice and human seems to be normal, but they are enlarged, probably because of decreased apoptosis of activated B cells. AID might not be directly involved in the actual SHM and CSR, but might edit mRNA encoding enzymes that are involved in SHM or CSR.[162]

1.3 B-CELL MALIGNANCIES

During differentiation, B cells can undergo malignant transformation, which can occur at various stages. B-cell malignancies can be classified according to the corresponding normal B-cell differentiation stage based on the immunophenotype and the immunogenotype, which is also reflected by the site of origin (Figure 9). However, a B-cell malignancy does not always exactly correspond with the normal counterpart, because despite the malignant transformation, the B cell can still proceed a bit further in differentiation before the actual clonal expansion takes place.

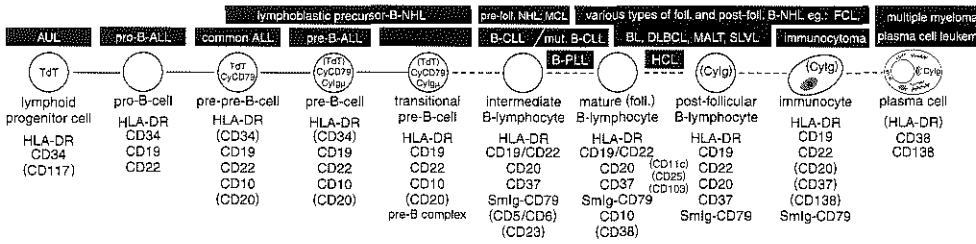


Figure 9. Classification of B-cell malignancies based on the immunophenotype and corresponding with normal B-cell differentiation stages.

Precursor-B-ALL

Precursor-B acute lymphoblastic leukemias (precursor-B-ALL) originate from malignant transformation of BM precursor-B cells. Precursor-B-ALL can be divided in three subgroups: pro-B-ALL, common ALL, and pre-B-ALL based on their immunophenotypes. All precursor B-ALL express CD19, CyCD79 and TdT. The three types of precursor-B-ALL can be discriminated with CD10 and CyIgμ expression. Pro-B-ALL is $CD10^-$ and $CyIg\mu^-$, common ALL is $CD10^+$ and $CyIg\mu^-$, and pre-B-ALL is $CD10^+$ and is further characterized by CyIgμ expression, which needs to be present in at least 15-20% of the cells for pre-B-ALL

classification. The immunophenotype of pro-B-ALL corresponds to a differentiation stage between pro-B cells and pre-B-I cells, because pro-B-ALL already express CD19, which is not found on normal pro-B cells, but do not yet express CD10 like pre-B-I cells. The immunophenotype of common ALL reflects that of pre-B-I cells and pre-B-ALL are comparable to pre-B-II cells. One of the characteristics of precursor-B-ALL is the continuous presence of the recombinase enzyme system.[163-165] Transitional pre-B-ALL form a small subgroup of pre-B-ALL which express CyIgμ as well as low levels of surface membrane bound (Sm) Igμ in the context of a pre-B-cell receptor.

Mature B-cell malignancies

Mature B-cell malignancies are clonal proliferations of B cells ranging from naive B cells to mature plasma cells. They can roughly be divided in three groups of origin: pre-follicular, follicular, post-follicular.

Naive B cells are small resting B cells, which circulate in the peripheral blood and are found in primary follicles and follicle mantle zones. B-cell malignancies that arise from these re-circulating B cells are B-cell chronic lymphocytic leukemias (B-CLL) and mantle cell lymphomas (MCL). It should be noted that not all B-CLL arise from naive B cells, but can also arise from recirculating memory B cells, which are characterized by SHM. B-CLL that originate from naive B cells and MCL do not contain SHM in the V regions, because these cells did not yet encounter antigen. These malignancies are regarded as pre-follicular malignancies.

Upon antigen encountering, naive B cells migrate to the center of a primary follicle, which develops a GC. In the GC, SHM and CSR occur. Follicular lymphoma arise from GC B cells and are therefore characterized by ongoing SHM and consequently show intraclonal diversity. Part of the diffuse large B-cell lymphoma (DLBCL) also originate from GC B cells and have ongoing SHM, whereas another part of DLBCL are of post-germinal center origin.

B-cell malignancies of post-germinal center origin are characterized by the presence of SHM in the V regions, but do not show intraclonal variation. As a result of CSR they can express non-IgM isotypes, e.g. IgG or IgA. They might arise from memory B cells or from plasma cells, which are terminally differentiated (end-stage) B cells that secrete Ig. Examples of memory B cell-derived post-follicular B-cell malignancies are hairy cell leukemias (HCL), B-CLL, Burkitt's lymphomas (BL), and prolymphocytic leukemias (PLL). Multiple myelomas (MM) and plasma cell leukemias are monoclonal proliferations of plasma cells, which are primarily found in the BM.

Example of immunogenotype-based classification

The presence or absence of SHM is an important immunogenotypic feature in classification of B-cell malignancies. An example of classification, which was changed based on this immunogenotypic characteristics is the assignment of Burkitt's leukemia for the entity which was previously referred to as B-cell acute lymphoblastic leukemias (B-ALL).[166]

B-ALL comprise 2-3 % of pediatric ALL and show similarities with Burkitt's lym-

phoma (BL) with respect to biology, oncogenic event (*c-MYC* translocation) and treatment response. We studied immunogenotypic characteristics of 12 B-ALL patients. Somatic mutations in the *IGH* genes were found with a frequency of 2.7±1.8%. The type of somatic mutations and their position did not provide evidence for antigen selection and also no intraclonal heterogeneity was observed. The presence of somatic mutations indicates that B-ALL do not arise from immature B-cells, as the name might suggest, but from a post-GC B cell. The presence of somatic mutations and low frequency of EBV positivity are comparable to sporadic BL (Table 2). B-ALL differ significantly from the other types of B-lineage ALL, which all represent precursor-B-ALL. In combination with the known similarities in biology, oncogenic event and treatment response between B-ALL and BL, the immunogenotypic data support the view to replace the term B-ALL by Burkitt's leukemia, which is fully in line with the WHO classification for lymphomas.[167] Analogous to the term T-ALL for T-lineage ALL, the term B-ALL could then be reserved for B-lineage ALL, i.e. ALL of B cell precursor origin.

Table 2. Characteristics of *IGH* gene rearrangements in B-ALL, precursor-B-ALL, and the three types of BL.

Disease category	B-ALL	Precursor-B-ALL	Sporadic BL	Endemic BL	AIDS associated BL
Number of cases[a]	9	50[b]	27 (15 cell lines)[c]	12 (9 cell lines)[c]	8 (5 cell lines)[c]
Somatic mutations in V_H	2.7±1.8%	0%	4.6±3.1%	7.9±3.9%	7.5±3.6%
(range)	(0.6-6.0%)	-	(0.3-13%)	(1.4-15%)	(3.1-13.3%)
Antigen selection in V_H[d]	0/9	0/50	1/27	0/12	1/8
Intraclonal heterogeneity	0/9	0/50	1/27	1/12	0/8

a. The 9 B-ALL cases as well as 4 sporadic BL and 1 endemic BL were analyzed in this study, whereas the other BL cases were reviewed by Chapman *et al.*[189]

b. Van der Burg *et al.*, unpublished results.

c. Cell lines were included, since Chapman *et al.* demonstrated that somatic mutations of V-genes do not occur *in vitro*.[190]

d. Absence or presence of antigen selection was deduced from the R/S ratios of the CDR and FR sequences.

Use of B-cell malignancies as "single cell" model system

B-cell malignancies can be used as clonal "single-cell" model system for the corresponding normal counterparts in different phases of B-cell differentiation. In Chapter 3 of this thesis, several types of B-cell malignancies were used as clonal "single-cell" model system to study the regulation of Ig light chain gene recombination during different stages of B-cell differentiation via detailed analysis of Ig gene configuration patterns. The advantage of using B-cell malignancies as model system is that they allow extensive analyses due to the high cell counts. In this way, both alleles of all three Ig loci can reliably be analyzed via Southern blot and PCR analysis, with a minimal risk of missing rearrangements. However, it cannot be completely ruled out that Ig recombination patterns in B-cell malignancies are influenced by oncogenic processes. Alternatively, normal B cells can be analyzed for these studies via a single-cell PCR approach, which has the inherent disadvantage of missing rearrangements due to false-negative PCR results. The advantage of single-cell analysis, however, is that normal B cells can be studied.

1.4 ONCOGENIC RECOMBINATION IN B CELLS

Chromosomal translocations play an important role in oncogenesis.[168,169] Two types of chromosomal translocations can be found in B-cell malignancies, i.e. translocations involving Ig loci that result in (over)expression of oncogenes and translocations that lead to the formation of fusion proteins.

Translocations involving Ig loci

Translocations involving Ig genes are particularly found in the more mature types of B-cell malignancies.[170] In precursor-B-ALL and CLL the frequency of these translocations is low, but in some disease categories specific Ig translocations are used as diagnostic marker, because of their high frequency. Examples are t(8;14)(q24;q32) involving the *IGH* locus and the *MYC* gene in BL, t(11;14)(q13;q32) involving *IGH* and *BCL1/CCND1* in mantle cell lymphoma (MCL), or t(14;18)(q32;q21) involving *IGH* and *BCL2* in follicular B-non Hogdkins lymphoma (B-NHL). In Table 3 the most frequent Ig translocations in the various B-cell malignancies are summarized. Translocations can not only involve the *IGH* locus, but can also involve the *IGK* or *IGL* locus. This has been described for translocations to *BCL1/CCND1*, *BCL2*, *C-MYC*, and *BCL6*.[171-179] However, these so-called variant translocations occur with a much lower frequency.

Table 3. Examples of the most frequent translocations involving the Ig loci.

Disease	Translocation	Involved gene	Frequency
Burkitt's lymphoma	t(8;14)(q24;q32)	*MYC*	100%
Follicular lymphoma	t(14;18)(q32;q21)	*BCL2*	80%
Mantel cell lymphoma	t(11;14)(q13;q32)	*CCND1/Cyclin D1*	95%
DLBCL	t(14;18)(q32;q21)	*BCL2*	20%
	t(8;14)(q24;q32)	*MYC*	10%
	t(3;14)(q27;q32)	*BCL6*	5-10%
Lymphoplasmacytoid lymphoma	t(9;14)(p13;q32)	*PAX5*	50%
CLL	t(14;18)(q32;q21)	*BCL2*	1-2%
Multiple myeloma	t(11;14)(q13;q32)	*CCND1/Cyclin D1*	20-25%
	t(4;14)(p16;q32)	*FGFR3*	20-25%

Translocations involving Ig loci occur as a result of errors in normal recombination processes.[180] Translocations can arise during V(D)J recombination, during which an oncogene translocates to the $(D_H)J_H$ region. Examples of these translocations are t(14;18)(q32;q21) involving the *BCL2* gene in B-NHL and t(11;14)(q13;q32) involving *IGH* and *BCL1/CCND1* MCL. Translocations can also arise during CSR, which results in translocation of oncogenes to S regions in the *IGH* locus. The *BCL6* translocation t(3;14)(q27;q32), which is found in DLBCL is an example of a CSR-mediated translocation.[181] The t(8;14)(q24;q32) involving the *C-MYC* gene can be mediated via V(D)J recombination or via CSR, dependent of the type of BL. The breakpoints in the *IGH* locus in endemic BL are found in the J_H region, which is indicative for the involvement of V(D)J recombinase machinery,

whereas in sporadic BL and AIDS related BL the breakpoints are found in the S regions.[182] In some translocations RSS like motifs are found at or near to the breakpoint in the oncogene. However, the presence of a switch region-like motif has not been identified. As somatic mutations are also mediated via DSBs, it has been demonstrated that particular translocations might arise as a "by-product" of the SHM process.[183,184] In these cases translocation breakpoints are identified within a rearranged V(D)J region or in the intron downstream of a rearrangement. In addition to a role of SHM in translocations involving Ig loci, SHM have also been shown in non-Ig genes, especially in genes which are expressed in GC B cells, such as *BCL6*.[185,186]

The molecular consequence of Ig translocations is the deregulated expression of the translocated gene. As a result of the translocations the translocated gene comes under control of Ig enhancers (Eμ) resulting in overexpression, which contributes to the oncogenic process. The translocation might interfere with regulatory mechanisms of apoptosis, cell cycle progression, signal transduction, or NF-κB activation.

The *MYC* gene, for example, is normally tightly linked to the early G_1 phase of the cell cycle. As a result of a t(8;14)(q24;q32) normal regulation of the gene is lost and Myc is expressed throughout the cell cycle. The precise function of Myc in the oncogenic process is unclear; however, Myc is known to act as transcriptional activator and repressor and can function as inducer of apoptosis or proliferation. The t(11;14)(q13;q23) in MCL results in overexpression of cyclin D1, which is involved in cell cycle regulation. Cyclin D1 overexpression leads to perpetuating transition from G_1 to S phase. Overexpression of Bcl2 as a result of t(14;18)(q32;q21) leads to inhibition of apoptosis.

Disturbances or aberrations in V(D)J recombination and SHM are not only involved in leukemogenesis, but have also been suggested to play a role in the emergence of autoimmune diseases. For example in patients with systemic lupus erythematosus higher frequencies of somatic mutations as compared to healthy controls have been reported in combination with disturbances in positive and negative selection.[193]

Non-Ig mediated translocations

The second type of translocations found in B-cell malignancies does not result in deregulated expression of genes, but in the formation of fusion genes. These fusion genes encode fusion proteins, which are also called chimeric proteins. The fusion protein has functional features different from the corresponding wild type proteins. In addition to the new features of the fusion protein, loss of wild type activity due to the translocation might also exert an (oncogenic) effect. This type of translocations is primarily found in precursor B-cell malignancies. The mechanism through which these translocations arise is largely unknown. The most frequently found chromosomal translocations in precursor-B-ALL are mentioned in Table 4. Translocations t(9;22)(q34;q11) and t(4;11)(q21;q23) encoding the fusion proteins BCR-ABL and MLL-AF4, respectively, are correlated with an unfavorable prognosis. Also t(1;19)(q23;p13), which results in expression of the fusion protein E2A-PBX1, is correlated with an unfavorable clinical outcome, although this is less clear than for t(4;11) and t(9;22).

The t(12;21)(p13;q23) resulting in the *TEL-AML1* fusion gene is correlated with a relatively favorable prognosis. As the different chromosome aberrations influence the clinical outcome, early detection of these chromosome translocation at diagnosis is important. In Chapter 4 of this thesis the development of rapid and sensitive split-signal FISH probes for detection of these translocations is described.

Table 4. Most frequently found chromosomal translocations in precursor-B-ALL.

Chromosomal translocation	Involved genes	Frequency	Prognosis
t(9;22)(q34;q11)	*BCR-ABL*	3-4%	Poor
t(4;11)(q21;q23)	*MLL-AF4*	2-3%	Poor
t(1;19)(q23;p13)	*E2A-PBX*	6% (25% of pre-B-ALL)	Unfavorable[a]
t(12;21)(p13;q22)	*TEL-AML1*	16-29%	Favorable

a. The t(1;19)(q23;p13) is generally correlated with an unfavorable prognosis, which can overcome with more intensive chemotherapy.[191,192]

1.5 OUTLINE OF THIS THESIS

In this thesis three main subjects are discussed. The first subject (Chapter 2) focusses on the human *IGL* locus. Two aspects of the *IGL* gene locus are discussed, i.e. isotype rearrangements in B-cell malignancies and the two human *IGL* light chain polymorphisms.

The second subject is the central theme of this thesis and deals with the regulation of Ig light chain gene recombinations (Chapter 3). Several types of B-cell malignancies were used as clonal "single cell" model to study rearrangement patterns of Ig light chain genes. Processes that might influence regulation of Ig light chain rearrangements, including the degree of order of Ig light chain gene rearrangements, allelic exclusion, and somatic mutations followed by receptor revision, are studied and discussed.

Oncogenic recombinations in B-cell malignancies are discussed Chapter 4. First, the considerations for the development of split-signal FISH for chromosome aberrations are described. The general principle of split-signal FISH was illustrated in translocations involving the *MLL* gene, which has a large number of partner genes. Furthermore, the influence of translocations involving the *E2A* gene, on antigen receptor gene rearrangement patterns in pre-B-ALL is discussed. Finally, split-signal FISH for the five most important translocations is summarized.

The General Discussion (Chapter 5) contains the main and integrated conclusions together with directions for future research.

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Chapter 2

THE HUMAN IG LAMBDA (*IGL*) LIGHT CHAIN LOCUS: POLYMORPHISMS AND REARRANGEMENT PATTERNS

2.1 The human Ig lambda (*IGL*) light chain locus.

2.2 Unraveling of the polymorphic Cλ2-Cλ3 amplification and the $Ke^{+}Oz^{-}$ polymorphism in the human immunoglobulin lambda locus.
J Immunol 2002: in press.

2.3 Immunoglobulin lambda isotype gene rearrangements in B-cell malignancies.
Leukemia 2001;15:121-127.

Chapter 2.1

THE HUMAN IG LAMBDA (*IGL*) LIGHT CHAIN LOCUS

THE HUMAN IG LAMBDA (*IGL*) LIGHT CHAIN LOCUS

The human immunoglobulin (*IGL*) lambda locus is located on chromosome 22q11.2 and spans a region of about 1 Mb.[1-3] The *IGL* locus contains 73-74 Vλ gene segments, of which 29-33 can be expressed.[3-8](http://imgt.cnusc.fr:8104) The Vλ gene segments are grouped in ten families, which are organized in three separate clusters: A, B, and C (Figure 1).[4] Figure 2 shows that the Vλ gene segments of cluster A, which is located most proximal to the J-Cλ gene regions, are most frequently used and that the expressed Vλ repertoire is strongly biased to five Vλ gene segments (Vλ2-14, Vλ1-40, Vλ2-8, Vλ1-44, Vλ3-21) that encode more than half of the repertoire.[9] This biased Vλ usage can be ascribed to either intrinsic genetic factors that lead to preferential rearrangement of certain Vλ gene segments or to expression-dependent events such as pairing of IgH with Igλ chain and positive or negative selection.[10] However, in the course of evolution certain Vλ gene segments with capacity to bind to a specific range of antigens might have acquired intrinsic mechanisms that ensure frequent usage.[10]

Downstream of the Vλ segments seven J-Cλ gene regions are located. Of these, only four J-Cλ gene regions are functional, i.e. J-Cλ1, J-Cλ2, J-Cλ3, and J-Cλ7; the regions

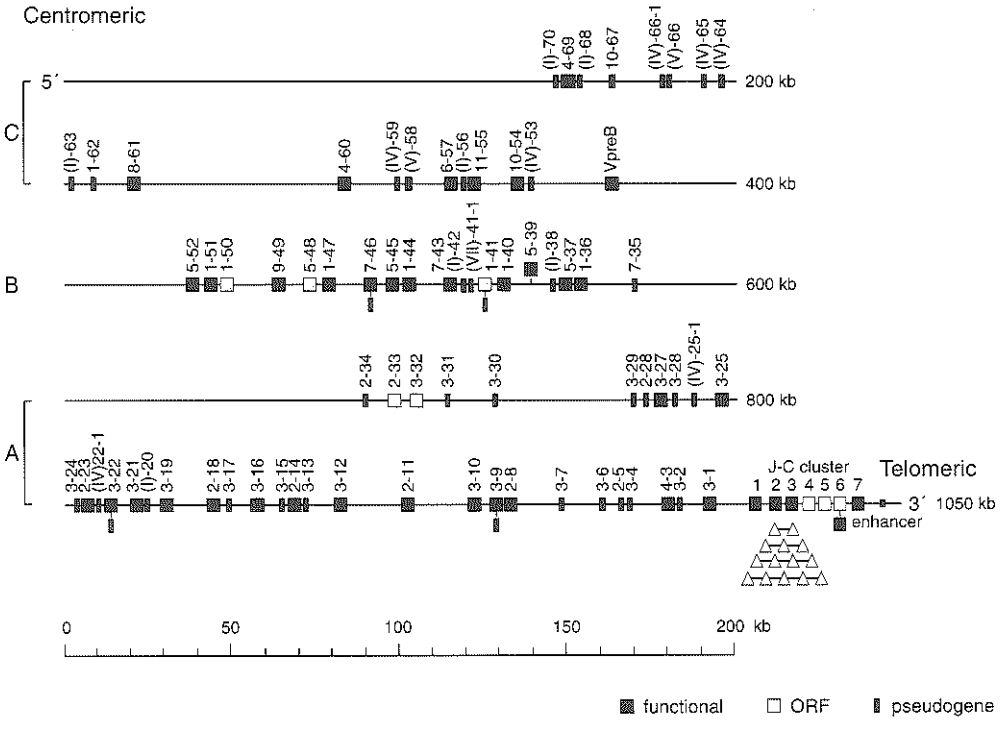

Figure 1. Human *IGL* locus with all Vλ gene segments. (Adapted form Lefranc *et al.*[6])

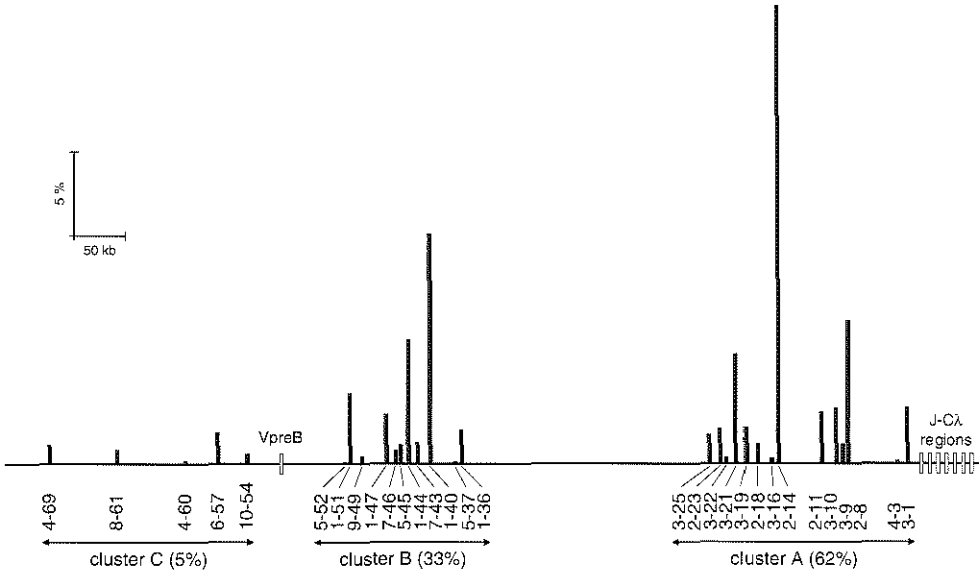

Figure 2. Frequency of expression of individual Vλ gene segments. (Adapted from Ignatovich *et al.*[9])

J-Cλ4, J-Cλ5, and J-Cλ6 are non-functional (pseudo) gene regions.[2,11,12] In Chapter 2.3 the contribution of the different J-Cλ gene regions to the expressed Igλ repertoire is discussed. Also Southern blot analysis of *IGL* isotype gene rearrangements in different B-cell malignancies is described in this chapter and the frequencies are compared to those of normal B cells.

The four functional types of Igλ light chains have also been identified in human serum using the serological isotype markers Mcg, Kern (Ke), Oz, and Mcp.[1,13-15] J-Cλ1, J-Cλ2, J-Cλ3, and J-Cλ7 encode the isotypes $Mcg^+Ke^-Oz^-$, $Mcg^-Ke^-Oz^-$, $Mcg^-Ke^-Oz^+$ and Mcp^+, respectively. A fifth isotype is the polymorphic $Mcg^-Ke^+Oz^-$, which is highly homologous to the J-Cλ2-encoded $Mcg^-Ke^+Oz^-$ isotype. However, this polymorphism is only known from serological analyses, but has not been determined at the DNA level. A second polymorphism in the human *IGL* locus concerns an amplification in the Cλ2-Cλ3 region, which can be present once, twice or three times per allele, giving rise to up to 10 J-Cλ gene regions per chromosome.[5,16-18]. Chapter 2.2 describes the precise unraveling of both human *IGL* polymorphisms at the DNA level and deals with the topic whether the two polymorphisms are linked, i.e. whether the $Mcg^-Ke^+Oz^-$ encoding Cλ segment is located on the amplified Cλ2-Cλ3 fragment. Furthermore, the structure of the Cλ2-Cλ3 amplification polymorphism was assessed and the influence of additional J-Cλ gene regions (as a result of the Cλ2-Cλ3 amplification) on the frequency of Igλ protein expression is described.

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Chapter 2.2

UNRAVELING OF THE POLYMORPHIC Cλ2-Cλ3 AMPLIFICATION AND THE $Ke^{+}Oz^{-}$ POLYMORPHISM IN THE HUMAN IMMUNOGLOBULIN LAMBDA LOCUS

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ABSTRACT

Two polymorphisms of the human immunoglobulin lambda (*IGL*) locus have been described. The first polymorphism concerns a single, two-fold or three-fold amplification of 5.4 kb of DNA in the Cλ2-Cλ3 region. The second polymorphism is the Mcg$^-$Ke$^+$Oz$^-$ isotype, which has only been defined via serological analyses in Bence-Jones proteins of multiple myeloma patients and was assumed to be encoded by a polymorphic Cλ2 segment, because of its high homology with the Mcg$^-$Ke$^-$Oz$^-$ Cλ2 isotype. It has been speculated that the Mcg$^-$Ke$^+$Oz$^-$ isotype might be encoded by a Cλ gene segment of the amplified Cλ2-Cλ3 region. We now unraveled both *IGL* gene polymorphisms. The amplification polymorphism appeared to result from a duplication, triplication or quadruplication of a functional J-Cλ2 region and is likely to have originated from unequal crossing over of the J-Cλ2 and J-Cλ3 region via a 2.2 kb homologous repeat. The amplification polymorphism was found to result in the presence of 1 to 5 extra functional J-Cλ2 per genome regions leading to decreased Igκ/Igλ ratios on normal peripheral blood B cells. Via sequence analysis, we demonstrated that the Mcg$^-$Ke$^+$Oz$^-$ isotype is encoded by a polymorphic Cλ2 segment that differs from the normal Cλ2 gene segment at a single nucleotide position. This polymorphism was identified in only 1.5% (2/134) of individuals (1.5%) without J-Cλ2 amplification polymorphism, and was not found in the J-Cλ2 amplification polymorphism of 44 individuals, indicating that the two *IGL* gene polymorphisms are not linked.

INTRODUCTION

The human immunoglobulin (*IGL*) lambda locus is located on chromosome band 22q11.2 and spans a region of about one megabase.[1] The *IGL* locus contains 73-74 Vλ gene segments, including 56-57 functional Vλ segments, which could be assigned to 11 subgroups based on nucleotide homology. [1-6](http://imgt.cnusc.fr:8104) Seven J-Cλ gene regions are located downstream of the Vλ segments. Each Cλ gene segment is preceded by a Jλ gene segment. Only four J-Cλ gene regions are functional: i.e. J-Cλ1, J-Cλ2, J-Cλ3, and J-Cλ7; the regions J-Cλ4, J-Cλ5, and J-Cλ6 are non-functional (pseudo) gene regions.[7-9] This is due to a deletion of ~1150 base pairs (bp) in the J-Cλ4 region and due to lack of some of the essential nucleotides in the recombination signal sequence (RSS) of the Jλ4 gene segment.[7,10] In the 3' end of the Cλ5 exon, 11 bp are deleted and the RSS of the Jλ5 segment also lacks some of the essential nucleotides.[7,10] Finally, the Cλ6 exon contains a duplication of four nucleotides which results in a premature stop codon.[7,9] Rearrangements to the J-Cλ6 segment can occur, but encode a truncated Igλ protein.[11]

The four different functional types of Igλ light chains have also been identified in human serum using the serological isotype markers Mcg, Kern (Ke), Oz, and Mcp.[11-14] These serological isotype markers are based on amino acid differences found in the Cλ regions of

various Bence-Jones proteins derived from multiple myeloma (MM) patients as well as from Igλ light chains isolated from intact immunoglobulins. The four functional J-Cλ gene regions J-Cλ1, J-Cλ2, J-Cλ3, and J-Cλ7 encode the isotypes Mcg$^+$Ke$^-$Oz$^-$, Mcg$^-$Ke$^-$Oz$^-$, Mcg$^-$Ke$^-$Oz$^+$ and Mcp$^+$, respectively. A fifth isotype, termed Mcg$^-$Ke$^+$Oz$^-$, is highly homologous to Mcg$^-$Ke$^-$Oz$^-$ and is assumed to be encoded by a polymorphic Cλ2 gene segment, but this has not yet been confirmed by sequence analysis at the DNA level.

The human *IGL* locus also contains a ~5.4 kb amplification polymorphism in the Cλ2-Cλ3 region. As the amplified region can be present once, twice, or three times, this amplification might result in up to 10 J-Cλ gene regions per allele.[3,15-17] The polymorphic Cλ2-Cλ3 amplifications can be identified via Southern blot (SB) analysis as *Eco*RI fragments of 13.7, 19.1, or 24.5 kb, representing one, two, or three amplifications, respectively.[15,18] In the absence of an *IGL* amplification polymorphism, only an 8.3 kb *Eco*RI fragment is detected. In *Hin*dIII digested DNA, the *IGL* polymorphism is visible as a 5.4 kb fragment, independent of the number of amplified regions.[18] It has been described that the frequency of the Cλ2-Cλ3 amplification polymorphism appeared to vary between populations of different geographical areas,[3,16,17] but this polymorphism has not been characterized in full detail.

Here, we studied both the Cλ2-Cλ3 amplification polymorphism and the Mcg$^-$Ke$^+$Oz$^-$ polymorphism. Our aim was to investigate whether the Mcg$^-$Ke$^+$Oz$^-$ isotype is encoded by a polymorphic Cλ2 gene segment and whether the Cλ segment encoding this isotype is located on the amplified Cλ2-Cλ3 fragment, implying that the two *IGL* polymorphisms might be linked, as speculated earlier.[8,14] Furthermore, we wished to assess the structure of the Cλ2-Cλ3 amplification polymorphism and determine whether the amplified region contains functional J-Cλ segments that might lead to higher frequencies of Igλ protein expression.

MATERIALS AND METHODS

Cell samples

Peripheral blood (PB) samples were obtained from 96 healthy individuals. The expression of Igκ and Igλ on PB B-cells was determined via Igκ/CD19 and Igλ/CD19 two color immunofluorescence in 69 cases. DNA was isolated from the granulocytes of all 96 healthy individuals using phenol/chloroform extraction.[18] In addition, DNA samples of 82 precursor-B-ALL samples and Igκ$^+$ B-CLL with both *IGL* alleles in germline configuration, were analyzed for the presence of the Mcg$^-$Ke$^+$Oz$^-$ polymorphism.[19,20]

Southern blot analysis

15 μg of DNA of 80 healthy controls (60 form Caucasoid origin and 20 from Chinese origin) was digested with *Eco*RI (Life Technologies, Rockville, MD, USA), separated in 0.7% agarose gels, and transferred by vacuum blotting to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany).[18] The filters were hybridized with the ^{32}P labeled λ-IVS probe.[15]

PCR analysis and fluorescent sequencing of the amplified Cλ2-Cλ3 region

Specific PCR primers for the Cλ2-Cλ3 amplification polymorphism (*IGL*amp-F and *IGL*amp-R) were designed, using the OLIGO 6.2 software program (Dr. W. Rychlik; Molecular Biology Insights, Inc., Cascade, CO) (Table 1). PCR analysis was performed with Expand™ Long Template PCR System (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. PCR products were analyzed on a 1% agarose gel and purified using a Qiagen PCR purification kit (Qiagen, Hilden, Germany) prior to fluorescent sequencing. PCR products were sequenced on an ABI 377 fluorescent cycle sequencer (Applied Biosystems, Foster City, CA, USA) with Big Dyes (Applied Biosystems) according to the manufacturer's instructions. The original PCR primers were used as sequence primers and subsequently new sequence primers were designed in the newly sequenced regions. An extra PCR was performed using the primers Up-F and Down-R to sequence the gap between the primers *IGL*amp-R and *IGL*amp-F (see also Table 1 and Figure 2b). The *IGL* gene sequence (accession number X51755) was used as reference sequence. Pairwise Alignment algorithms were used of the EMBL, European Bioinformatics Institute (Cambridge, United Kingdom) (http://www.ebi.ac.uk/emboss/align/).

Table 1. Sequences of used primers for PCR analysis and/or sequencing of the *IGL* locus.

Primer	Sequence (5'-3')
*IGL*amp-F	TTGTGCTGCCACCAGGAT
*IGL*amp-R	TGCCCAGGGAACTGAGAAC
Up-F	GTGGGTTCTCAATTTGTGGT
Down-R	TGCCAGATGGATAAATGTGAC
Cλ2-F	CCTGCCCCTCATCCACC
Cλ2-R	GAGAGCTCACCAGAGTCACTGG
Cλ2-Rseq	TCAGGCGTCAGGCTCAGATAG

Analysis of the Cλ2 gene region for the presence of the Mcg^{-}Ke^{+}Oz^{-} polymorphism

The Cλ2 region was amplified by PCR using the primers Cλ2-F and Cλ2-R (Table 1), using the TaqGold amplification system (Applied Biosystems). The obtained PCR products were sequenced with the Cλ2-F primer and/or the Cλ2-Rseq primer (Table 1), as described above.

RESULTS

Southern blot analysis of Cλ2-Cλ3 amplification polymorphism in Caucasoid and Chinese individuals

The *IGL* locus of 80 healthy controls (60 of Caucasoid origin and 20 of Chinese origin) was analyzed with respect to the number of amplifications using the λ-IVS probe in com-

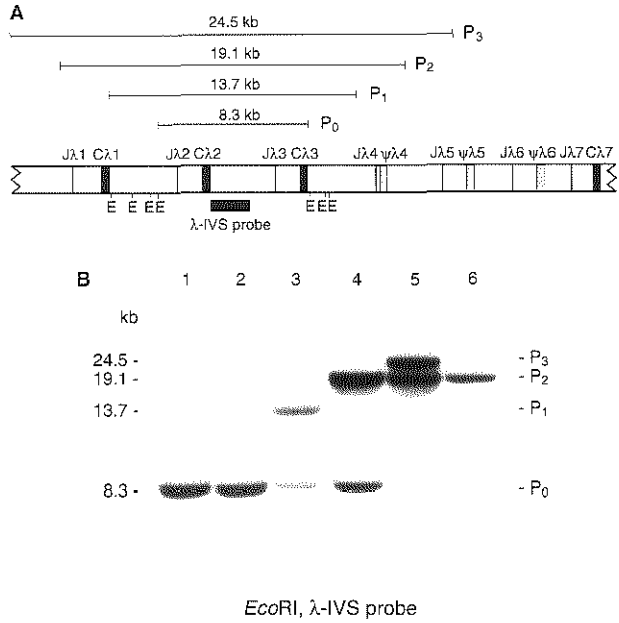

Figure 1. Detection of the *IGL* amplification polymorphism of the Cλ2-Cλ3 region. **A.** Schematic representation of the *IGL* locus with the amplification polymorphism of the Cλ2-Cλ3 region. The *Eco*RI restriction sites, the position of the λ-IVS probe and the relevant restriction fragments are indicated. In cases without an *IGL* amplification polymorphism (P_0) the λ-IVS probe detects an 8.3 kb band. Dependent on the number of amplified regions (P_1, P_2, or P_3), a 13.7, 19.1, or 24.5 band is detected, respectively. **B.** Southern blot analysis of *Eco*RI digested DNA of six healthy controls with the λ-IVS probe. Lane 1 and 2: no *IGL* amplification polymorphism on both alleles (P_0/P_0). Lane 3,4 and 6: one allele without *IGL* amplification polymorphism together with a polymorphic allele, P_0/P_1, P_0/P_2, and P_0/P_2, respectively. Lane 5: two polymorphic alleles (P_2/P_3).

bination with *Eco*RI digests (Figure 1a). Examples of Southern blot results of two individuals without a Cλ2-Cλ3 amplification polymorphism and four individuals with amplification polymorphisms are shown in Figure 1b. 72% of the Caucasians did not have the Cλ2-Cλ3 amplification polymorphism, in contrast to only 15% of the Chinese individuals (Table 2). The *IGL* locus of individuals without a Cλ2-Cλ3 amplification polymorphism on both alleles is described as P_0/P_0. In the group of Caucasoid individuals the frequency of monoallelic amplifications was 7%, 15%, and 7% for one, two, or three amplifications, respectively (P_0/P_1, P_0/P_2 and P_0/P_3). These percentages were comparable to the percentages in the Chinese individuals. However, 55% of the Chinese individuals had biallelic amplifications (P_1/P_2, P_1/P_3, P_2/P_2, and P_2/P_3), which were not observed in the Caucasoid individuals.

Sequencing strategy of the Cλ2-Cλ3 amplification polymorphism

So far, the polymorphic Cλ2-Cλ3 amplification has never been sequenced, probably because of the complexity of the *IGL* locus, especially because of the high homology between the J-Cλ2 region and the J-Cλ3 region. The primers (*IGL*amp-F and *IGL*amp-R) were

Table 2. Configuration of the IGL locus with respect to the number of IGL amplifications as determined by Southern blot analysis.

IGL locus configuration	Caucasians (n=60)	Chinese (n=20)	Total (n=80)
P_0/P_0	43 (71.6%)	3 (15%)	46 (57.5%)
P_0/P_1	4 (6.7%)	1 (5%)	5 (6.3%)
P_0/P_2	9 (15%)	4 (20%)	13 (16.2%)
P_0/P_3	4 (6.7%)	1 (5%)	5 (6.3%)
P_1/P_1	-	-	-
P_1/P_2	-	2 (10%)	2 (2.5%)
P_1/P_3	-	1 (5%)	1 (1.2%)
P_2/P_2	-	7 (35%)	7 (8.8%)
P_2/P_3	-	1 (5%)	1 (1.2%)
P_3/P_3	-	-	-

designed tail-to-tail, just upstream of the *Hin*dIII restriction site, such that in situations without polymorphic Cλ2-Cλ3 amplifications (P_0/P_0), no PCR product is formed (Figure 2a, c). In cases with a polymorphic amplification, a PCR product of about 5.4 kb was generated, independent of the number of amplifications (Figure 2b, 2c). The size of the PCR product was comparable to the size of the amplified region as it was determined via Southern blot analysis using *Hin*dIII digests.[18] The amount of PCR product was quantified using semi-quantitative PCR with sampling after different cycles (7, 10, 13, 16, 19, and 22 cycles), followed by hybridization with the J-Cλ2 specific IGLC2D probe.[19] The obtained hybridization signals correlated with the number of amplifications as determined via Southern blot analysis (data not shown). This result implies that in case of multiple Cλ2-Cλ3 regions, all regions were represented in the sequence analysis. The 5.4 kb PCR product of 4 individuals with the genotypes P_1/P_2, P_2/P_2, P_2/P_2, and P_0/P_3 were completely sequenced. Sequence analysis was started with the *IGL*amp-F and *IGL*amp-R primers. Subsequently, sequence primers were designed in the newly generated sequence ("gene walking"). Finally, an extra primer set was designed to analyze the region between the two primers *IGL*amp-R and *IGL*amp-F by PCR and sequencing. The forward primer (Up-F) was designed in the newly generated sequence and the reverse primer (Down-R) downstream of the *IGL*amp-F primer (Figure 2b). These primers also annealed in the homologous region just downstream of the J-Cλ2 region (Figure 2b). The total sequenced region is indicated in Figure 2b.

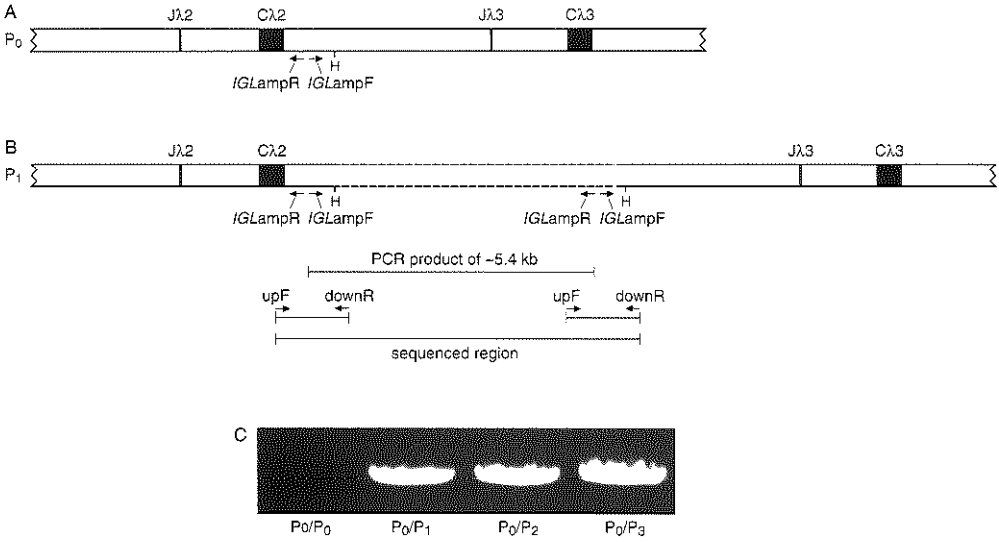

Figure 2. Strategy for PCR and sequencing analysis of the *IGL* amplification polymorphism. **A.** Cλ2-Cλ3 region without *IGL* amplification polymorphism (P_0) with the primers *IGL*amp-R and *IGL*amp-F positioned just upstream of the HindIII restriction site. **B.** Cλ2-Cλ3 region with one polymorphic *IGL* amplification (P_1) with the positions of the two *IGL*amp primers. PCR analysis with the primers *IGL*amp-F and *IGL*amp-R results in the generation of a fragment of ~5.4 kb. The PCR product generated with the *IGL*amp-F and *IGL*amp-R primers was totally sequenced starting with the PCR primers and subsequently with sequence primers designed in the newly generated sequence ("gene walking"). To analyze the region between the *IGL*amp-F and *IGL*amp-R primers, sequence analysis was performed using PCR products generated with primers Up-F and Down-R. The total sequenced region is indicated. **C.** PCR analysis with the primers *IGL*amp-F and *IGL*amp-R of one healthy individual without *IGL* amplification polymorphism (P_0/P_0) and three with an *IGL* amplification polymorphism (P_0/P_1; P_0/P_2; P_0/P_3).

Structure of the Cλ2-Cλ3 amplification polymorphism

The region of the *IGL*amp-F *IGL*amp-R product together with the extended region that fills the "gap" between the ends of the two primers was further analyzed for the presence of restriction sites (Figure 3a, white area). This sequence was subsequently aligned with the standard *IGL* gene sequence (P_0) to determine the structure of the amplification. The first 2.5 kb of the sequenced region appeared to be homologous to the region downstream of Cλ2 gene segment (Figure 3a, dotted lines) with 99.3% homology. The second part of the sequenced region (2.9 kb) shared the highest homology with the J-Cλ2 region (Figure 3a, dashed lines), which was also 99.3%. It should be noted that there is a highly homologous region of 2.2 kb between the J-Cλ2 and J-Cλ3 region with a similarity of 98.7%. These homologous regions are indicated as gray areas. Figure 3a represents the experimental approach for characterization of the *IGL* amplification, but does not reflect the mechanism. In Figure 3b the actual positioning of the amplification polymorphism is depicted in the total structure of a P_1 allele. The amplified region starts with a 2.2 kb homologous region and ends just before the 2.2 kb

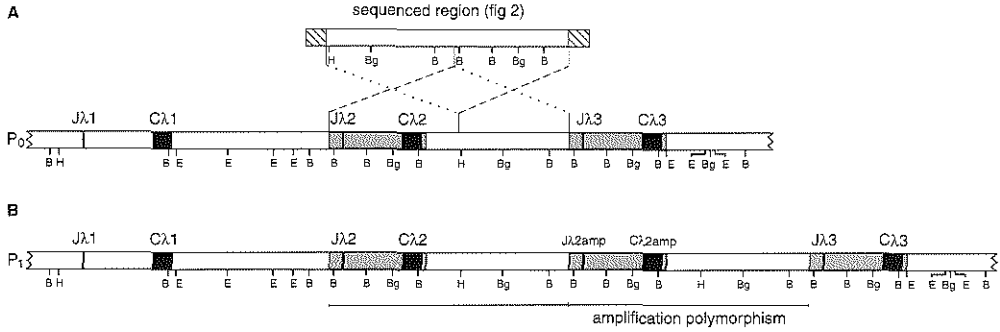

Figure 3. Experimental approach for characterization of the *IGL* amplification polymorphism resulting in the actual positioning within the *IGL* locus. **A.** Schematic representation of the total sequenced region, as defined in Figure 2b. For further sequence analysis the region of the *IGL*amp-F *IGL*amp-R product together with the extended region to fill the "gap" between the ends of the two primers was used (white area), the remaining sequence was excluded (dashed area). *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), and *Hin*dIII (H) restriction sites are indicated. The homologous repeats of the J-Cλ2 and J-Cλ3 regions with a similarity of 98.7% in the standard sequence (P_0) are marked as gray areas. The first part of the sequence could be aligned with the region downstream of the Cλ2 gene segment (indicated with dotted lines) with a homology of 99.3%. The second part of the sequence was aligned with the J-Cλ2 region (indicated with dashed lines) and had also a homology of 99.3%. **B.** The Cλ1-Cλ2-Cλ3 region of a polymorphic allele (P_1) with the actual positioning of the polymorphic amplified region.

homologous region of J-Cλ3.

Due to the polymorphic amplification a new *Hin*dIII is generated (Figure 3b). SB analysis of *Hin*dIII digested DNA of individuals with the amplification polymorphism will therefore show the 5.4 kb polymorphic *Hin*dIII fragment when using the λ-IVS probe. Consequently, two and three (identical) repeats of the amplified region (P_2 or P_3) also result in 5.4 kb *Hin*dIII restriction fragments.

Sequence analysis of the J-Cλ region within the *IGL* amplification polymorphism

The J-Cλ region of the amplified *IGL* region shares the highest homology with the J-Cλ2 region. The Jλ2amp gene segment is identical to the Jλ2 except for one amino acid (Figure 4). The recombination signal sequence of the Jλ2amp was compared to the RSS of the seven other RSS and appeared to be identical to that of Jλ2 and Jλ3 and contains all essential nucleotides (Table 3). The donor and acceptor splice sites of the J-Cλ2amp region are intact. The Cλ2amp gene segment is fully identical to the Cλ2 gene segment and also codes for the Mcg$^-$Ke$^-$Oz$^-$ (Table 4, comparison to all isotypes). So, the *IGL* amplification polymorphism does not encode the Mcg$^-$Ke$^+$Oz$^-$ polymorphism and constitutes a different *IGL* polymorphism. Based on these data we conclude that the J-Cλ2amp region is functional. This conclusion was supported by homology search of the Jλ2amp segment using nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST), which resulted in perfect match with more than fifteen sequences of human mRNA for Igλ chains.

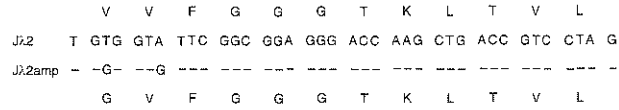

Figure 4. The Jλ gene segment of the *IGL* amplification polymorphism (Jλ2amp) compared to the normal Jλ2 region. The segments differ at two nucleotide positions of which only one results in an amino acid substitution (V→ G).

Table 3. RSS of all Jλ gene segments as compared to the consensus RSS.

	Nonamer[a]	Spacer	Heptamer[a]
Consensus RSS	GGTTTTTGT	12	CACTGTG
Jλ1	- - - - - - G- -	12	- - - - - - -
Jλ2	- - - - - - - - -	12	- - - A - - -
Jλ2amp	- - - - - - - - -	12	- - - A - - -
Jλ3	- - - - - - - - -	12	- - - A - - -
Jλ4	A- - - - - - - -	12	- - - C - C A
Jλ5	- - - - - - - - -	12	- - - A - C A
Jλ6	- - - - - G- - -	12	- - - A - - -
Jλ7	- - - - - G- - -	12	- - - - - - -

a. Essential nucleotides are underlined.

Effect of the number of IGL amplifications on the Igκ/Igλ ratio on PB B cells

As sequence analysis of the J-Cλ region on the *IGL* amplification showed that this region can undergo gene rearrangements and can be expressed, we wanted to study the effect of the number of functional J-Cλ regions on the frequency of Igλ positive B lymphocytes, by determining the Igκ/Igλ ratio on normal PB B cells. The combined Southern blot and Igκ/Igλ ratio data are summarized in Table 5. The human *IGL* locus contains four functional J-Cλ regions, meaning that in cases without *IGL* amplification polymorphisms (P_0/P_0) a total of eight functional J-Cλ regions are present. In cases with the *IGL* amplification polymorphism, every amplified region represents one additional functional J-Cλ region. The Igκ/Igλ ratio of cases with an amplification (resulting in 9-13 functional J-Cλ gene regions) was found to be significantly lower than the Igκ/Igλ ratio of cases without amplification (8 J-Cλ gene regions): 1.37±0.18 versus 1.56±0.26 (t-test; $p<0.001$). Already one additional J-Cλ gene region appeared to be sufficient for a decrease in the Igκ/Igλ ratio (Table 5).

Frequency of Mcg$^-$Ke$^+$Oz$^-$ Cλ2 polymorphism

The sequence data demonstrated that the *IGL* amplification polymorphism does not coincide with the Mcg$^-$Ke$^+$Oz$^-$ polymorphism. Therefore, it is most likely that the Mcg$^-$Ke$^+$Oz$^-$ polymorphism is encoded by a polymorphic Cλ2 gene segment. This implies that at the DNA level the codon AGC for serine (S) at position 152 must be changed in GGC for glycine (G), which is the serological marker Ke$^+$. The Cλ2 region of 178 individuals was analyzed for the presence of the Mcg$^-$Ke$^+$Oz$^-$ polymorphism. The group consisted of 96 healthy

Table 4. The five Igλ isotypes described by the serological markers Mcg, Ke, Oz, and Mcp.

Cλ segment	Serological marker[a]	Amino acid position						
		112	114	152	190	136	157	195
		Mcg		Ke	Oz	Mcp		
Cλ1	$Mcg^+Ke^+Oz^-$	N	T	G	R	I	A	Q
Cλ2	$Mcg^-Ke^-Oz^-$	A	S	S	R	I	A	Q
Cλ2amp	$Mcg^-Ke^-Oz^-$	A	S	S	R	I	A	Q
Cλ2P[b]	$Mcg^-Ke^+Oz^-$	A	S	G	R	I	A	Q
Cλ3	$Mcg^-Ke^-Oz^+$	A	S	S	K	I	A	Q
Cλ7	$Mcp^+Ke^+Oz^-$	A	S	G	R	V	V	R

a. The serological marker Mcg is determined based on amino acid N at position 112 and T at position 114; Ke is based on a G at position 152; Oz is based on K at position 190; and Mcp by V at position 136 and 157 and R at position 195.

b. Cλ2P; polymorphic Cλ2 region.

controls (granulocyte DNA) as well as 82 precursor-B-ALL and $Igκ^+$ B-CLL samples, without *IGL* gene rearrangements as determined by Southern blot analysis.[19,20] All samples were first screened with the *IGL*amp-F and *IGL*amp-R primers to define cases with the *IGL* amplification polymorphism. Twenty-five percent of cases (44/178) appeared to have the *IGL* amplification polymorphism. In cases without the *IGL* amplification polymorphism, the Cλ2 region was analyzed by PCR and sequencing. The Cλ2amp regions of individuals with the *IGL* amplification polymorphism were also sequenced. For this purpose the PCR products generated with the *IGL*amp-F and *IGL*amp-R primers were sequenced with the Cλ2-F and Cλ2-Rseq primers.

In two of the 134 analyzed P_0/P_0 cases (1.5%) a heterozygous peak of the nucleotides A and G was observed in the codon encoding the serological marker Ke. This implies that these individuals are heterozygous for the $Mcg^-Ke^+Oz^-$ polymorphism. In the Cλ2amp region of the 44 individuals with an *IGL* amplification polymorphism, we did not detect the $Mcg^-Ke^+Oz^-$ polymorphism, indicating that the two polymorphisms are not linked.

Table 5. Mean Igκ/gλ ratios for different numbers of functional J-Cλ gene regions.

Number of J-Cλ gene regions	Mean Igκ/Igλ ratio	SD	Number of analyzed cases
8	1.56[a]	0.26	38
9-13	1.37[a]	0.18	31
9	1.30	0.35	4
10	1.36	0.17	13
11	1.39	0.14	6
12	1.40	0.15	7
13	1.42	-	1

a. The Igκ/Igλ ratio in cases without amplification is significantly higher than in cases with amplification (t-test; $p<0.001$).

DISCUSSION

Two polymorphisms have been described in the human *IGL* locus. However, they have never been sequenced at the DNA level. The first polymorphism concerns an *IGL* amplification polymorphism of 5.4 kb of DNA in the Cλ2-Cλ3 region. The second polymorphism is the Mcg$^-$Ke$^+$Oz$^-$ isotype, which was only known from serological analyses of Bence-Jones proteins of multiple myeloma patients.[11-14] Due to the high homology with the Mcg$^-$Ke$^-$Oz$^-$ isotype, which is encoded by the J-Cλ2 region, it was proposed that the Mcg$^-$Ke$^+$Oz$^-$ polymorphism is encoded by a polymorphic J-Cλ2 region that differs from the normal J-Cλ2 region at a single nucleotide position. It has been speculated that the two polymorphisms might be linked in such a way that the Cλ region on the polymorphic amplification encodes the isotype Mcg$^-$Ke$^+$Oz$^-$.[8,14]

In this study, we were able to sequence the 5.4 kb *IGL* amplification polymorphism. The *IGL* amplification polymorphism shares a homology of more than 99% with the J-Cλ2 region. The first 2.5 kb of the amplification, containing the Jλ and Cλ gene segments, coincides with a 2.2 kb region, which is also highly homologous to a 2.2 kb region in the J-Cλ3 cluster (98% homology) (Figure 3b). Taub *et al.* proposed already in 1983 a mechanism for the generation of this *IGL* polymorphism.[15] Based on SB data and restriction fragment analysis, they proposed unequal crossing over between two 8 kb *Eco*RI allelic fragments through homologous recombination between two large homologous repeats. Our sequence information of the polymorphic *IGL* amplification together with the germline sequence of the complete *IGL* locus, shows that the homologous regions are 5.4 kb apart in the germline (P_0) situation. Therefore, we confirm that the amplification most probably occurred during meiosis via unequal homologous recombination between the two 2.2 kb homologous J-Cλ2 and J-Cλ3 regions.

The Cλ2 and Cλ3 gene region might be susceptible to duplication, because of its high homology, but especially because of the two homologous J-Cλ regions of 2.2 kb. The maximum number of amplifications is three, which suggests that the region is indeed susceptible to duplication, but this phenomenon apparently has its limitations. The different amplification variants appeared to be exactly identical. No differences were observed in multiple amplifications on the same allele, which suggests that during evolution the various amplifications occurred simultaneously or shortly after each other.

The J-Cλ region of the polymorphic amplification appeared to have the highest homology with the J-Cλ2 region and contains all essential elements, which are required for recombination and expression, i.e. a functional RSS, intact donor and acceptor splice-sites, as well as functional Jλ and Cλ gene regions. Since the amplified J-Cλ2 region does not encode the Mcg$^-$Ke$^+$Oz$^-$ isotype, we concluded that the human *IGL* locus has two separate polymorphisms.

As known from literature, the *IGL* amplification polymorphism is found up to three times per allele.[15,18] Our sequence analysis showed that this polymorphism concerns identical

amplifications, implying that in cases with a P_2 amplification the first amplified region does not differ from the second. Screening of healthy controls showed that the frequency of the *IGL* amplification was 28% in Caucasoid individuals and involved only one allele. In Chinese individuals, the frequency was much higher (85%) and involved both alleles in the majority of cases.

As the polymorphic amplified regions contain functional J-Cλ2 regions, we investigated the effect of the total number of J-Cλ regions on the frequency of $Ig\lambda^+$ B lymphocytes by studying the distribution between Igκ and Igλ light chain expression (Igκ/Igλ ratio). The presence of additional functional J-Cλ gene regions resulted in decreased Igκ/Igλ ratios of blood B lymphocytes as compared to individuals without the J-Cλ2 amplification. Already one additional J-Cλ gene region was sufficient to decrease the Igκ/Igλ ratio, suggesting that this effect is dominant and that the addition of extra J-Cλ gene regions has little effect on the Igκ/Igλ ratio.

The Igκ/Igλ ratio can not only be decreased by extra copies of J-Cλ regions, but also by a decrease in *IGK* gene copies. In a patient with a heterozygous *de novo* deletion of chromosome 2 region p11.2p13, including the *IGK* locus (2p12), the Igκ/Igλ ratio was strongly decreased to 0.7.[21] This decrease is stronger than found in individuals with extra J-Cλ regions. This difference in effect on the Igκ/Igλ ratio can be explained by the fact that Ig light chain rearrangements take place in an ordered fashion, starting with rearrangements in the *IGK* locus followed by *IGL* gene rearrangements, if no functional *IGK* rearrangement took place.[20] If there is only one *IGK* allele, the chance of a functional rearrangement resulting in Igκ expression is approximately 50% reduced. Consequently, the rearrangement process will shift to the *IGL* locus in an earlier stage, which is in line with a reduction of the Igκ/Igλ ratio from 1.4 to 0.7. On the other hand, there is a limited time period or limited number of attempts for acquiring functional Ig gene rearrangements.[22,23] If the B cell is not able to generate an Ig molecule within that time frame or that number of attempts, the cell will die by apoptosis.[22,23] Finally, the presence of multiple functional J-Cλ regions also implies that consecutive *IGL* rearrangements with Vλ-Jλ replacements can occur on one allele.

The polymorphic $Mcg^-Ke^+Oz^-$ isotype is the second type of *IGL* polymorphism and was only known from serological analyses. Our sequencing data now show that this isotype is encoded by a polymorphic Cλ2 gene segment. The polymorphic Cλ2 region was detected in only two individuals, which were both heterozygous for this *IGL* polymorphism. The reported frequency of the $Mcg^-Ke^+Oz^-$ polymorphism in 70 multiple myeloma patients was 6%,[13,19,24] which exceeds the frequency of 1.5% in the 134 analyzed individuals, which did not carry the *IGL* amplification polymorphism. However, this difference is not statistically significant (χ^2-test, $p>0.05$). No $Mcg^-Ke^+Oz^-$ polymorphism was detected in the J-Cλ2 amplification polymorphism of 44 individuals, but this might be due to the low number of analyzed cases. Nevertheless, the obtained data indicate that the J-Cλ2 amplification polymorphism and the Cλ2 polymorphism are independent. The two polymorphisms might theoretically be present in one individual, but the frequency of such event will be low (<0.5%).

In conclusion, two separate polymorphisms in the human *IGL* locus exist: the *IGL*

amplification polymorphism and the Cλ2 polymorphism resulting in the Mcg$^-$Ke$^+$Oz$^-$ isotype. Both polymorphisms involve the Cλ2 region, but they are not linked, although they might theoretically be both present in one individual. The presence of the *IGL* amplification polymorphism results in the presence of extra functional J-Cλ regions, which appeared to decrease the Igκ/Igλ ratio of normal blood B lymphocytes.

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Chapter 2.3

IMMUNOGLOBULIN LAMBDA ISOTYPE GENE REARRANGEMENTS IN B-CELL MALIGNANCIES

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ABSTRACT

The human immunoglobulin lambda (*IGL*) locus contains seven J-Cλ gene regions of which only J-Cλ1, J-Cλ2 J-Cλ3 and J-Cλ7 encode the four Igλ isotypes, i.e. Mcg, $Ke^{-}Oz^{-}$, $Ke^{-}Oz^{+}$, and Mcp, respectively. We used isotype specific DNA probes for detection of *IGL* gene rearrangements in 212 B-cell malignancies: 76 precursor B-cell acute lymphoblastic leukemias (precursor B-ALL), 74 $Ig\lambda^{+}$ chronic B-cell leukemias (CBL), 34 $Ig\lambda^{+}$ non-Hodgkin lymphomas (B-NHL), and 28 $Ig\lambda^{+}$ multiple myelomas (MM). The J-Cλ3 gene region was most frequently involved (50%), followed by J-Cλ2 (38%) and J-Cλ1 (9%). There was no involvement of J-Cλ4 and J-Cλ5 gene regions. Rearrangements to J-Cλ6 (n=4) were exclusively found in precursor B-ALL (19% of all *IGL* rearrangements in precursor B-ALL) and only a single J-Cλ7 recombination was detected in an $Ig\lambda^{+}$ B-NHL. In the group of $Ig\lambda^{+}$ malignancies a significant shift was observed from predominant J-Cλ3 usage (54%) in mature surface $Ig\lambda^{+}$ malignancies (CBL and B-NHL) to 60% J-Cλ2 usage in $Ig\lambda^{+}$ secreting MM. The distribution of *IGL* isotype rearrangements found in MM reflected the Igλ isotype protein expression reported in MM patients.

Based on these extensive Southern blot data, we suggest that a rapid and efficient detection of clonal *IGL* gene rearrangements can be obtained when a single *Bgl*II digest is used in combination with the IGLJ2 probe, which detects clonality in >95% of cases with an $Ig\lambda^{+}$ malignancy. Higher percentages (>98%) can be reached by including a second digest (*Hin*dIII) that reduces the chance of comigration rearranged and germline bands. In case of precursor B-ALL we recommend to include the IGLJ6 probe for the detection of rearrangements to J-Cλ6.

INTRODUCTION

The human immunoglobulin lambda light chain gene (*IGL*) has been mapped to chromosome 22q11.[1-3] The locus contains 36 Vλ gene segments with an open reading frames (ORFs) (but only 31 of them have been found in functional rearrangements) 33 pseudo gene segments, and seven J-Cλ gene regions.[3,4] A genetic amplification polymorphism of the Cλ2-Cλ3 region can lead to the presence of up to ten J-Cλ gene regions per allele.[5] The Vλ gene segments are grouped in ten families, which are organized in three separate clusters.[6] Cluster A is located proximal to the J-Cλ regions and contains families VλII and III, cluster B contains family VλI, one member of family IV, and families V, VII, and IX, whereas the remaining members of family VλIV as well as families VI, VIII, and X are grouped in cluster C.[6]

The gene regions J-Cλ1, J-Cλ2, J-Cλ3, and J-Cλ7 are functional, while J-Cλ4, J-Cλ5, and J-Cλ6 are non-functional (pseudo) gene regions.[2,7,8] The J-Cλ4 region has a deletion of ~1150 bp and in the 3' end of the Cλ5 exon 11 bp are deleted.[2] Moreover, the recombination

signal sequences (RSS) of the Jλ4 and Jλ5 gene segments do not contain all essential nucleotides.[9] The Cλ6 region contains a duplication of four nucleotides which results in a premature stop codon.[2,8] Stiernholm *et al.* demonstrated that rearrangements to the J-Cλ6 segment can occur, but encode a truncated Igλ protein.[10]

Initially, four different isotypes of Igλ light chains have been identified in human serum: Mcg, Ke^-Oz^-, Ke^-Oz^+, and Ke^+Oz^-.[1] The serological isotype markers Mcg, Kern (Ke) and Oz are based on amino acid differences found in the Cλ region of various Bence-Jones proteins from multiple myeloma (MM) patients and Igλ chains isolated from intact immunoglobulins.[1,11,12] The four isotypes were initially assumed to be encoded by the four functional J-Cλ gene regions J-Cλ1, J-Cλ2, J-Cλ3, and J-Cλ7, respectively.[1,8] However, Niewold *et al.* discovered another isotype, Mcp, and demonstrated that Mcp and not Ke^+Oz^- is encoded by J-Cλ7.[13] Based on the high homology of the Ke^-Oz^- and Ke^+Oz^- amino acid sequences, it might be that Ke^+Oz^- is encoded by a polymorphic J-Cλ2 gene segment. However this is not yet proven by DNA sequence analysis.

Since at least one third of all B-cell malignancies have rearranged *IGL* genes, J-Cλ region specific Southern blot probes were developed to detect and identify clonal *IGL* rearrangements in these malignancies.[14] In this study, these "isotype-specific" DNA probes were used to determine the frequency of J-Cλ gene rearrangements in a large series of 212 B-cell malignancies: 76 precursor-B acute lymphoblastic leukemias (precursor B-ALL), 74 $Ig\lambda^+$ chronic B-cell leukemias (CBL), 34 $Ig\lambda^+$ B-cell non-Hodgkin lymphomas (B-NHL), and 28 $Ig\lambda^+$ MM. The results of the J-Cλ rearrangement patterns in the MM are compared with the reported frequencies of Igλ isotype protein expression. We assume that the Igλ isotype rearrangement patterns in the various types of B-cell malignancies can give insight into Igλ isotype usage and/or Igλ isotype selection during B-cell differentiation.

MATERIALS AND METHODS

Cell samples

For this study 212 B-cell malignancies were selected based on the availability of sufficient cells for DNA extraction, on their tumor load (i.e. > 70% for leukemias and B-NHL and >25% for multiple myelomas) and on the membrane or cytoplasmic expression of Igλ in case of mature Ig^+ malignancies. In this way 212 patient samples were selected, including the 40 patients who have previously been described [14]: 76 precursor B-ALL, 74 $Ig\lambda^+$ chronic B-cell leukemias CBL, 34 $Ig\lambda^+$ B-NHL, and 28 $Ig\lambda^+$ MM. The series of 74 $Ig\lambda^+$ CBL consisted of 63 B-cell chronic lymphocytic leukemias (B-CLL), 6 B-cell prolymphocytic leukemias (B-PLL), and 5 hairy cell leukemias (HCL). Mononuclear cells (MNC) were isolated from 117 peripheral blood (PB) and 78 bone marrow (BM) samples by Ficoll-Paque density centrifugation (density 1.077 g/l; Pharmacia, Uppsala, Sweden). Cell suspensions were prepared from 17 lymph node samples of $Ig\lambda^+$ B-NHL patients; the remaining 17 $Ig\lambda^+$ B-NHL were leukemic so that PB samples could be used.

Southern blot analysis

DNA was isolated as previously described.[15] Fifteen micrograms of DNA were digested with the appropriate restriction enzymes (Pharmacia). The restriction fragments were size fractionated in 0.7% agarose gels and transferred by vacuum blotting to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany). The membranes were hybridized with ^{32}P-random oligonucleotide labeled probes.[15]

DNA probes and *IGL* gene restriction map

In order to identify clonal *IGL* rearrangements in the J-Cλ gene locus, a set of seven isotype-specific DNA probes were used, which specifically recognize the seven J-Cλ regions: the IGLC1D probe for the J-Cλ1 region, the IGLJ2 probe for the highly homologous J-Cλ2 and J-Cλ3 regions, the IGLC2D probe, which exclusively recognizes the J-Cλ2 gene region, and the IGLC4D, IGLJ5, IGLJ6 and the IGLJ7 probes for the last four J-Cλ gene regions.[14] These probes were used in combination with *Hin*dIII, *Bgl*II, *Bam*HI/*Hin*dIII, *Eco*RI/*Hin*dIII restriction enzyme digests.[14] The restriction map of the J-Cλ gene region is given in Figure 1. The 'general' Cλ probe (IGLC3) was used in combination with *Eco*RI/*Hin*dIII digests and is also indicated in Figure 1.[16]

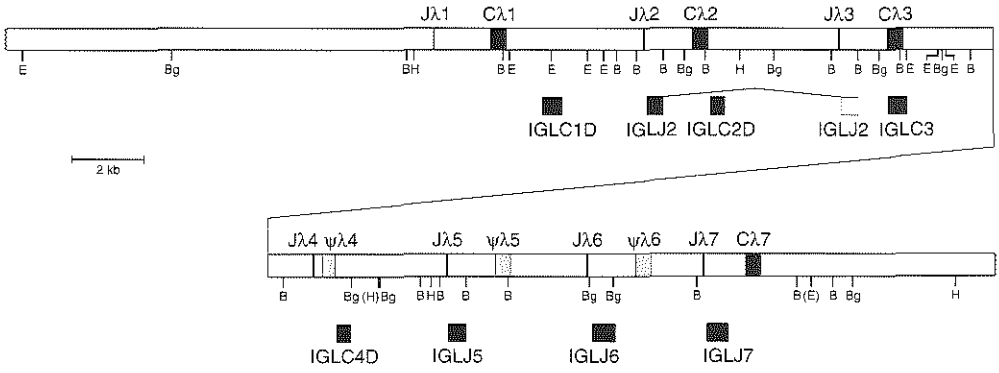

Figure 1. Structure and restriction map of the J-C region of the human *IGL* gene complex.[2,8,14] The location of the relevant *Hin*dIII (H), *Bgl*II (Bg), *Bam*HI (B), and *Eco*RI (E) sites are indicated. The partially resistant *Hin*dIII and *Eco*RI restriction sites downstream of the ψCλ4 and Cλ7 exons, respectively, are in parentheses. The solid boxes represent the functional Cλ exons and the shaded boxes represent the non-functional pseudo Cλ exons. The probes are indicated as solid bars. These probes are specific for the corresponding J-Cλ gene segments, except for the IGLJ2 probe, which recognizes both the J-Cλ2 and J-Cλ3 gene regions (98% homology), and the general IGLC3 which recognizes all Cλ exons (85% homology) of the classical *IGL* locus[15] and the Cλ exons of the surrogate λ-like gene complexes 14.1, 16.1, 16.2, and 18.2.[16] The recognition site of the IGLJ2 probe in the J-Cλ3 region is indicated with an open bar.

RESULTS AND DISCUSSION

Although about 40% of all B-cell malignancies have rearranged *IGL* genes that can be employed for clonality studies,[17] *IGL* gene rearrangements are rarely studied in B-cell malignancies because of the complex structure of the human *IGL* locus. This is the first detailed study on *IGL* isotype rearrangements in a large series of 212 B-cell malignancies using a set of seven *IGL* isotype probes.[14] The use of these isotype-specific *IGL* probes together theoretically detects all *IGL* gene rearrangements.

Frequency of *IGL* gene rearrangements

In all 136 Igλ+ B-cell malignancies at least one *IGL* gene rearrangement was found by Southern blot analysis; in 35% (47/136) of cases both *IGL* alleles were rearranged and in one B-CLL even three *IGL* rearrangements were identified (Table 1).

In contrast, in only 20% (15/76) of precursor B-ALL *IGL* rearrangements were found. This frequency is similar as described previously.[18] In ten precursor B-ALL a single *IGL* gene rearrangement was found, in four cases biallelic *IGL* gene rearrangements, and one case even had three rearranged *IGL* alleles.

Precursor B-ALL arise from B-cell precursor cells that are transformed at an immature differentiation stage in which the recombinase enzyme system is continuously active.[19] This results in the occurrence of ongoing Ig gene rearrangements in 30 to 40% of cases and cross-lineage T-cell receptor gene rearrangements in ~90% of cases.[20-23] Despite this continuous activity of the recombinase enzyme system, we observed only a low frequency of 20% *IGL* rearrangements. This might be caused by a lower accessibility of the *IGL* locus for recombination at this immature differentiation stage.[17]

The frequency of *IGL* gene rearrangements in precursor B-ALL is higher than in Igκ+ CBL, which have *IGL* rearrangements in only 5% of cases.[24] CBL arise from B-cells that are transformed after the Ig gene rearrangement process has been completed successfully, resulting in surface Ig expression. These malignancies show the expected Ig light chain gene rearrangement pattern, which can be described by the ordered model of Ig light chain rearrangements: start of recombination in the Ig kappa (*IGK*) locus, followed by *IGK* deletion and *IGL* gene rearrangements, and a stop of recombination as soon as a functional protein is expressed on the cell surface.[24-26] The low frequency of *IGL* gene rearrangements in Igκ+ CBL is probably caused by inaccessibility of the *IGL* locus and down regulation of the recombinase enzyme system as soon as a functional Igκ protein is formed. The continuous recombinase activity in precursor B-ALL apparently partly overcomes the inaccessibility of the *IGL* locus, resulting in higher frequencies of the *IGL* gene rearrangements than found in mature Igκ+ B-cell malignancies.

Table 1. Igλ isotype gene rearrangements in 212 B-cell malignancies.

B-cell malignancies	J-Cλ1	J-Cλ2	J-Cλ3	J-Cλ4	J-Cλ5	J-Cλ6	J-Cλ7	Unidentified rearrangements	Total number of rearranged alleles
Igλ+ CBL	10	32	53	0	0	0	0	1	96
(n=74)	(10%)	(33%)	(55%)	(0%)	(0%)	(0%)	(0%)	(1%)	
Igλ+ B-NHL	7	16	27	0	0	0	1	1	52
(n=34)	(13%)	(30%)	(51%)	(0%)	(0%)	(0%)	(2%)	(2%)	
Igλ+ MM	1	21	13	0	0	0	0	0	35
(n=28)	(3%)	(60%)	(37%)	(0%)	(0%)	(0%)	(0%)	(0%)	
Total Igλ+ malignancies	18	69	93	0	0	0	1	2	183
(n=136)	(10%)	(38%)	(51%)	(0%)	(0%)	(0%)	(0.5%)	(1%)	
Precursor B-ALL[a]	0	9	8	0	0	4	0	0	21
(n=76)	(0%)	(43%)	(38%)	(0%)	(0%)	(19%)	(0%)	(0%)	
Total B-cell malignancies	18	78	101	0	0	4	1	2	204
(n=212)	(9%)	(38%)	(50%)	(0%)	(0%)	(2%)	(0.5%)	(1%)	

a. Fifteen of the 76 precursor B-ALL (20%) contained at least one rearranged *IGL* allele.

J-Cλ usage

Most rearrangements in our series of 212 B-cell malignancies involved the J-Cλ3 region (50%), followed by J-Cλ2 (38%) and J-Cλ1 (9%), while rearrangements to the J-Cλ6 and J-Cλ7 regions were rare (2% and 0.5%, respectively) (Table 1). In one CBL and one B-NHL patient the *IGL* gene rearrangements could not be identified precisely, which might be caused by e.g. chromosome aberration t(18;22). Rearrangements to J-Cλ4 and J-Cλ5 were not found (Table 1), which could be expected from the lack of several essential nucleotides in the heptamer sequences of the Jλ4 and Jλ5 RSS.[9] Rearrangements involving the J-Cλ6 region (n=4) were exclusively found in precursor B-ALL and represented 19% of all *IGL* gene rearrangements in this group of malignancies (Figure 2A). The absence of J-Cλ6 rearrangements in mature Igλ+ B-cell malignancies might be explained by the fact that the J-Cλ6 region can only encode a truncated protein, which cannot contribute to a functional Ig molecule.[10]

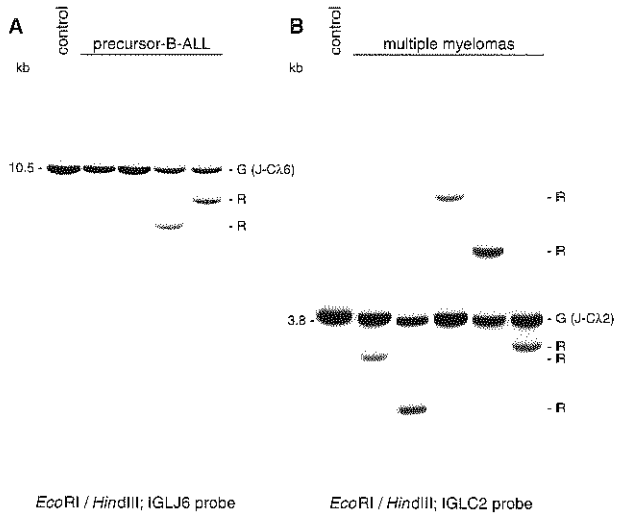

Figure 2. Southern blot analysis of *IGL* genes in B-cell malignancies. DNA samples from precursor B-ALL and MM patients were digested with *Eco*RI/*Hin*dIII, size fractionated in agarose gels, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled DNA probes. **A.** J-Cλ6 gene rearrangements can occur in precursor B-ALL. **B.** J-Cλ2 rearrangements are rather frequent in MM.

The percentages of J-Cλ usage in our series of Igλ+ B-cell malignancies are in agreement with the results of Ignatovich *et al.*, which are based on unbiased Igλ cDNA libraries from PB of five healthy individuals (Table 2).[27] However, Farner *et al.* recently reported a much higher percentage of Jλ7 usage (60% in productive rearrangements and 54% in non-productive rearrangements) by single-cell PCR on purified CD19+/IgM+/CD5+ and CD5- PB B-cells (Table 2).[28] This unexpectedly high frequency of Jλ7 usage in normal CD5+ and CD5- PB B-lymphocytes is in full contrast to the report of Ignatovich et al. (0.6%),[27] to our

Table 2. Jλ isotype usage in normal and malignant B-cells.

	Normal B-cells[a]		Normal B-cells[b]		Malignant B-cells[c]			
	Five cDNA libraries 7600 clones		Non-productive 55 alleles	Productive 172 alleles	SmIgλ+ 148 alleles		Igλ+ MM 35 alleles	
Jλ1	27%		6%	7%	12%		3%	
Jλ2	38%	72%	35%	39%	33%	88%	60%	97%
Jλ3	34%				55%		37%	
Jλ7	0.6%		60%	54%	<1%		0%	

a. Data from Ignatovich *et al.*[27]
b. Data from Farner *et al.*[28]
c. Presented study.

results in B-cell malignancies (0.5%) and to literature data on Igλ isotype usage in MM patients (<1%).[1,11,12,29] This discrepancy cannot easily be explained from an immunobiological point of view, but it might well be caused by a technical pitfall, such as inappropriate primer design.

The low frequency of rearrangements to the J-Cλ6 and J-Cλ7 gene regions might partly be attributed to the larger distance from the Vλ gene segments as compared to the J-Cλ1, J-Cλ2, and J-Cλ3 regions. However, proximity is probably not the only factor influencing the J-Cλ usage,[30] as was demonstrated by the results in precursor B-ALL where 19% of the rearrangements did involve the J-Cλ6 region.

In the surface Igλ+ B-cell malignancies (CBL and B-NHL) rearrangements occurred most frequently to the J-Cλ3 gene region (54%), followed by J-Cλ2 (32%) and J-Cλ1 (11%), while rearrangements in Igλ+ MM most frequently involved the J-Cλ2 region (60%) (Figure 2B), followed by J-Cλ3 (37%) and J-Cλ1 (3%). The two-fold increase of J-Cλ2 rearrangements in MM coincided with a four-fold decrease of J-Cλ1 rearrangements and a 1.5 fold decrease of J-Cλ3 rearrangements (Table 2). This statistically significant shift ($p<0.0064$) (χ^2 test, with $p<\alpha$, and $\alpha=0.05$) from preferential J-Cλ3 rearrangements in CBL and NHL to preferential J-Cλ2 rearrangements in MM might be due to Igλ isotype selection processes during terminal B-cell differentiation.

When comparing J-Cλ isotype usage in normal PB B-cells (Ignatovitch *et al.*) and SmIgλ+ malignant B cells, there is a significant shift form J-Cλ1 (27% vs 12%) to J-Cλ3 (34% vs 55%). This might reflect an editing process in the malignant B cells mediated via secondary rearrangements using downstream Jλ gene segments. However, receptor editing only takes place if during antigen selection unfavorable somatic mutations occurred resulting in loss of Ig light chain expression or autoreactivity. Like most PB B-cells also many CBL and a part of B-NHL do not have somatic mutations.[31,32] Therefore, it is unlikely that receptor editing induced by unfavorable somatic mutations is the sole explanation for the observed significant difference in J-Cλ usage.

Correlation between *IGL* isotype gene rearrangements and Igλ isotype protein expression in multiple myeloma

The distribution of Igλ isotype gene rearrangements in MM resembles the reported

Igλ isotype pattern of Bence Jones proteins. Cλ2 (Ke^-Oz^-) occurs most frequently, followed by Cλ1 (Mcg), Cλ3 (Ke^-Oz^+), and Cλ7 (Mcp) (Table 3).[12,29] However, in the Bence Jones protein studies the frequency of Cλ1 (Mcg) expression appeared to be higher (23%) than the frequency of J-Cλ1 isotype gene rearrangements that we observed in our multiple myeloma series (3%) (Table 3). It might be that the studied series of Bence Jones proteins was a non-random selection or that in our random series Cλ1 was underrepresented. Alternatively, it might be that the formation of Bence Jones proteins is influenced by the isotype of the Igλ chain.

The used *IGL* specific isotype probes can not discriminate between rearrangements in normal J-Cλ2 regions and polymorphic J-Cλ2 regions. However, the reported combined frequency of Ke^-Oz^- and Ke^+Oz^- Bence Jones proteins (57%) is comparable to our frequency of J-Cλ2 rearrangements (60%) in MM (Table 3), which would favor the assumption that Ke^+Oz^- proteins might be encoded by a polymorphic J-Cλ2 region.

Table 3. Correlation between Igλ isotype protein expression and the Igλ isotype gene rearrangement in multiple myeloma.

Multiple myeloma	J-Cλ1 (Mcg)	J-Cλ2 (Ke^-Oz^-)	Polymorphic J-Cλ2 (Ke^+Oz^-)[a]	J-Cλ3 (Ke^-Oz^+)	J-Cλ7 (Mcp)
Igλ isotype protein expression (n=70)[b]	16/70 (23%)	36/70 (51%)	4/70 (6%)	14/70 (20%)	<1%[c]
Igλ isotype gene rearrangements (n=35), this study	1/35 (3%)	21/35[d] (60%)		13/35 (37%)	0/35 (0%)

a. It is assumed, but not yet proven, that Ke^+Oz^- Igλ proteins are derived from the polymorphic Cλ2 region.
b. Based on literature data by Fett and Deutsch and Walker *et al.*[12,29] The number of studied Bence Jones proteins in the study by Walker *et al.* is corrected for duplicates with the study by Fett and Deutsch.
c. According to Niewold et al. the frequency of Mcp isotype is <1%.[13]
d. The IGLJ2 and IGLC2D probes can not discriminate between rearrangements in normal and polymorphic J-Cλ2 regions.

Strategies for probe/enzyme combinations in analysis of *IGL* gene rearrangements

Based on our data of *IGL* gene rearrangements in B-cell malignancies, several strategies can be proposed for clonality studies. The Southern blot data indicate that 97% (197/204) of the rearrangements occurred to the J-Cλ1, J-Cλ2, and J-Cλ3 region, while rearrangements to the remaining J-Cλ regions were rare (2%, 5/204) with another two rearrangements (1%, 2/204) remaining unidentified.

In this study the seven isotype-specific probes were used in fifteen probe-enzyme combinations. As a consequence of this extensive analysis all *IGL* gene rearrangements were detected and 99% of the rearranged *IGL* alleles were identified (Table 4). However, we wanted to reduce the high number of hybridizations. For this purpose, it was determined to what extent the general Cλ probe (IGLC3) detected all *IGL* rearrangements. In combination with *Eco*RI/*Hin*dIII digests this probe detected rearrangements in 97% of the 151 B-cell malignancies with rearranged *IGL* genes and 94% of the rearranged *IGL* alleles (Table 4). However, a disadvantage of this approach is the difficult interpretation of the complex banding pattern, with germline and rearranged bands of different density, which is caused by vari-

Table 4. Comparison of four strategies for detection of clonal *IGL* gene rearrangements in B-cell malignancies.

B-cell malignancies (Patients/alleles)	All seven isotype probes (fifteen hybridizations)		IGLC3 probe (*Eco*RI/*Hin*dIII; one hybridization)		IGLC1D, IGLC2D, and IGLJ2 probes (*Hin*dIII, *Bgl*II, *Eco*RI/*Hin*dIII; five hybridizations)		IGLC1D and IGLJ2[a] (*Bgl*II; two hybridizations)	
	patients	alleles	patients	alleles	patients	alleles	patients	alleles
Igλ+ CBL (n=74/n=96)	100%	99%	95%	93%	97%	97%	97%	97%
Igλ+ B-NHL (n=34/n=52)	100%	98%	100%	96%	100%	98%	97%	94%
Igλ+ MM (n=28/n=35)	100%	100%	100%	97%	100%	100%	93%	91%
Subtotal (n=136/n=183)	100%	99%	97%	95%	99%	97%	96%	95%
Precursor-B-ALL (n=15/n=21)	100%	100%	93%	90%	73%	81%	71%	76%
Total (n=151/n=204)	100%	99%	97%	94%	96%	96%	94%	93%

a. If no identification is required it is possible to restrict Southern blot analysis to a single hybridization of IGLJ2 probe, because IGLC1D and IGLJ2 reside on the same *Bgl*II restriction fragment.

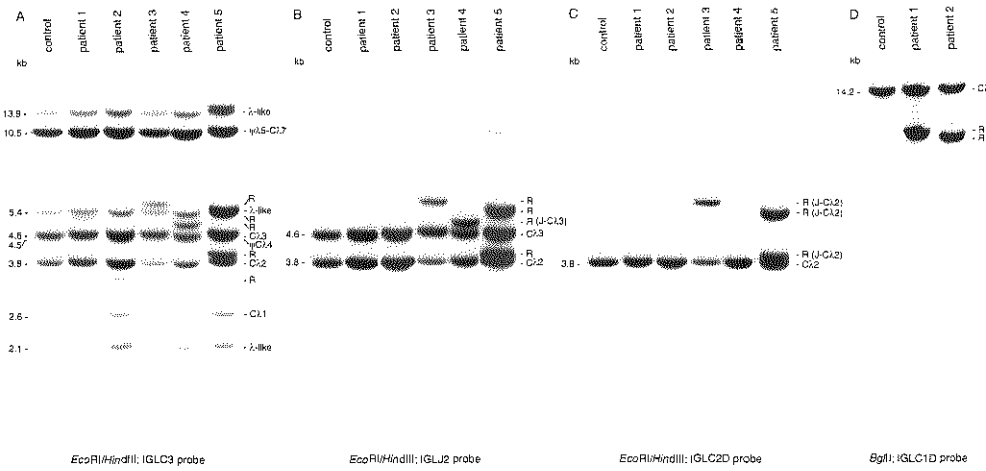

Figure 3. Comparative Southern blot analysis in B-cell malignancies. Control DNA, and DNA from five Igλ+ malignancies were digested with *Eco*RI/*Hin*dIII or *Bgl*II, and hybridized with ^{32}P-labeled IGLC3, IGLJ2, IGLC2D, and IGLC1D probes. **A.** Hybridization of the 'general' IGLC3 probe to *Eco*RI/*Hin*dIII digests results in multiple germline bands and rearranged bands of different density. **B.** The IGLJ2 probe identifies rearrangements to both the J-Cλ2 and J-Cλ3 gene regions. **C.** The IGLC2D probe only identifies rearrangements to the J-Cλ2 region. The germline and rearranged J-Cλ3 bands (as seen in panel B) are absent. **D.** Rearrangements to the J-Cλ1 region can easily be identified with the IGLC1D probe. It should be noted that J-Cλ1 gene rearrangements frequently give only a faint band if hybridization is performed with the 'general' IGLC3 probe (panel A, lanes 2 and 3) because of the lower homology (93%) between the Cλ1 exon and the other Cλ exons.

able degrees of hybridization of the IGCL3 probe to Cλ gene segments of the classical and surrogate *IGL* loci (Figure 3A).[14,16] Particularly in case of lower tumor loads (<75%) the detection of rearranged bands is less efficient due to faint signals. For example, rearrangements to the J-Cλ1 region can easily be missed (Figure 3A, D), because of reduced homology (93%) between the Cλ1 gene sequence and the IGLC3 probe.[14]

Since 97% of all *IGL* rearrangements occurred to the first three J-Cλ regions, we evaluated the use of IGLC1D, IGLC2D, and IGLJ2 probes in combination with *Eco*RI/*Hin*dIII, *Bgl*II and *Hin*dIII digests (see Figure 1). During the first round of hybridization the IGLC2D probe can be hybridized to *Eco*RI/*Hin*dIII filters and the IGLC1D probe to *Bgl*II and *Hin*dIII filters and in a second round hybridization the IGLJ2 probe can be applied to *Eco*RI/*Hin*dIII and *Bgl*II filters. These five hybridizations resulted in identification and detection of 96% of all *IGL* gene rearrangements (Table 4, Figure 3 B,C,D). With this combination 99% of patients with an Igλ+ malignancy could be identified. Only in precursor B-ALL approximately 27% of the patients were missed (Table 4), mainly because they had J-Cλ6 rearrangements. In contrast to the IGLC3 probe, the IGLC1D probe easily identified all J-Cλ1 rearrangements (Figure 3A, D).

In case of limited amounts of DNA, one might decide to restrict the analyses to a sin-

gle *Bgl*II digest and successive hybridization with the IGLJ2 probe and the IGLC1D probe. This approach allowed detection and identification of 93% (190/204) of all *IGL* gene rearrangements, and 94% (142/151) of the patients, but discrimination between J-Cλ2 and J-Cλ3 rearrangements was difficult if the tumor load was not high (<75%) and the four J-Cλ6 rearrangements in precursor B-ALL were missed. Most clonality studies are needed for supporting the diagnosis of mature B-cell malignancies (not for precursor B-ALL) and for such clonality studies it is generally not necessary to identify the *IGL* gene rearrangement as involving J-Cλ1, J-Cλ2, or J-Cλ3. Therefore it is in principle possible to restrict the Southern blot analysis to application of the IGLJ2 probe in *Bgl*II digests, because this probe hybridizes to both the Jλ2 and Jλ3 regions and also detects rearrangements in the Jλ1 region, since J-Cλ1 and J-Cλ2 are located on the same *Bgl*II restriction fragment (see Figure 1). According to our data, this single probe-enzyme approach results in detection of 95% (174/183) of all *IGL* gene rearrangements in Igλ+ B-cell malignancies and clonality detection in 96% (131/136) of the patients (Table 4). The reason for missing some rearrangements was comigration of two rearranged bands or a rearranged and germline band, which is prevented by use of a second digest (*Hin*dIII). An important advantage of this approach is that the same *Bgl*II filter can be used for analysis of gene rearrangements in the *IGH* locus with the IGHJ6 probe and for rearrangements in the *IGK* locus with the IGKJ5 and IGKDE probes.[14,15,33,34] This would imply that a single *Bgl*II digest in combination with four successive hybridizations (IGHJ6, IGKJ5, IGKDE, and IGLJ2) would provide insight in the configuration of the *IGH, IGK,* and *IGL* genes. Generally we advice to use two restriction enzyme digests per probe for reliable Southern blot analysis.[15,33-35] Nevertheless, our data indicate that *Bgl*II digests are informative in the vast majority of cases (>95%) and rarely show restriction fragment length polymorphisms in the relevant *IGH*, *IGK* and *IGL* regions.[14,15,33,34]

CONCLUSION

Our extensive data on *IGL* isotype specific gene rearrangements in a large series of 212 B-cell malignancies show that in >96% of cases the J-Cλ1, J-Cλ2 or J-Cλ3 gene region was involved, while rearrangements to J-Cλ6 and J-Cλ7 were rare and J-Cλ4 and J-Cλ5 recombinations were completely absent. Moreover, J-Cλ6 rearrangements were exclusively found in precursor B-ALL. For efficient and reliable Southern blot analysis, we advice to use a *Bgl*II digest in combination with the IGLJ2 probe, which allows detection of J-Cλ1, J-Cλ2, and J-Cλ3 rearrangements, covering 99% of the rearrangements in Igλ+ B-cell malignancies. Use of a second digest (*Hin*dIII) will exclude the chance of missing a rearranged allele due to comigration of rearranged and germline bands. For precursor B-ALL we recommend to include the IGLJ6 probe in addition to the above mentioned probes, because J-Cλ6 recombinations represented 19% of the *IGL* gene rearrangements in precursor B-ALL.

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Chapter 3

REGULATION OF IG LIGHT CHAIN GENE RECOMBINATION

3.1 Regulation of Ig light chain gene recombination.

3.2 Ordered recombination of immunoglobulin light chain genes occurs at the *IGK* locus, but seems less strict at the *IGL* locus.
Blood 2001;97:1001-1008.

3.3 Immunoglobulin light chain gene rearrangements display hierarchy in absence of selection for functionality in precursor-B-ALL.
Leukemia 2002;16: in press.

3.4 Immunoglobulin light chain gene recombination patterns in Mantle cell lymphoma as clonal model system for naive B cells.

3.5 Biased Igλ expression in hypermutated IgD multiple myelomas does not result from receptor revision.
Leukemia 2002;16: in press.

Chapter 3.1

REGULATION OF IG LIGHT CHAIN GENE RECOMBINATION

REGULATION OF IG LIGHT CHAIN GENE RECOMBINATION

Proper regulation of V(D)J recombination at the Ig loci is required for the generation of monospecific B cells. V(D)J recombination is regulated at different levels ensuring restriction of V(D)J recombination to lymphoid cells, ensuring lineage specificity, i.e. V(D)J recombination of Ig loci in B cells and TCR gene recombinations in T cells, and aiming at allelic exclusion resulting in the presence of a functional rearrangement at only one allele.[1] In Chapter 3 of this thesis we focused on the regulation of allelic exclusion of Ig light chain genes. In addition to allelic exclusion, also isotypic exclusion applies to Ig light chain loci. Isotypic exclusion is defined as the regulation process for single *IGK* or *IGL* expression. Two models have been proposed for the regulation of isotypic exclusion. The ordered model proposes that the *IGK* genes rearrange prior to *IGL*, while the stochastic model postulates that, in principal, the two types of Ig light chain genes rearrange totally independent, but that other factors render *IGL* gene rearrangements more difficult.[2-7]

In our studies we used B-cell malignancies as clonal model system to study regulation of isotypic and allelic exclusion of Ig light chain genes during several stages of differentiation by analysis of *IGK* and *IGL* gene configuration patterns. B-cell malignancies are derived from B cells, which were malignantly transformed at a certain point during B-cell differentiation. Although these B cells underwent malignant transformation, they might still be used as clonal "single-cell model system" studying Ig gene rearrangement patterns. However, results derived from B-cell malignancy models always require careful evaluation to see to what extent the data might be influenced by the malignant transformation. A major advantage of using B-cell malignancies over normal single B cells is that the Ig gene configuration patterns of both *IGK* and both *IGL* alleles can be reliably and completely analyzed.

In Table 1, the four B-cell malignancy models, which were used in this Chapter, are summarized together with the specific characteristics regarding expression of Ig molecules.

Table 1. Ig characteristics of the four B-cell malignancy models, which are used in Chapter 3.

B-cell malignancy model	Normal B-cell subpopulation	Specific characteristics
Precursor-B-ALL - pro-B-ALL - common ALL - pre-B-ALL	Precursor B-cells - Pro-B - Pre-B-I - Pre-B-II	- No surface Ig expression - Continuous RAG activity
MCL	Naive B cells	- Surface Ig expression - No somatic mutations
CBL	Mature B cells	- Surface Ig expression - Partly somatically mutated
MM	Plasma cells	- Ig secretion - SHM and CSR processes completed

This study was initiated in chronic B-cell leukemias (CBL), because they allowed analysis of regulation of Ig light chain gene rearrangements at the DNA, RNA and protein levels (Chapter 3.2). Subsequently, Ig light chain gene recombination patterns were analyzed in precursor-B-ALL in order to investigate how allelic exclusion is induced in precursor-B cells (Chapter 3.3). As almost half of the CBL have somatic mutations in the V gene regions, mantle cell lymphomas (MCL) were studied as a model system without somatic mutations (Chapter 3.4).

Somatic mutations might theoretically influence *IGK* and *IGL* gene configuration patterns via induction of receptor revision, which is defined as secondary rearrangements in the periphery. During the last years, it was suggested that the *RAG* genes can be re-expressed in peripheral B lymphocytes in the germinal center leading to secondary Ig gene rearrangements (receptor revision).[8-12] Somatic mutations might be involved in the induction of this process in two ways. First, somatic mutations might result in decreased affinity of the Ig molecule for the antigen and second, somatic mutations might result in complete loss of assembly of the Ig molecule. In both situations secondary rearrangements might rescue the B lymphocyte. Receptor revision is, however, a controversial phenomenon, because other studies suggest that the *RAG* genes are not re-expressed, but simply were not down-regulated at the time the B cell left the bone marrow.[13-16]

To further investigate whether there are indications for somatic-mutation induced receptor revision, four IgDλ MM were studied (Chapter 3.5). IgD MM form a small proportion of the total group of MM and they have three main characteristics. First, they have undergone an IgM to IgD switch via homologous recombination of two 442 bp direct repeats (σμ and Σμ) (Figure 1).[17,18] In hairy cell leukemia, a second type of IgM to IgD switch has been identified, which is mediated via recombination of Sμ and a switch-like sequence upstream of the Cδ gene segment (σδ) (Figure 1).[18,19] Both IgM to IgD switch mechanisms are also found in normal germinal center B cells.[18] A second characteristic of IgD MM is their high level of somatic mutations and finally, they display biased Igλ expression. Based on this high frequency of somatic mutations in combination with the biased Igλ expression,

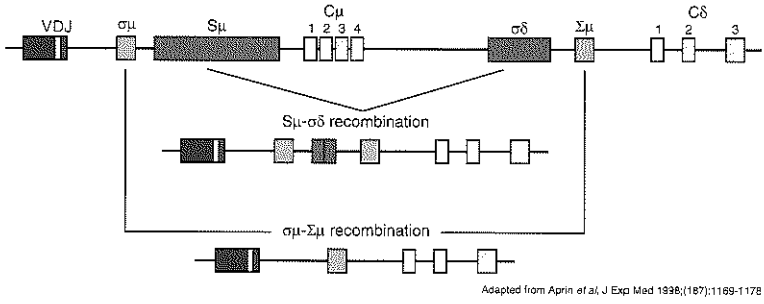

Figure 1. Schematic diagram of the two types of IgM to IgD switch mechanisms, which are found in normal germinal center B cells. Homologous recombination of the two 442 bp repeat sequences σμ and Σμ is found in IgM$^-$IgD$^+$ MM, whereas Sμ-σδ recombination has been described in hairy cell leukemia.[17-19]

we hypothesized that unfavorable somatic mutations might occur, which then might have resulted in induction of receptor revision. In the General Discussion (Chapter 5) the combined data of these four studies are discussed in detail.

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Chapter 3.2

ORDERED RECOMBINATION OF IMMUNOGLOBULIN LIGHT CHAIN GENES OCCURS AT THE *IGK* LOCUS, BUT SEEMS LESS STRICT AT THE *IGL* LOCUS

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ABSTRACT

Regulation of allelic and isotypic exclusion of human Ig light chain genes was studied in 113 chronic B-cell leukemias (CBL) as "single cell" model that allowed complete analysis of each light chain allele. Our data show that monospecific Ig light chain expression is in ~90% of cases determined by ordered recombination: *IGK* gene rearrangements, followed by *IGK* deletions and *IGL* rearrangements, resulting in the presence of only one functional Ig light chain rearrangement. In ~10% (10 cases) two functional Ig light chain rearrangements (*IGK/IGL* or *IGL/IGL*, but no *IGK/IGK*) were identified. This might be explained by the fact that regulation of the ordered recombination process is not fully strict, particularly when the *IGL* locus is involved. Unfavorable somatic mutations followed by receptor editing might have contributed to this finding. Eight of these ten cases indeed contained somatic mutations. In cases with two functional Ig light chain rearrangements both alleles were transcribed, but monospecific Ig expression was still maintained. This suggests that in these cases allelelic exclusion is not regulated at the mRNA level, but either at the level of translation or protein stability, or via preferential pairing of Ig light and Ig heavy chains. Nevertheless, ordered rearrangement processes are the main determinant for monospecific Ig light chain expression.

INTRODUCTION

In normal and malignant human B-cells, functional expression of immunoglobulin (Ig) kappa (*IGK*) genes occurs more frequently than functional expression of Ig lambda (*IGL*) genes, resulting in an Igκ/λ distribution of approximately 1.4.[1] The observed ratio between Igκ and Igλ expressing B-cells in mice is significantly higher (~10).[2] This difference in κ/λ ratio between mice and man might be related to differences in the organization of the *IGK* and *IGL* loci. While the human *IGK* locus is organized like the murine *IGK* locus, the organization of the *IGL* loci differs.[3,4] Mice only have three Vλ and four Jλ segments, each followed by a Cλ segment, which are arranged in two clusters.[5,6] In man, ~30 functional Vλ gene segments are located upstream of four functional J-Cλ clusters.[7,8] Thus, the theoretical combinatorial repertoire of Vλ-Jλ rearrangements in man is tenfold higher than in mice, which might explain the difference in κ/λ ratio.

Two models have been proposed for explaining the relative "over-representation" of *IGK* genes in both species: the ordered model and the stochastic model.[9-12] The ordered model proposes that *IGK* genes rearrange prior to *IGL*, while the stochastic model postulates that, in principal, the two types of Ig light chain genes rearrange totally independent, but that other factors render *IGL* gene rearrangements more difficult.[13,14] The latter would imply that *IGL* gene rearrangements can occur in the absence of *IGK* gene rearrangements and vice-versa.

Alt and Baltimore have first postulated the ordered model of Ig gene rearrangements in mice.[15,16] They suggested that the Ig gene rearrangement process starts within the Ig heavy chain locus (*IGH*) and that the rearrangement process is terminated as soon as a productive (in-frame) IgH chain is expressed on a pre-B-cell, resulting in allelic exclusion of the *IGH* locus.[16] They also postulated the ordered model for Ig light chain gene rearrangements, starting with *IGK* gene recombination, only followed by rearrangement in the *IGL* locus if no functional combination is formed.[15] The ordered model assumes a feed-back mechanism, which implies that the V(D)J recombinase system is downregulated upon surface Ig expression. This feedback mechanism explains the establishment of allelic exclusion (expression of one heavy chain and one Ig light chain), including isotypic exclusion (Igκ or Igλ expression).

In mice, monoallelic *IGK* demethylation ensures the ordered process of *IGK* rearrangements, thereby establishing allelic exclusion of the *IGK* alleles.[17] By analogy, Engel et al. described in their murine hit-and-run model that *IGK* and *IGL* are activated for recombination at consecutive developmental stages.[3] Also in man it is believed that *IGK* genes rearrange prior to *IGL*,[9,18] which is supported by recent studies in a human immature B-cell line showing that the *IGK* enhancer, but not the *IGL* enhancer is accessible for DNAseI.[19] However, it is known that an *IGL* gene rearrangement can occasionally be present, while the *IGK* genes are in germline configuration.[20]

Until recently, allelic exclusion was generally regarded to be a safe mechanism that guarantees the expression of a single type of antigen receptor on each lymphocyte. However, during the last years, several reports have indicated that dual receptor expression might occur in B-lymphocytes as well as in T-lymphocytes because of ongoing rearrangements after a functional receptor gene has been formed and expressed.[21-26] It was found that a single T-lymphocyte might express two different T-cell receptor (TCR)β chains and two different TCRα chains, indicating that both *TCRB* alleles as well as both *TCRA* alleles are functionally rearranged and expressed.[24,25] By analogy, Giachino et al. demonstrated that in 0.2-0.5% of human B-lymphocytes dual expression of Igκ and Igλ occurs.[21,22,27] This would imply that dual Ig light chain expression might be even higher owing to dual Igκ/Igκ and dual Igλ/Igλ expression, which would theoretically occur in 0.3-0.6% and ~0.1%, respectively. Although it will be difficult to prove the presence of dual Igκ/Igκ and dual Igλ/Igλ expression by immunophenotyping due to lack of Vκ and Vλ specific antibodies, the estimated dual Ig light chain expression might thus even be as high as 1%.

Here we have studied human chronic B-cell leukemias (CBL) for the presence of functional *IGK* and *IGL* gene rearrangements. Clonal leukemic proliferations allow a complete and extensive analysis of both *IGK* and both *IGL* alleles, so that CBL can be regarded as the ideal "single cell model" of human B-cells, in which regulation of allelic and isotypic exclusion of light chain genes can be studied in detail.

MATERIALS AND METHODS

Cell samples, immunophenotyping, DNA and RNA isolation

Mononuclear cells (MNC) were obtained from peripheral blood (PB) or bone marrow (BM) samples by Ficoll-Paque (density 1.077 g/l, Pharmacia, Uppsala, Sweden) centrifugation from a series of 113 patients with chronic B cell leukemia, including 107 B-cell chronic leukemia (B-CLL), 5 B-cell prolymphocytic leukemia (B-PLL) and a single hairy cell leukemia (HCL). MNC were used for detailed immunophenotyping.[28,29] In a few cases, lymph node suspensions were used. In all samples the tumor load was at least 75%.

DNA and RNA were isolated from MNC or lymph node cells, as described.[30] cDNA was prepared from RNA using either AMV reverse transcriptase (Promega, Madison, WI, USA) or Superscript RT enzyme (Life Technologies, Paisley, UK) according to the manufacturers instructions.

Southern blot analysis

15 μg of DNA was digested with the appropriate restriction enzymes (Life Technologies, Rockville, MD, USA), separated in 0.7% agarose gels and transferred by vacuum blotting to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany).[30] The filters were hybridized with ^{32}P labeled probes. Probes specific for either Jκ (IGKJ5), Cκ (IGKC) and Kde (IGKDE) (DAKO Corporation, Carpinteria, CA, USA) were used in combination with *Bgl*II and *Bam*HI/*Hin*dIII restriction enzyme digests to determine the *IGK* gene configuration.[31] For the *IGL* alleles, isotype-specific probes (IGLC1D, IGLJ2, IGLC2D, IGLC4D, IGLJ5, IGLJ6, IGLJ7) were used in combination with *Hin*dIII, *Bgl*II, *Bam*HI/*Hin*dIII, and *Eco*RI/*Hin*dIII digests.[32]

(RT)-PCR heteroduplex analysis

The (RT)-PCR mixture of 100 μl contained 0.2 mM dNTPs (Pharmacia), 13 pmol of each primer, 1U Ampli Taq GOLD polymerase in Buffer II (PE Biosystems, Foster City, CA, USA), 1.5 mM $MgCl_2$, and 100 ng genomic DNA or 5μl cDNA (derived from 0.25 μg total RNA). The family specific Vκ and Vλ primers, the Jκ and Jλ primers, and the Cκ and Cλ exon primers are listed in Table 1. PCR conditions were 10 min at 94°C followed by 40 cycles of 1 min. 94°C, 1 min. 72°C and a final extension of 7 min. at 72°C. PCR products were further analyzed by heteroduplex analysis to determine whether the PCR products were derived from clonal or polyclonal rearrangements.[33]

Sequencing analysis

Clonal (RT)-PCR products were directly sequenced on an ABI 377 fluorescent cycle sequencer (PE Biosystems) with Dye Terminator mix or Big Dyes (PE Biosystems) according to the manufacturers instructions. Vκ, Jκ, Vλ and Jλ segments were identified using DNAPLOT software (W. Müller, H-H. Althaus, University of Cologne, Germany) via

VBASE and IMGT databases (http://imgt.cnusc.fr:8104)[34] Subsequently, the frame of the rearrangement and the mutation status were determined. A case was classified as somatically mutated if the involved V gene segment had less than 98% homology with the most related V gene segment.

Table 1. Primers for PCR analysis of *IGK* and *IGL* gene rearrangements.

Primers	Sequence (5'-3')
IGK locus	
leaderVκI+II	GGTCCCCGCTCAGCTCCT
leaderVκIV	TCTCTGTTGCTCTGGATCTCT
VκI	GTAGGAGACAGAGTCACCATCACT
VκII	TGGAGAGCCGGCCTCCATCTC
VκIII	GGGAAAGAGCCACCCTCTCCTG
VκIV	GGCGAGAGGGCCACCATCAAC
VκV	CCAGGAGACAAAGTCAACATCTCC
VκVI	CTGTGACTCCAAAGGAGAAAGTC
VκVII	AGGACAGAGGGCCACCATCACC
Jκ1,2,4	CCCTGGTTCCACCTCTAGTTTGCA
Jκ3	GGGACCAAAGTGGATATCAAACGT
Jκ5	GGGACACGACTGGAGATTAAACGT
Cκ	ACTTTGGCCTCTCTGGGATA
IGL locus	
leaderVλI	GCCCAGTCTGTGCTGAC
leaderVλII	CTGGGCTCTGCTCCTCCT
leaderVλIII	GTGACCTCCTATGTGCTGACT
VλI	GGCAGAGGGTCACCATCTC
VλII	ATCTCCTGCACTGGAACCA
VλI+II	ATTCTCTGGCTCCAAGTCTGGCA
VλIII	ATTCTCTGGCTCCAACTCTGGGAA
Jλ1	AGGCTGGGAAAGGTTGAG
Jλ2,3	AGAGGGGAGAAGAGACTCAC
Cλ	TTGACGGGGCTGCTATCT

RESULTS

Southern blot analysis of the CBL samples

In order to study the complete and exact configuration of both *IGK* and both *IGL* alleles in a cohort of 113 CBL, detailed Southern blot analysis was performed. In a previous study, the configuration of the *IGK* alleles (i.e. germline, Vκ-Jκ rearrangement, or deletion of Jκ and/or Cκ) of most CBL was determined.[31] The data of the Igκ$^+$ and Igλ$^+$ CBL are summarized in Table 2. In our series of CBL half of the Igκ$^+$ CBL (25/53) had one rearranged *IGK* allele, while the others had biallelic *IGK* gene rearrangements (11/53) or one rearranged and one deleted *IGK* allele (17/53). 94% (50/53) of them showed both *IGL* genes in germline configuration (Table 2). On the other hand, all 60 Igλ$^+$ CBL had at least one deleted *IGK*

Table 2. ***IGK*** **and** ***IGL*** **gene configurations in 113 CBL (53 Igκ+ and 60 Igλ+ CBL) as determined by Southern blot analysis.**

	Igκ+ CBL			Igλ+ CBL		
	IGL gene configuration					
IGK gene configuration	G/G	G/R	R/R	G/G	G/R	R/R
G/G	-	-	-	-	-	-
R/G	47% (25/53)	-	-	-	-	-
R/R	19% (10/53)	2% (1/53)	-	-	-	-
D/R	28% (15/53)	2% (1/53)	2% (1/53)	-	10% (6/60)	1.5% (1/60)
D/D	-	-	-	-	57% (34/60)	21.5% (13/60)
G/D	-	-	-	-	7% (4/60)	3% (2/60)

G= germline configuration, R= rearranged allele, D= deletion of Jκ and/or Cκ gene segments.

allele and 88.5% (53/60) of them even had either both *IGK* alleles deleted or one deleted *IGK* allele with the other in germline configuration. This supports the general idea that *IGL* gene rearrangements are preceded by *IGK* gene deletions. Table 2 demonstrates the hierarchical order in Ig light chain gene rearrangements: the light chain gene rearrangement process starts at the *IGK* locus, followed by *IGK* deletion and subsequent *IGL* gene rearrangement.

As the presence of a rearranged band in Southern blot analysis does not necessarily imply the presence of a V-J joining, no distinction was possible between physiological V-J rearrangements and other events like translocations. Moreover, Southern blot analysis cannot discriminate between functional and non-functional V-J rearrangements. Therefore, CBL with two or more rearranged Ig light chain alleles were studied in more detail by PCR heteroduplex analysis and sequencing.

Igκ+ CBL with *IGL* gene rearrangements and Igλ+ CBL with *IGK* gene rearrangements

6% (3/53) of Igκ+ CBL contained *IGL* gene rearrangements and 11.5% (7/60) of the Igλ+ CBL had *IGK* gene rearrangements based on Southern blot analysis. The configurations of the *IGK* and *IGL* genes of these ten cases are summarized in Table 3. The *IGK* and *IGL* alleles of the three Igκ+ B-CLL and seven Igλ+ B-CLL were further analyzed by PCR heteroduplex analysis and sequencing to determine the frame of the rearrangements, the involved gene segments, and the mutation status of the V genes.

In two of the three Igκ+ CBL (patients 1 and 3) an in-frame *IGL* rearrangement was detected at the DNA level. The junctional regions of all in-frame rearrangements were analyzed for the presence of stop codons. In patient 3 a functional transcript of the *IGL* rearrangement was detected by RT-PCR. Lack of material precluded further analysis in patient 1. In

Table 3. Combined Southern blot and sequence data of the Igκ+ CBL with *IGL* gene rearrangements and Igλ+ CBL with *IGK* gene rearrangements.

			Igκ+ CBL								
			IGL alleles							Somatic mutations[e]	
Patient	*IGK* gene configuration[a]	*IGL* gene configuration	Vλ	Junctional region[b]			Jλ	Frame[c]	RT-PCR[d]	*IGK*	*IGL*
1	R/VJ-Kde	R/G	Vλ3-21	0	GGGG	-3	Jλ1	+	ND	+	-
2	R/VJ-Kde	R/R	1st Vλ2-33	-3	AAA	-2	Jλ2	-[f]	ND	+	-
			2nd not found								
3	R/R[g]	R/G	Vλ1-40	0	-	0	Jλ3	+	+	NI	+
			Igλ+ CBL								
			IGK alleles							Somatic mutations	
	IGK gene configuration	*IGL* gene configuration	Vκ	Junctional region			Jκ	Frame	RT-PCR	*IGK*	*IGL*
4	R/VJ-Kde	R/R[h]	1st not found							NI	+
			2nd Vκ1-39	-6	GA	-9	Jκ4	-	-		
5	R/V-Kde	R/G	Vκ2-30	-10	TGACC	-4	Jκ2	-	ND	-	NI
6	R/V-Kde	R/G	Vκ2-28	-4	TCACT	-6	Jκ4	-	ND	-	-
7	R/V-Kde	R/G	Vκ1-33	-1	ACC	-6	Jκ4	-	ND	-	-
8	R/V-Kde	R/G	Vκ4-1	-3	CCTG	-2	Jκ2	+	+	-	-
9	R/VJ-Kde	R/G	1st Vκ1-33	-3	ATC	-2	Jκ3	+	+	-	-
			2nd Vκ4-1	-3	CT	0	Jκ4	-	-		
10	R/VJ-Kde	R/G	1st Vκ1-12	-3	-	0	Jκ3	+	+	+	+
			2nd Vκ1-33	-3	AT	0	Jκ3	-	-		

a. R, *IGK* gene rearrangement; V-Kde, Vκ-segment rearranged to kappa deleting element; VJ-Kde, Vκ-Jκ and IntronRSS-Kde rearrangement on the same allele.

b. The junctional region is described as the number of bases lost at the 3' end of the V segment followed by randomly inserted nucleotides and the number of bases lost from the 5' end of the J segment.

c. Frame of the rearrangement: +, in-frame, without stop codons in the junctional region; -, out-of-frame.

d. ND: not determined; +, present; -, absent.

e. +, V segments with >2% somatic mutations; -, <2% somatic mutations; NI, not identified, because the rearrangement could not be amplified.

f. A deletion of 36 nucleotides was found, starting form the last two codons of the Jλ2 gene segment up to 30 nucleotides in the J-Cλ intron, thereby deleting the Jλ splice site.

g. For biallelic *IGK* gene rearrangements, see Table 4.

h. For biallelic *IGL* gene rearrangements, see Table 5.

patient 2 only one out-of-frame *IGL* rearrangement was found. The second rearrangement could not be identified, which might be caused by the inability of the used primers to recognize the involved gene segments, or by a chromosomal aberration involving chromosome 22.

The seven Igλ+ B-CLL (patients 4 to 10) had one rearranged and one deleted *IGK* allele (Table 3). In patients 4, 9 and 10 the Kde was rearranged to the intron RSS, thereby deleting the Cκ exon, but retaining the Vκ-Jκ rearrangement on the same allele (VJ-Kde).[31] This rearrangement does not give rise to a functional transcript, because of deletion of the Cκ exon. Therefore, in these four patients two Vκ-Jκ rearrangements can be detected at the DNA level, but only the *IGK* rearrangement on the allele without the Cκ deletion can be detected by RT-PCR. In patients 5, 6, 7, and 8 a V-Kde deletion was detected, which resulted from a rearrangement of a Vκ-segment to the Kde in which the Jκ and Cκ segments are deleted together with the preexisting Vκ-Jκ rearrangement.

In patient 4 only one Vκ-Jκ rearrangement was found. To determine whether this rearrangement was located at the non-deleted or deleted allele a primer was designed overlapping the junctional region. Long range PCR analysis using the junction-specific primer and the Kde primer (and the Cκ primer as control) showed that the identified Vκ-Jκ rearrangement was located on the allele with the Cκ deletion. This rearrangement was indeed not found by RT-PCR. The identified Vκ-Jκ rearrangements of patients 5, 6 and 7 were out-of-frame. Patients 8, 9 and 10 had a functional (in-frame) *IGK* rearrangement on the rearranged allele, as confirmed by detection of Vκ-Cκ transcripts and sequencing.

Somatic mutations in the V genes were determined. Patients 1, 2, 3, 4, and 10 were somatically mutated. Three of the five patients with two in-frame light chain rearrangements (patients 1, 3, and 10) carried somatic mutations either on one or both alleles against two of the five patients with a single in-frame light chain rearrangement (patients 2 and 4). In patient 3 the functional rearrangement was not found, but the *IGL* rearrangement was mutated although not expressed on the membrane.

In patients 3, 8, 9, and 10 presenting with double in-frame *IGK* and *IGL* gene rearrangements at both the DNA and RNA level, flow cytometric analysis of surface membrane Ig light chains was repeated. No dual Ig light chain expression was observed in the four patients (Figure 1). The Igλ expression of patient 10 was lower as compared to the Ig light chain expression in the other 3 patients (Figure 1D), but no Igκ expression was found and therefore also this CBL was concluded to be monospecific. No remaining cells were available for reanalysis of Ig light chain expression in patient 1, but the original data did not show dual Ig light chain expression in the Igκ+ CBL.

To further study whether isotypic exclusion is regulated at the translational level or by post-translational modification, the cytoplasmic Ig expression of the four CBL was analyzed on cytocentrifuge preparations.[29] However, the amount of cytoplasmic Ig in CBL was too low to be detectable; even the isotype which was expressed on the cell surface could not be detected in the cytoplasm by fluorescence microscopy (data not shown).

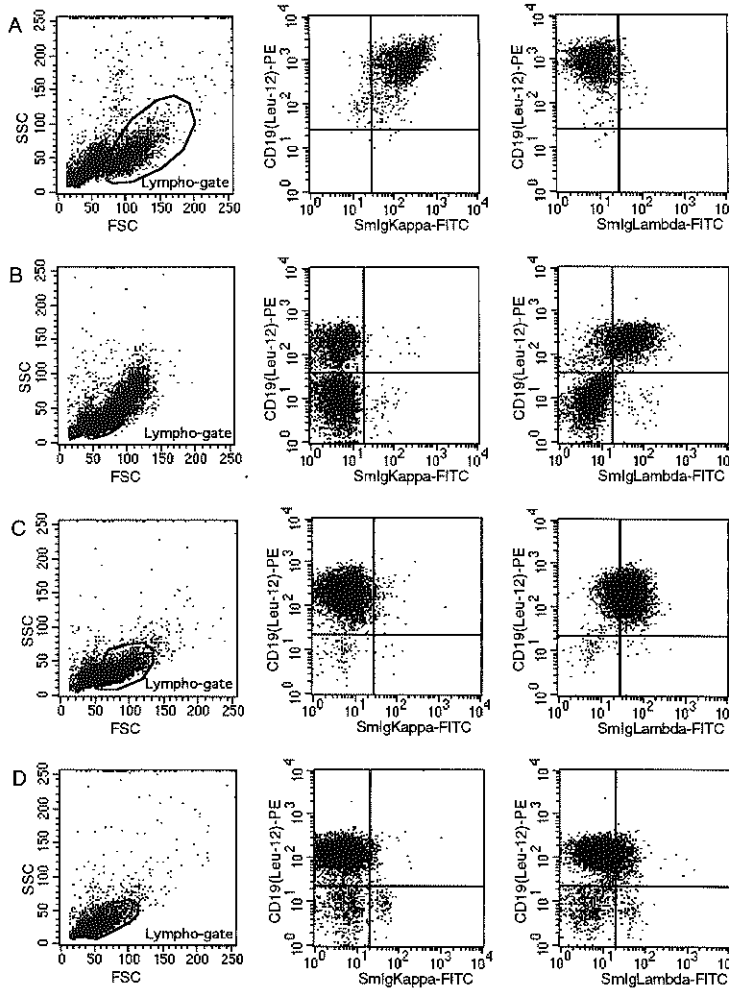

Figure 1. Flow cytometric immunophenotyping of lymphocytes of four B-CLL patients with in-frame *IGK* and *IGL* gene rearrangement. **A.** CD19+ B-lymphocytes of patient 3 express Igκ, but not Igλ. Patient 8 **(B)** and patient 9 **(C)** express Igλ on CD19+ B-lymphocytes, but there is no Igκ expression detectable. **D.** Patient 10 has low Igλ expression, but Igκ expression is absent.

Analysis of CBL with biallelic *IGK* or biallelic *IGL* rearrangements

In this series of CBL 19% (10/53) of Igκ+ CBL had biallelic *IGK* gene rearrangements with *IGL* genes in germline configuration (Table 2) and 25% (15/60) of the Igλ+ CBL had biallelic *IGL* rearrangements with *IGK* genes deleted or in germline configuration (Table 2). In addition, patients 3 (Igκ+ CBL) and patient 4 (Igλ+ CBL) had biallelic *IGK* and *IGL* gene rearrangements, respectively, in combination with a rearrangement of the other isotype (Table 3). Sufficient cell material for (RT)-PCR heteroduplex analysis and sequencing was

Table 4. CBL with potentially functional biallelic Vκ-Jκ rearrangements as determined by Southern blotting.

Patient	Vκ	Junctional region[a]			Jκ	Frame[b]	RT-PCR[c]	Somatic mutations[d]
3	Vκ1-37	-3	CT	-6	Jκ4	-	+	-
	NI							NI
11	Vκ1-5	-	-	-	Jκ4	+	+	-
	NA							NA[e]
12	Vκ2-30	-	-	-3	Jκ1	+	+	-
	Vκ1-39	-3	GG	-3	Jκ2	-	-	-
13	Vκ1-5	-3	-	-3	Jκ1	+	+	-
	NI							NI
14	Vκ1-5	-3	CGC	-1	Jκ2	+	+	-
	Vκ2-28	-	GA	-4	Jκ1	-	+	-
15	Vκ3-20	-1	G	-1	Jκ2	+	+	+
	Vκ3-11	-	T	-	Jκ4	-	-	-
16	Vκ1-39	0	A	-1	Jκ1	+	ND	+
	Vκ1-5	-13	-	-	Jκ4	-	ND	-
17	Vκ3-20	-	-	-	Jκ5	+	+	-
	Vκ1-37	-	TT	-	Jκ3	-	-	-
18	Vκ3-20	-1	GA	-4	Jκ1	+	+	-
	Vκ4-1	-	C	-5	Jκ4	-	+	-
19	Vκ1-27	-3	-	-	Jκ1	+	+	-
	NI							NI

a. The junctional region is described as the number of bases lost at the 3' end of the V segment followed by randomly inserted nucleotides and the number of bases lost from the 5' end of the J segment.

b. Frame of the junctional region: +, in-frame, without stop codons in the junctional region; -, out-of-frame .

c. ND, not determined; NI, not identified; +, present; -,absent.

d. +, V segments with >2% somatic mutations; -, <2% somatic mutations; NI, not identified, because the rearrangement could not be amplified.

e. NA, not applicable, because only one intact chromosome 2 was present, although two rearrangements seemed to be present in Southern blot analysis (see text).

available of ten out of eleven Igκ+ CBL with two *IGK* rearrangements and fourteen out of sixteen Igλ+ CBL with two *IGL* rearrangements. The results are shown in Tables 4 and 5, respectively.

In none of the ten Igκ+ CBL, two in-frame *IGK* rearrangements were detected. In patient 3 no in-frame *IGK* rearrangement could be identified, although the CBL clearly showed Igκ positivity. In patient 11 only one rearrangement could be detected by PCR, although two rearrangements seemed to be present in Southern blot analysis. By karyotyping of the clonal cells of patient 11 only one intact chromosome 2 was detected, which might explain why only one Vκ-Jκ rearrangement could be amplified. Apparently, part of the other chromosome 2 with the *IGK* locus was still present in the genome. Also in patients 13 and 19, only one rearrangement could be identified, possibly due to primer mismatching or a chromosome aberration.

The RT-PCR results showed that out-of-frame *IGK* rearrangements can be transcribed (3 out of 6 cases tested). In two of the six completely analyzed cases (patient 15 and 16) the V gene segment of the in-frame rearrangement was mutated.

Table 5. CBL with potentially functional biallelic Vλ-Jλ rearrangements as determined by Southern blotting.

Patient	Vλ	Junctional region[a]			Jλ	Frame[b]	Somatic RT-PCR[c]	mutations[d]
4	Vλ1-44	0	G	-1	Jλ3	+	+	+
	Vλ2-5[e]	-4	TGG	-1	Jλ3	-	+	+
20	Vλ1-40	-4	TCTA	0	Jλ3	+	+	+
	Vλ3-12	0	-	-3	Jλ3	+	+	+
21	Vλ3-21	0	-	0	Jλ3	+	ND	-
	Vλ1-47	0	A	0	Jλ3	-	ND	-
22	Vλ2-11	-15	26 nt of Vλ2-14	-7	Jλ3	+	+	+
	Vλ1-47	-4	C	0	Jλ3	+	+	+
23	Vλ1-40	-2	CGAG	-2	Jλ3	+	+	-
	Vλ3-21	-2	-	-2	Jλ3	-	+	-
24	Vλ3-21	0	CCCT	-1	Jλ3	+	+	-
	Vλ2-5[e]	-4	-	-1	Jλ2	(+)[e]	+	-
25	Vλ3-21	0	-	0	Jλ3	+	ND	-
	Vλ1-51	-11	CCC	0	Jλ3	-	ND	-
26	Vλ1-40	-5	AG	0	Jλ3	+	ND	+
	Vλ3-10	-6	GGGA	-4	Jλ1	-	ND	+
27	Vλ3-21	0	-		Jλ3	+	+	-
	NI			Jλ3[f]		NI	NI	
28	Vλ2-18	-3	GGT	0	Jλ1	+	+	+
	Vλ4-60	-2	-	-1	Jλ3	+	+	-
29	Vλ2-11	-3	CA	-3	Jλ3	+	+	+
	Vλ1-44	-2	-	0	Jλ3	+	+	-
30	Vλ2-14	-3	GG	-3	Jλ1	+	+	+
	NI			Jλ3[f]		NI	NI	
31	Vλ1-47	-6	-	-3	Jλ3	+	+	-
	NI			Jλ2[f]		NI	NI	
32	Vλ3-21	0	-	0	Jλ3	+	+	+
	Vλ1-47	-2	-	-1	Jλ3	+	+	+

a. The junctional region is described as the number of bases lost at the 3' end of the V segment followed by randomly inserted nucleotides and the number of bases lost from the 5' end of the J segment.
b. Frame of the junctional region: +, in-frame, without stop codons in the junctional region; -, out-of-frame.
c. ND, not determined; NI, not identified; +, present; -, absent.
d. +, V segments with >2% somatic mutations; -, <2% somatic mutations; NI, not identified, because the rearrangement could not be amplified.
e. Vλ2-5 is a pseudo gene, and therefore not functional.
f. Based on Southern blot data.

Five of the Igλ+ CBL with biallelic *IGL* gene rearrangements (patients 4, 21, 23, 25, and 26) had one in-frame and one out-of-frame *IGL* junctional region (Table 5). In six other Igλ+ CBL (patients 20, 22, 24, 28, 29, and 32) two in-frame *IGL* rearrangements were found. The presence of both transcripts was demonstrated by RT-PCR in all six patients. All used Vλ segments were functional except for the second allele of patient 24. This Vλ2-5 segment is a pseudogene,[35] implying that this patient has only one functional *IGL* gene rearrangement. In patients 27, 30 and 31 only one in-frame rearrangement could be identified by Vλ-Jλ PCR analysis. Somatic mutations were found in seven of the eleven completely analyzed cases. Five of them (patients 20, 22, 28, 29 and 32) had two in-frame *IGL* gene rearrangements. Patients 20, 22, and 32 carried mutations on both alleles. The V regions of both *IGL*

rearrangements of patient 26 were mutated and the out-of-frame rearrangement contained a stop codon in the junctional region, which might be the result of a somatic mutation, although this cannot be proven.

DISCUSSION

Ordered Ig light chain gene rearrangements

The configuration of the *IGK* and *IGL* genes was investigated in a series of 113 CBL (53 Igκ$^+$ and 60 Igλ$^+$) to study the mechanism of allelic and isotypic exclusion of human Ig light chain genes. CBL were chosen as "single cell" model, because large clonal cell populations allow complete analysis of both alleles of the *IGK* and *IGL* genes. Our data confirm the hypothesis of the hierarchical order in human Ig light chain gene rearrangements: the gene rearrangement process starts at the *IGK* locus, followed by *IGK* deletion and subsequent *IGL* gene rearrangement (Table 2).[12] Since not a single CBL had one or two rearranged *IGL* alleles with both *IGK* genes in germline configuration, the data completely fit with the ordered model. However, the ordered model was not stringent in all cases, because in some cases *IGL* gene rearrangements apparently had started before both *IGK* alleles were deleted. Curiously, three Igκ$^+$ CBL cases had *IGL* rearrangements: one case in the group with biallelic *IGK* rearrangements and two cases in the group with one rearranged and one deleted *IGK* allele. One of the latter two cases even had biallelic *IGL* rearrangements. To include these cases in the ordered model, it must be slightly adapted, although the principle of the ordered Ig light chain rearrangement processes is retained: rearrangement of one *IGK* allele (R/G) → further *IGK* gene rearrangements (R/R) → one *IGK* allele deleted (D/R) and occasionally one *IGL* allele rearranged (R/G) → both *IGK* alleles deleted (D/D) and one or two *IGL* rearrangements (R/G or R/R) (Figure 2). In other words, the original ordered model applies to *IGK* gene rearrangements, but *IGL* gene recombination processes seem to be less strictly controlled.

Regulation of isotypic exclusion in cases with two in-frame Ig light chain rearrangements of distinct isotype

In 90% of the CBL (103/113) either *IGK* or *IGL* rearrangements were present, indicating that ordered Ig light chain gene rearrangements ensured isotypic exclusion in these cases. Therefore, we conclude that monospecific Ig light chain expression is primarily determined by ordered rearrangement processes. Based on a computer simulation model of murine Ig light chain rearrangements, Mehr et al. also suggested that allelic exclusion in B-cells is maintained, if recombination occurs in an ordered rather than a random process.[36]

Nevertheless, our data show that the ordered rearrangement process of Ig light chain genes is not absolute, and that *IGK* and *IGL* rearrangements can coexist (10/113 cases). In such cases a different level of regulation of monospecific Ig light chain expression (i.e. allelic and isotypic exclusion) should be expected. In five of these ten CBL, both an in-frame *IGK* and an in-frame *IGL* rearrangement were detected at the DNA level (Table 6). Four of these

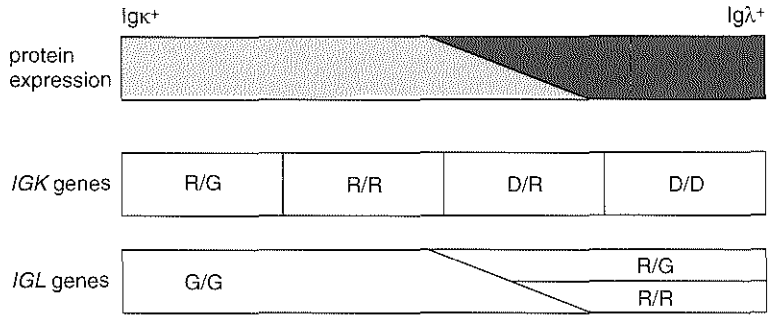

Figure 2. Schematic diagram of Ig light chain protein expression and Ig light chain gene rearrangements according to the ordered model. Ig light chain gene rearrangements start with rearrangement of one *IGK* allele (R/G) → further *IGK* gene rearrangements (R/R) → one *IGK* allele deleted (D/R) and occasionally one *IGL* allele rearranged (R/G) → both *IGK* alleles deleted (D/D) and one or two *IGL* rearrangements (R/G or R/R). Due to the fact that the ordered rearrangement process is not fully strict, *IGK* and *IGL* rearrangements might coexist (in 6% of Igκ+ cases and in 12% of Igλ+ cases of the presented CBL series). (G = Germline, R = Rearrangement, D = Deletion).

five cases could be analyzed by RT-PCR and all four showed bitypic functional transcripts (i.e. in-frame transcripts without stop codons due to somatic mutations). Therefore, there is no indication for regulation of isotypic exclusion at the level of transcription in these cases. Other possibilities include regulation at the level of translation, at the level of protein stability, or via preferential Ig light chain assembly with the Ig heavy chain. Although we did not find dual Igκ/Igλ chain expression in our CBL series (see also Figure 1), it has been described to occur in normal and malignant human B-lymphocytes.[21-23,26]

Allelic exclusion in cases with biallelic *IGK* or biallelic *IGL* rearrangements

The mechanism of allelic exclusion was further studied using 24 CBL with biallelic *IGK* or biallelic *IGL* rearrangements. The allelic exclusion of biallelic *IGK* rearrangements was solely regulated at the DNA level because only one functional rearrangement was present in all six evaluable cases (Table 6). Theoretically, 1/3 of the rearrangements on the second *IGK* allele in Igκ+ CBL could be in-frame. However, the murine experiments by Mostoslavsky et al. showed that undermethylation of an *IGK* allele is required for and precedes a rearrangement.[17] Moreover, they demonstrated that *IGK* gene demethylation takes place preferentially on only one allele in each cell, resulting in differential accessibility of the two *IGK* alleles for the recombinase system. If no productive rearrangement is obtained, a second rearrangement of the same allele involving an upstream Vκ and a downstream Jκ gene segment can take place.[17] Alternatively, the second allele is demethylated and rearranged. On top of the methylation-induced differential accessibility of *IGK* alleles, also the feedback mechanism of down regulation of the V(D)J recombinase system probably plays a role in the maintenance of allelic exclusion. This is in line with the ordered rearrangement model of Ig light chain genes and fits with the *IGK* gene data of our CBL series.

Table 6. Summary of data of CBL with two complete Ig light chain rearrangements, as identified by (RT-)PCR analysis and sequencing.

	Double *IGK*	*IGK/IGL*	Double *IGL*	Total
Patients (see Tables 3 to 5)	12, 14-17	1-3[a], 5-10	4[b], 20-26, 28, 29, 32	n=26
Two functional rearrangements	0/6	5/9	5/11	10/26
Somatic mutations in cases with:				
- one functional rearrangement	2/6	1/4	2/6	5/16
- two functional rearrangements	-	3/5	5/5	8/10
Both functional rearrangements transcribed	-	4/4	5/5	10/10
Double Ig light chain protein expression	-	0/5	Not evaluable	0/5

a. Patient 3 was classified in the *IGK/IGL* category, because this Igκ+ CBL contained a functional *IGL* rearrangement.

b. Patient 4 (Igλ+ CBL) was classified in the *IGL/IGL* category, because both *IGL* rearrangements were identified, but the *IGK* rearrangement was not found.

In eleven Igλ+ CBL, two Vλ-Jλ rearrangements could be identified. Six of them had two in-frame junctions (without stop codons) of which five were functional, and in one case a pseudo Vλ segment was used. So, in five cases two Igλ protein chains may be expressed, but it was not possible to make a distinction between the expression of one or two Igλ chains, because there are no Vλ family specific antibodies available. These five CBL were all somatically mutated on either one allele (2 cases) or both alleles.

Possible mechanism explaining regulation of monospecific Ig light chain expression

The start of Ig light chain gene recombination seems to be strictly regulated by ordered accessibility of the *IGK* locus ensuring complete allelic exclusion at the DNA level. Igκ+ CBL with two functionally rearranged *IGK* genes did not occur in our CBL series, suggesting that dual Igκ/Igκ expressing B-cells do not occur or are rare. However, when *IGL* rearrangements were involved, two in-frame (functional) rearrangements and transcripts (*IGK/IGL* or *IGL/IGL*) were occasionally detected (Table 6), implying the possibility of dual Ig light chain expression (Igκ/Igλ or Igλ/Igλ).

Several mechanisms might operate to regulate ordered recombination, including demethylation of one allele, remodeling of chromatin structure, or selective accessibility of recombination machinery through differential presence of transcription factors (such as Rel/NF-κB or E12/E47 bHLH factors).[37] Demethylation indeed appeared to be necessary for rearrangement of Ig and TCR genes. In the mouse, monoallelic demethylation has been shown to occur at the *IGK* locus.[17] In contrast to *IGK* genes, the ordered demethylation process might be less strict for the human *IGL* genes.

The occurrence of somatic mutations and subsequent receptor editing might have contributed to the presence of two functional Ig light chain rearrangements in our study. B-CLL can arise either from pre- or post germinal center B-cells.[38] In the latter case the V regions carry somatic mutations (~50% of cases).[38] Theoretically, the somatic mutation process can result in unfavorable mutations leading to receptor editing via secondary rearrangements and light chain replacement.[39,40] It is not yet clear whether the secondary rearrangements for

receptor editing follow an ordered pattern, comparable to primary rearrangements. In cases with two functional rearrangements one should expect somatic mutations in the in-frame rearrangement that is not expressed, whereas the expressed in-frame rearrangement can be mutated depending on whether the B-cell underwent a second germinal center reaction. Indeed eight out of ten CBL (80%) with two functional *IGK/IGL* or *IGL/IGL* rearrangements contained somatic mutations in contrast to five out of sixteen cases (~30%) of CBL with two complete Ig light chain gene rearrangements, one functional and one non-functional (Table 6). Nevertheless, this study indicates that isotypic and allelic exclusion is regulated by ordered rearrangement of Ig light chain genes in ~90% of cases. Even in cases with two functional Ig light chain transcripts, monospecific Ig expression can still be maintained, probably via mechanisms at the protein level, such as differential protein stability and preferential pairing of Ig chains.

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Chapter 3.3

IMMUNOGLOBULIN LIGHT CHAIN GENE REARRANGEMENTS DISPLAY HIERARCHY IN ABSENCE OF SELECTION FOR FUNCTIONALITY IN PRECURSOR-B-ALL

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ABSTRACT

The general order of the immunoglobulin (Ig) gene rearrangement process in human precursor-B cells is largely known. However, the exact Ig rearrangement patterns reflecting this process, especially those of the Ig light chain genes, are not well established. This requires detailed analysis of the gene configuration of all six *IGH, IGK,* and *IGL* alleles at the single cell level. As such extensive analyses are difficult to perform in a reliable way within a single normal precursor-B cell, we used 169 precursor-B-ALL (i.e. 6 pro-B-ALL, 112 common ALL, and 51 pre-B-ALL) as clonal "single cell" model system. The Ig gene recombinations show hierarchy starting with *IGH* gene rearrangements in all cases, followed by *IGK* rearrangements, *IGK* deletions and/or *IGL* rearrangements in 71% of cases. *IGK* deletions were found in the absence of *IGL* rearrangements in 34% of cases, which might be explained by the continuous recombinase activity in precursor-B-ALL, resulting in "end-stage" *IGK* rearrangements, together with an apparently limited accessibility of the *IGL* locus. Remarkably, in 5% of cases *IGL* rearrangements took place in the absence of *IGK* rearrangements. In addition we found that in-frame *IGH* rearrangements are not necessarily required for the induction of Ig light chain gene rearrangements and that *IGL* rearrangements can be induced irrespective of the frame of the accompanying *IGK* rearrangements. In conclusion, precursor-B-ALL constitute a model system for studying Ig gene rearrangement processes without selection for functionality of the rearrangements or the influence of somatic hypermutations. Nevertheless, the hierarchy of *IGH, IGK,* and *IGL* rearrrangements is apparent in precursor-B-ALL.

INTRODUCTION

During normal human B-cell differentiation immunoglobulin (Ig) genes undergo V(D)J recombination in order to express a unique antigen specific receptor. This process is tightly regulated with respect to the stage of B-cell differentiation and the accessibility of *IGH, IGK,* and *IGL* loci.[1-3] The Ig gene recombination process starts at the pre-B-I cell stage with a D_H-J_H rearrangement followed by V_H to D_H-J_H joining.[4,5] As soon as a complete in-frame V_H-D_H-J_H rearrangement is formed, Igμ is expressed in the cytoplasm (CyIgμ), which is characteristic for the pre-B-II stage, and the recombination activating genes (*RAG* genes) are down regulated. Subsequently, a pre-B-cell receptor (pre-BCR) consisting of the Igμ heavy chain and the surrogate light chain is expressed on the membrane. After a proliferation phase, the surrogate light chain is down regulated. During this stage (small pre-BCR$^-$ pre-B-II cells), the *RAG* genes are re-expressed and Ig light chain gene rearrangements take place. As soon as a functional rearrangement results in Ig light chain protein expression (Igκ or Igλ) and subsequent pairing with the Ig heavy chain, the cell further differentiates into a surface IgM (sIgM) positive immature B cell.

Although the general order of Ig gene rearrangements is known, the exact configuration patterns of the Ig light chain genes during precursor-B-cell differentiation are not known. This would require analyses of both *IGH* alleles and particularly both alleles of the *IGK* and *IGL* loci at the single cell level. Such extensive analyses are difficult to carry out reliably at the single cell level, but can be performed on human clonal lymphoid cell populations. These can be regarded as "single cell" model systems allowing complete and exact analysis of all Ig gene alleles by Southern blot analysis, PCR, and sequencing.

In a previous study, chronic B-cell leukemias (CBL) were used as single cell model for Ig expressing mature B cells, about half of which contain somatic mutations in the rearranged V regions.[6,7] We found an ordered model for Ig light chain gene rearrangements: the Ig light chain gene recombination process starts within the *IGK* locus, followed by *IGK* deletion(s) and rearrangements in the *IGL* genes. This order of the rearrangements guaranteed allelic exclusion of Ig light chain genes in >90% of CBL cases.

In this study we investigated whether or to what extent the different subgroups of precursor-B-acute lymphoblastic leukemias (precursor-B-ALL) can be used as a "single cell" model system for early stages of B-cell differentiation (pro-B cells and pre-B cells) in the bone marrow with respect to the patterns of Ig gene recombination. Precursor-B-ALL can be divided in three subgroups: pro-B-ALL, common ALL, and pre-B-ALL based on their expression profiles. The immunophenotype of pro-B-ALL corresponds with a differentiation stage between pro-B cells and pre-B-I cells, because pro-B-ALL already express CD19, which is not found on normal pro-B cells, but do not yet express CD10 like pre-B-I cells. The immunophenotype of common ALL reflects that of pre-B-I cells and pre-B-ALL are comparable to pre-B-II cells. One of the characteristics of precursor-B-ALL is the continuous presence of the recombinase enzyme system.[8,9] This implies that the immunogenotypes (Ig rearrangement patterns) of precursor-B-ALL might slightly differ from normal precursor-B cells.

MATERIAL AND METHODS

Patient samples

A series of 169 precursor-B-ALL consisting of 6 pro-B-ALL, 112 common ALL and 51 pre-B-ALL were studied. The configuration of *IGH, IGK*, and *IGL* genes was reported in previous studies for 74, 111, and 76 patients, respectively.[10-13]

Southern blot analysis of Ig gene rearrangements

Southern blot analysis of 169 precursor-B-ALL was performed to determine the configuration of *IGH, IGK,* and *IGL* loci. 15 μg of DNA was digested with *Bgl*II or a combination of *Bam*HI and *Hin*dIII (Life Technologies, Rockville, MD, USA), separated in 0.7% agarose gels and transferred by vacuum blotting to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany). The filters were hybridized with ^{32}P labeled probes. The

probes were specific for the regions of J_H (IGHJ6), Jκ (IGKJ5), Cκ (IGKC) and Kde (IGKDE) (DAKO Corporation, Carpinteria, CA, USA).[11,12] To study *IGL* alleles, IGLC1D and IGLJ2 probes were used.[13]

PCR heteroduplex analysis

The 100 µl PCR mixture contained 0.2 mM dNTPs (Pharmacia, Uppsala, Sweden), 13 pmol of each primer, 1U Ampli*Taq* GOLD polymerase in Buffer II (Applied Biosystems, Foster City, CA, USA), 1.5 mM $MgCl_2$, and 100 ng genomic DNA. Family specific V_H, Vκ and Vλ primers were used in combination with consensus J_H, Jκ and Jλ primers, respectively.[6] PCR conditions were 10 min at 94°C followed by 40 cycles of 1 min. at 94°C, 1 min. at 60°C, 2 min. at 72°C and a final extension of 7 min. at 72°C. PCR products were further analyzed by heteroduplex analysis to determine whether the PCR products were derived from clonal or polyclonal rearrangements.[14] A selected number of cases were studied by RT-PCR analysis using the same family specific V primers in combination with Cµ, Cκ or Cλ exon primers.

Sequencing

A total of 68 clonal PCR products from complete *IGH* rearrangements derived from 2 pro-B-ALL, 28 common ALL, and 17 pre-B-ALL were directly sequenced on an ABI 377 fluorescent cycle sequencer (Applied Biosystems) with Dye Terminator mix or Big Dyes (Applied Biosystems) using family-specific V_H primers and a J_H consensus primer. All sequences were derived from two independent PCR products.

From the randomly selected series of 13 common ALL and 10 pre-B-ALL not only the clonal V_H-J_H rearrangements were sequenced, but also the clonal light chain rearrangements (Vκ-Jκ and/or Vλ-Jλ). Segments were identified using DNAPLOT software (W. Müller, H-H. Althaus, University of Cologne, Germany) via VBASE and IMGT databases (http://imgt.cnusc.fr:8104).[15] Subsequently, the frame of the rearrangement and the mutation status were determined.

RESULTS

***IGH* gene configurations**

In 86% (146/169) of precursor-B-ALL both *IGH* alleles were rearranged. In the pre-B-ALL group even 100% had biallelic *IGH* rearrangements (Table 1). One of the six pro-B-ALL had one rearrangement in combination with an *IGH* deletion. 80% of common ALL had biallelic *IGH* gene rearrangements. *IGH* gene deletions were found in 20 common ALL on one allele (16 cases) or both alleles (4 cases).

In 39% of cases with two rearrangements oligoclonality was observed in concordance with what was previously described.[16,17] The frequency of oligoclonality was significantly higher in pre-B-ALL (51%) as compared to common ALL (31%) (P=0.006); the pro-B-ALL series was too small to draw any statistical conclusion.

Table 1. *IGH* gene configuration of the three categories of precursor-B-ALL.

	R/R (% oligoclonality)	R/G	R/D	D/D
Pro-B-ALL (n=6)	5 (60%)	-	1	-
Common ALL (n=112)	90 (31%)	1	16	4
Pre-B-ALL (n=51)	51 (51%)	-	-	-
Precursor-B-ALL (n=169)	146 (39%)	1	17	4

Analysis of somatic mutations in *IGH* gene rearrangements

A total of 68 complete *IGH* rearrangements derived from 2 pro-B-ALL, 28 common ALL, and 17 pre-B-ALL patients, were sequenced. Thirty percent of these rearrangements appeared to be in-frame; they were found in both pre-B-ALL and common ALL. No somatic mutations were found in any of these 68 rearrangements. The generated sequences will be available upon request.

IGK and *IGL* gene configurations

At first glance no apparent recombination pattern emerged from the combined *IGK* and *IGL* Southern blot data in precursor-B-ALL (Table 2). To evaluate whether recombinations in the Ig light chain loci occur in an ordered way, the *IGK/IGL* recombination patterns were categorized. Six categories were defined according to the ordered recombination model found in CBL (Table 3):[6] category A: both *IGK* and both *IGL* alleles in germline configuration; category B: at least one rearrangement at the *IGK* locus with the second allele in germline configuration, rearranged or deleted; category C: cases without functional *IGK* rearrangements, but with one or two *IGK* deletions; category D: one or two *IGK* alleles rearranged and at least one *IGL* allele rearranged; category E: at least one *IGK* allele deleted and at least one *IGL* allele rearranged; category F: cases without *IGK* rearrangements or deletions, but with *IGL* rearrangements.

Twenty-nine percent of precursor-B-ALL did not contain any Ig light chain rearrangements (category A) (Table 3). *IGK* rearrangements without *IGL* rearrangements (category B) were found in 13% of the precursor-B-ALL. Category C was approximately 34% in the total group of precursor-B-ALL as well as in the common and pre-B-ALL subgroups. Only 4% showed rearrangements in both Ig light chain loci (category D) without *IGK* deletions. The frequency of precursor-B-ALL cases showing *IGL* rearrangements with at least one *IGK* deletion (category E) was higher, being 14%. The highest frequency was observed in common ALL (18%). The last category (F), showing *IGL* rearrangements without any *IGK* (deletional) rearrangement appeared to be small with frequencies of 6%, 2%, and 5% in common, pre-B and precursor-B-ALL, respectively. One out of six pro-B-ALL cases had a single *IGL* rearrangement.

Table 2. ***IGK*** **and** ***IGL*** **gene configurations as determined by Southern blot analysis in the three separate subgroups pro, common, and pre-B-ALL and the total number of precursor-B-ALL.**

Pro-B-ALL n=6 (%)	*IGL* gene configuration			Common ALL n=112 (%)	*IGL* gene configuration		
IGK gene configuration	G/G	R/G	R/R	*IGK* gene configuration	G/G	R/G	R/R
G/G	4 (67)	1 (16.5)	-	G/G	27 (24.1)	5 (4.5)	2 (1.8)
R/G	-	-	-	R/G	9 (8.0)	5 (4.5)	-
R/R	-	-	-	R/R	2 (1.8)	-	-
D/R	-	-	-	D/R	2 (1.8)	5 (4.5)	-
D/D	-	-	1 (16.5)	D/D	17 (15.2)	6 (5.3)	7 (6.2)
G/D	-	-	-	G/D	23 (20.5)	1 (0.9)	1 (0.9)
Pre-B-ALL n=51 (%)	***IGL*** **gene configuration**			**Precursor-B-ALL n=169 (%)**	***IGL*** **gene configuration**		
IGK gene configuration	G/G	R/G	R/R	*IGK* gene configuration	G/G	R/G	R/R
G/G	18 (35.3)	-	1 (2.0)	G/G	49 (29.0)	6 (3.5)	3 (1.8)
R/G	5 (9.8)	2 (3.9)	-	R/G	14 (8.3)	7 (4.1)	-
R/R	2 (3.9)	-	-	R/R	4 (2.4)	-	-
D/R	3 (5.9)	-	1 (2.0)	D/R	5 (3.0)	5 (3.0)	1 (0.6)
D/D	11 (21.5)	-	-	D/D	28 (16.6)	6 (3.5)	8 (4.7)
G/D	6 (11.8)	2 (3.9)	-	G/D	29 (17.1)	3 (1.8)	1 (0.6)

Table 3. Frequencies of the six Ig light chain gene configuration categories given in percentages.

	Category					
	A	B	C	D	E	F
IGK gene	G/G	R/G;R/R;D/R	D/D;D/G	R/G;R/R	D/R;D/D;D/G	G/G
IGL gene	G/G	G/G	G/G	R/G;R/R	R/G;R/R	R/G;R/R
Pro-B-ALL (n=6)	67.0	0	0	0	16.5	16.5
Common ALL (n=112)	24.1	11.6	35.7	4.5	17.9	6.2
Pre-B-ALL (n=51)	35.3	19.6	35.3	3.9	3.9	2
Precursor-B-ALL (n=169)	29.0	13.6	34.3	4.2	13.6	5.3
- Oligoclonal (n=57)[a]	35.1	15.8	28.1	5.2	12.3	3.5
- Monoclonal (n=112)	25.9	12.5	37.5	3.6	14.2	6.3

a. No statistical differences between the frequencies in oligoclonal and monoclonal precursor-B-ALL.

The precursor-B-ALL series was further divided based on oligoclonality at the *IGH* locus, to see whether Ig light chain recombination patterns are influenced by the presence of oligoclonality at the *IGH* locus (lower part of Table 3). Oligoclonality might hamper the assignment of rearrangements as belonging to the same clone. No statistically significant differences were found in the Ig light chain recombination categories between precursor-B-ALL with and without *IGH* oligoclonality. Figure 1 summarizes the relative frequencies of the *IGK/IGL* gene rearrangement categories of precursor-B-ALL as compared to CBL.

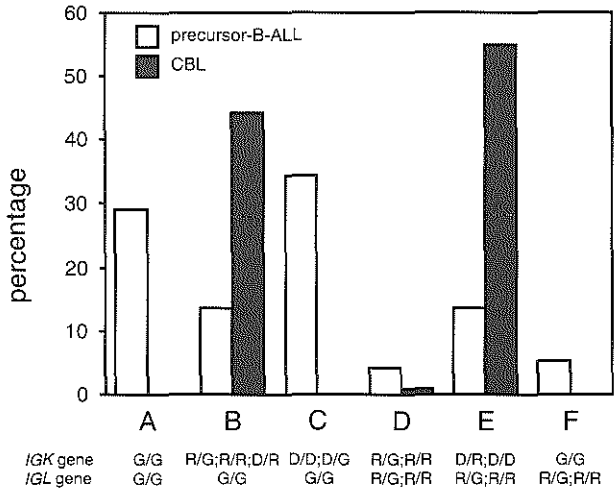

Figure 1. Relative frequencies of the *IGK/IGL* gene rearrangement categories of precursor-B-ALL as compared to CBL. See Table 3 for definition of the six categories.

Relation between in-frame *IGH* gene rearrangements and the occurrence of Ig light chain gene rearrangements

Thirteen common ALL and ten pre-B-ALL with or without Ig light chain gene rearrangements were randomly selected for sequencing of the *IGH, IGK,* and *IGL* gene rearrangements. Figure 2 shows the Southern blot analysis of the *IGH* loci of 12 out of the 13 analyzed common ALL patients. Oligoclonal bands were only present in patient 5289. In the 13th patient no oligoclonality was found either (data not shown). Based on the lack of oligoclonality in these twelve common ALL cases, we could reliably assign heavy and light chain rearrangements as belonging to the same clone. The frame of the Ig heavy and light chains gene rearrangements of the common ALL and pre-B-ALL samples are summarized in Table 4. All rearranged alleles, except for one *IGK* gene rearrangement, could be identified and no somatic mutations were found in any of the *IGH, IGK,* and *IGL* gene rearrangements. In six of the thirteen studied common ALL at least one in-frame *IGH* rearrangement was found, whereas in all 10 pre-B-ALL an in-frame rearrangement was present, which was expected based on the fact that pre-B-ALL express cytoplasmic Igμ molecules. No relation-

Figure 2. Southern blot analysis of *IGH* rearrangements of twelve of the thirteen common ALL patients that were randomly selected for sequencing of *IGH, IGK,* and *IGL* gene rearrangements. DNA was digested with *Bgl*II and hybridization was performed with the IGHJ6 probe. All patients, except for patient 5289, only show monoclonal bands.

ship was found between the presence of an in-frame *IGH* rearrangement and the presence or absence of *IGK* or *IGL* gene rearrangements in common ALL. In two pre-B-ALL, but also in one common ALL, an in-frame Ig light chain rearrangement was found in addition to an in-frame *IGH* rearrangement. RT-PCR analysis followed by heteroduplex analysis showed that these Ig light chain gene rearrangements were not transcribed, reflecting a block in differen-

Table 4. Randomly selected series of common ALL and pre-B-ALL studied by PCR and sequencing to determine the frame of the *IGH* and *IGK* or *IGL* gene rearrangements[a].

			IGK and/or *IGL*			
			Germline	In-frame (or deleted)	Out-of-frame	NI[b]
Common ALL (n=13)						
IGH	In-frame	6	1	1	3[c]	1[b]
	Out-of-frame	7	1[d]	2	4	-
Pre-B-ALL (n=10)						
IGH	In-frame	10	5	2	3	-
	Out-of-frame	-	-	-	-	-

a. All *IGH, IGK,* and *IGL* gene rearrangements found by Southern blot analysis in the 23 precursor-B-ALL could be identified, except for one *IGK* gene rearrangement.
b. NI, not identified. The *IGK* gene rearrangement of this common ALL could not be identified.
c. One of these three patients is Patient 3 in figure 2 with oligoclonal *IGH* gene rearrangements.
d. Southern blot data of this common ALL not shown in Figure 2.

tiation. This might explain that no surface Ig expression was found in pre-B-ALL, despite the fact that in-frame *IGH* as well as in-frame *IGK* or *IGL* gene rearrangements were present.

Relation between frame of Vκ-Jκ rearrangements and occurrence of *IGL* rearrangements

The Southern blot data of *IGK* and *IGL* genes show that in 95% of cases rearrangements in the *IGK* locus took place before *IGL* rearrangements. To determine whether the frame of Vκ-Jκ rearrangements influences this process, the Vκ-Jκ rearrangements of cases with concurrent *IGK* and *IGL* gene rearrangements (categories D and E) were analyzed. Thirteen of the thirty cases (43%) of categories D and E showed at least one *IGK* rearrangement, whereas DNA was available in eight of them. In six samples Vκ-Jκ rearrangements were out-of-frame, but two were in-frame. This shows that *IGL* rearrangements can occur in the presence of both out-of-frame or in-frame Vκ-Jκ rearrangements.

DISCUSSION

In this study we investigated whether precursor-B-ALL can be used as a "single cell" model system for precursor-B-cell differentiation with respect to the immunoglobulin gene status. The immunogenotype of all six *IGH, IGK* and *IGL* alleles was carefully evaluated in the three precursor-B-ALL subgroups: pro-B-ALL, common ALL, and pre-B-ALL.

During normal human B-cell differentiation the recombination process is assumed to start at the pre-B-I cell stage with rearrangements in the *IGH* locus followed by Ig light chain recombinations in the pre-B-II cell stage. In all three subgroups of precursor-B-ALL *IGH* gene rearrangements were observed. Moreover, *IGK* and *IGL* gene rearrangements were not restricted to pre-B-ALL, but were found in all three subgroups. From these data we conclude that the immunogenotypes of pro-B-ALL, common ALL and pre-B-ALL might not completely reflect the immunogenotypes of their normal counterparts. One possible explanation is that the recombinase enzyme system is continuously active *before* diagnosis in precursor-B-ALL.[8,9] Such prolonged recombinase activity might also affect clonal evolution *after* diagnosis, resulting in differences in rearrangement patterns between diagnosis and relapse.[18-20] However, a more likely explanation for the differences observed between genotypes in normal versus malignant precursor-B-cells, is the lack of proper selection for functionality of the rearrangements due to the malignant status of the cell. We therefore believe that precursor-B-ALL rather constitute a model system for studying Ig recombination processes without selection for functionality of the rearrangements based on Ig protein expression and without the influence of somatic hypermutations. The latter was proven by the absence of somatic mutations in 68 sequenced V_H-J_H rearrangements.

IGH gene rearrangements were identified in all precursor-B-ALL. In 10% of cases monoallelic *IGH* deletions and in 2% of cases even biallelic *IGH* deletions were identified with Southern blot analysis. The occurrence of these *IGH* deletions has been reported

before,[17,21,22] but the underlying mechanism is unknown and could not be explained by loss of chromosome 14. About 30% of all precursor-B-ALL, had both *IGK* and *IGL* alleles in germline configuration. This confirms the notion that *IGH* rearrangements precede Ig light chain recombination. In 39% of the precursor-B-ALL series oligoclonal *IGH* rearrangements were found, which most probably have resulted from ongoing rearrangements during the expansion phase after the final malignant hit. However, as the *IGK* and *IGL* gene configuration patterns did not differ significantly between oligoclonal and monoclonal precursor-B-ALL, we do not believe that ongoing rearrangements very much influenced the observed *IGK* and *IGL* configuration patterns.

To determine whether a relation exists between in-frame *IGH* gene rearrangements and the occurrence of Ig light chain gene rearrangements, a randomly selected series of 23 precursor-B-ALL (13 common ALL and 10 pre-B-ALL) was completely analyzed. As pre-B-ALL express CyIgμ, they have an in-frame *IGH* rearrangement. Therefore, it was not surprising to find Ig light chain rearrangements in pre-B-ALL. However, also in common ALL, which do not have CyIgμ expression, Ig light chain gene rearrangement were present, independent of the frame of the *IGH* rearrangements. So, Ig light chain gene rearrangements do not require the presence of an in-frame *IGH* gene rearrangement.

In a previous study on CBL, we found a hierarchical order in Ig light chain rearrangements: *IGK* rearrangements before *IGK* deletions and *IGL* rearrangements. This ordered model was used for defining the categories of *IGK* and *IGL* gene configuration patterns (see Table 3 and Figure 1). A strictly ordered model would be described by categories A, B, and E. Category D (*IGK* and *IGL* rearranged without *IGK* deletions) was added for CBL, because the ordered model appeared to be not completely strict, as *IGL* rearrangements can occur without prior *IGK* deletions. This was explained by a less strict regulation of *IGL* locus accessibility. In CBL categories B and E were 44% and 55%, respectively, and category D was 1%, whereas category A logically did not exist (Figure 1).

Here, category C was introduced as an exceptional category containing cases with only *IGK* deletions, but without any *IGL* rearrangement. As CBL have been selected during their differentiation process for having a functional Ig light chain gene rearrangement, category C logically does not exist in CBL. In precursor-B-ALL, however, this category was rather large comprising 34%. In these cases *IGK* was accessible for recombination, which proceeded until an end-stage had been reached. *IGK* deletions mediated via Vκ-Kde or intronRSS-Kde rearrangements are regarded as end-stage rearrangements, because they preclude further *IGK* rearrangements.[23-25] One could argue whether category C fits in the ordered model, because cases belonging to this category do not have Vκ-Jκ rearrangements without an intron-Kde rearrangement on the same *IGK* allele or Vλ-Jλ rearrangements. However, the Ig light chain gene rearrangement process started at the *IGK* locus and one would expect that in these cases normally the *IGL* locus would rearrange. This "delay" in *IGL* gene rearrangements might be explained by an overall limited *IGL* locus accessibility, reflected by the low frequency of *IGL* rearrangements in the total group of precursor-B-ALL (23%). Due to the block in differentiation the germline transcription of the *IGL* locus might not have

been started. Category C might also exist in normal bone marrow differentiation, although the time frame that a differentiating B cell remains in this stage might be rather short. Cases belonging to category F, having *IGL* gene rearrangements in the absence of *IGK* gene rearrangements or deletions, definitely do not fit in the ordered model. However, this category was found in only 5% in precursor-B-ALL, and was not observed in CBL.

The precursor-B-ALL data generally show a hierarchy in rearrangements of the three Ig loci (*IGH, IGK,* and *IGL*). However, there is no causal relationship in this hierarchy, because functional *IGH* rearrangements are not necessarily required for the induction of Ig light chain rearrangements. Likewise, the *IGK* rearrangement does not necessarily have to be out-of-frame before *IGK* deletion or *IGL* rearrangement can be initiated. Precursor-B-ALL therefore constitute a model system to study the Ig recombination process itself without any form of selection for the functionality of the rearrangements, because of the malignant status of the cell that results in block of differentiation. So, the Ig gene recombination process follows a certain hierarchy, which is most probably based on ordered accessibility of the Ig loci. During normal precursor-B-cell differentiation functional rearrangements would result in (surface) Ig protein expression upon which further selection of precursor-B cells takes place. This selection includes up and down regulation of expression of the recombinase enzymes, mediating subsequent Ig gene rearrangements. Although such selection mechanisms are absent in our precursor-B-ALL model system, the hierarchy of *IGH, IGK,* and *IGL* recombination remains apparent.

Based on the combined data, we believe that the Ig gene recombination process can be divided into two parts, the first being hierarchy in accessibility of the Ig loci and the second being selection based on Ig protein expression, which is responsible for regulation of the recombinase enzyme system. This is confirmed by recent studies in which transcription factor-induced accessibility of antigen receptor loci and recombinase activity are the two only prerequisites for inducing V(D)J recombination.[26-28] However, a definite answer to this question should come from single cell PCR studies on sorted precursor-B-cells, similar as has been performed on naive B-cells.[29] Such studies should reveal whether hierarchical accessibility is an important contributor to Ig light chain recombination patterns in early B-cell differentiation.

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Chapter 3.4

IMMUNOGLOBULIN LIGHT CHAIN GENE RECOMBINATION PATTERNS IN MANTLE CELL LYMPHOMA AS CLONAL MODEL SYSTEM FOR NAIVE B CELLS

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ABSTRACT

Regulation of immunogobulin (Ig) light chain gene recombination processes can be studied via analysis of Ig gene configuration patterns. Two approaches can be followed, i.e. single cell PCR on normal B cells or the use of clonal B-cell malignancies. In previous studies, we used precursor-B-ALL as clonal model system for precursor-B cells and chronic B-cell malignancies (CBL) as model for mature B cells, which are partly somatically mutated. In this study we analyzed a series of 35 mantle cell lymphomas (MCL) as model system for naive B cells with Ig expression, but lacking somatic hypermutations. The Ig kappa (*IGK*) light chain gene configuration patterns of MCL were compared to data from single cell PCR analysis of normal circulating naive B cells, which were recently published. From both MCL and normal naive B cells it could be concluded that Ig light chain gene rearrangements in naive B cells display an hierarchical order. The only observed difference between normal and malignant naive Igλ+ B cells was the relative distribution of cases with one or two *IGK* deletions. These appeared to be equally distributed in normal naive Igλ+ B cells and skewed towards biallelic *IGK* deletions in Igλ+ MCL. Ig light chain gene configuration patterns of MCL were also compared to patterns of CBL. The rearrangement hierarchy in MCL and CBL was comparable, except for a slightly higher frequency of *IGL* gene rearrangements in Igκ+ MCL. This similarity suggests that the influence of somatic mutations on Ig light chain gene configuration patterns is very limited, implying that somatic mutation-induced secondary rearrangements (receptor revision) that result in deleting of *IGK* and a switch to *IGL* expression does not seem to play a significant role.

INTRODUCTION

Immunoglobulin (Ig) gene recombinations occur in bone marrow precursor-B cells. During this process V, (D,) and J segments are assembled, resulting in the expression of an antigen-specific B-cell receptor, which is composed of two identical Ig heavy chains and two identical Ig light chains, either Ig kappa (Igκ) or Ig lambda (Igλ)[1,2] V(D)J recombination is stage- and tissue-specific, which is achieved via strict regulation of the recombination activation genes (*RAG1*, and *RAG2*) on one hand and accessibility of the Ig loci (*IGH, IGK, IGL*) on the other hand.[3-10] Ig gene recombinations occur with a hierarchy, starting with rearrangements at the *IGH* locus followed by light chain gene rearrangements.[8,11,12] According to the ordered model, the Ig light chain gene rearrangement process starts at the *IGK* locus.[13] If no functional *IGK* gene rearrangements are formed, the cell can escape apoptosis by deleting the Jκ-Cκ region with or without the preceding Vκ-Jκ rearrangements via a recombination of the kappa deleting element (Kde), followed by recombination at the *IGL* locus.[14,15]

To study whether this mechanism is indeed commonly used in the differentiation of naive B cells, we performed analysis of Ig recombination patterns. Two approaches can be

followed to investigate the regulation of Ig gene rearrangement processes via analysis of Ig recombination patterns, i.e. single cell analysis of normal B cells and the use of clonal B-cell malignancies.[13,15-18] Both systems have their inherent advantages and disadvantages. The obvious advantage of single-cell analysis is that normal B-cells can be studied; however, the disadvantage of the single-cell PCR approach is the risk of missing rearrangements due to false-negative PCR results. In contrast, B-cell malignancy model systems allow a complete and exact analysis of both alleles of all three Ig loci by Southern blot and PCR analysis, with a minimal risk of missing rearrangements. However, it cannot be completely ruled out that Ig gene recombination patterns in B-cell malignancies are influenced by oncogenic processes.

To study the regulation of the Ig light chain gene recombination process in early developing B-cells, we previously analyzed the Ig recombination patterns of a large series of precursor-B acute lymphoblastic leukemia (precursor-B-ALL).[18] Precursor-B-ALL (pro-B-ALL, common ALL, and pre-B-ALL) were used as clonal model system for precursor-B-cells at three stages of differentiation (pro-B, pre-B-I, and pre-B-II). However, the data showed that precursor-B-ALL do not form the perfect model system for early developing B-cells due to continuous recombination activity. It rather gave insight into the rearrangement process itself, without any form of selection for functionality of the rearrangements.

Furthermore, chronic B-cell leukemias (CBL) were used as clonal model system for mature B cells.[15] Approximately half of CBL cases are derived from B cells that have not been selected by antigen (Ag) based on the absence of somatic mutations, whereas the other half carry somatic mutations.[19] We assumed that CBL can indeed be used as model system for B cells, because the gene rearrangement process is most probably completed at the time of malignant transformation. The Ig gene rearrangements in CBL clearly showed a hierarchy in Ig light chain gene recombinations.[15]

To complete the insight into Ig light chain recombination during B-cell differentiation, starting with clonal precursor-B cells without any form of selection for functionality of the rearrangement and continuing with in clonal mature B cells, which (partly) carry somatic mutations, in this study we determined the Ig rearrangement patterns of naive B cells. These cells are selected for having a functional Ig molecule, but have not yet been subjected to the somatic hypermutation process.

For this purpose we collected a series of mantle cell lymphomas (MCL), as they are derived from naive cells, presumable mantle cells that are present at the rim of the germinal centers in the peripheral lymphoid organs.[20,21] Depending on the detection method 70 to virtually all MCL carry the translocation t(11;14) involving the *IGH* locus and *BCL1/CCND1*.[21,22] As a result of this translocation the *CCND1* gene is overexpressing the cyclin D1 protein, which is a cell cycle regulatory protein that is involved in the regulation of the G1 to the S phase of the cell cycle. We compared the Ig configuration patterns of MCL with the results of a recent study on single cell analysis of sorted human naive $Ig\kappa^+$ and $Ig\lambda^+$ B cells ($CD27^-IgD^+$), which can be regarded as the normal counterpart of MCL.[16] In addition, the Ig configuration patterns of MCL were compared to those of CBL to understand whether somatic mutations influence Ig gene rearrangement patterns.

MATERIALS AND MEHTODS

Patient samples

A total of 68 frozen tissue sections were obtained from 42 patients diagnosed with MCL, according to the WHO classification.[20,21] DNA extraction was successfully performed in 51 biopsies of 40 patients. Membrane Ig light chain expression was determined using direct immunofluorescence detection methods on frozen sections. In addition, Cyclin D1 overexpression was determined on the corresponding paraffin embedded tissue. From a total of 35 MCL sufficient frozen material was available for Southern bot analysis.

Southern blot analysis

Fifteen μg of DNA was digested with *Bgl*II and *Hin*dIII (Life Technologies, Rockville, MD, USA), separated in 0.7% agarose gels and transferred by vacuum blotting to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany).[23] The filters were hybridized with ^{32}P labeled probes. Probes specific for Jκ (IGKJ5), Cκ (IGKC) and Kde (IGKDE) (DAKO Corporatio, Carpinteria, CA, USA) were used to determine the *IGK* gene configuration.[24] For the *IGL* locus, the IGLC1D and IGLJ2 probes were used.[25]

RESULTS AND DISCUSSION

A series of 35 MCL selected on the availability of sufficient frozen tissue could reliably be analyzed with respect to Ig light chain expression and Ig gene configurations via Southern blot analysis. Of these, eighteen MCL were Igκ positive and the other seventeen MCL were Igλ positive. All cases showed cyclin D1 overexpression.

IGK and *IGL* gene configurations in MCL

The *IGK* and *IGL* gene rearrangements were determined via Southern blot analysis. The combined data are summarized in Table 1. The *IGK* locus can either be in germline configuration (G), rearranged (R), or deleted (D). In the latter case a rearrangement of the intron recombination signal sequence (intron RSS) to the kappa deleting element (Kde) could have occurred resulting in the deletion of the Cκ gene segment.[24] Alternatively, a rearrangement of an upstream Vκ gene segment to the Kde could have occurred resulting in the deletion of the preexisting Vκ-Jκ exon as well as the Jκ and Cκ gene segments.[24] The *IGL* locus can either be germline (G) or rearranged (R).

According to the ordered model, Ig light chain recombination starts at the *IGK* locus. If no functional rearrangement occurred on both alleles, deletion of *IGK* genes is followed by recombinations at the *IGL* locus. Indeed, in 72% (13/18) of the Igκ$^+$ MCL, both *IGL* alleles were in germline configuration and in 95% (16/17) of the Igλ$^+$ MCL at least one *IGK* deletion was found (Table 1). In 82% (13/17) of Igλ$^+$ cases even both *IGK* alleles were deleted. As predicted by the ordered model, none of the Igλ$^+$ cases had both *IGK* alleles in germline configuration.

Table 1. *IGK* and *IGL* gene configuration patterns of 18 Igκ+ and 17 Igλ+ MCL.

	Igκ+ MCL (n=18)			Igλ+ MCL (n=17)		
	IGL gene configuration					
IGK gene configuration	G/G	R/G	R/R	G/G	R/G	R/R
G/G	-	-	-	-	-	-
R/G	7 (39%)	2 (11%)	-	-	-	-
R/R	3 (17%)	1 (6%)	-	-	1 (6%)	-
D/R	4 (22%)	1 (6%)	-	-	1 (6%)	-
D/D	-	-	-	-	7 (41%)	6 (35%)
D/G	-	-	-	-	1 (6%)	1 (6%)

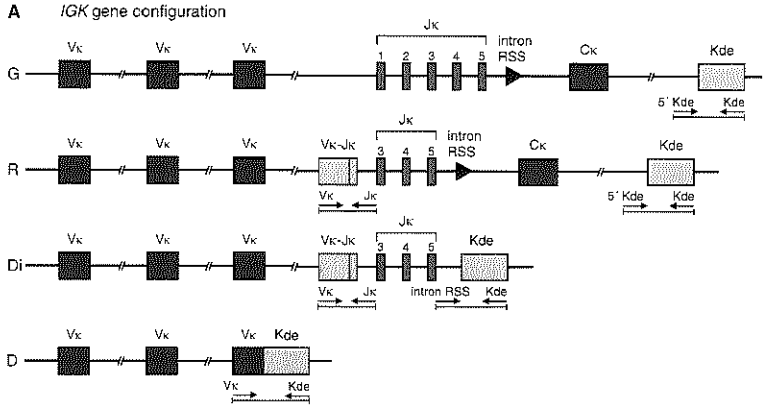
G, germline; R, rearrangement; D, deletion (Vκ-Kde or IntronRSS-Kde).

Comparison of *IGK* gene configuration profiles of MCL and naive B cells

In a recently published study by Bräuninger *et al.*, single cell analysis of Ig light chain gene configurations of human naive Igκ+ and Igλ+ B-cells (CD27-IgD+) is described.[16] Single cells were sorted and after genomic DNA amplification, PCR and sequence analysis of the Ig light chain gene rearrangements was performed. Figure 1 shows the used PCR approach. *IGK* gene configuration data were shown of 138 Igκ+ cells of two healthy individuals and 175 Igλ+ cells of three healthy individuals. Data from 17 Igκ+ and 29 Igλ+ cells that lacked information of the Kde region were excluded from the comparative analysis. Unfortunately, no information was available of the *IGL* rearrangements in the normal naive B cells. The results permitted for some categories two or more possible interpretations (summarized in Figure 1B). Figure 2 shows a comparison of the *IGK* gene configurations of the Igκ+ and Igλ+ naive B cells, MCL, and CBL. For the normal naive B cells, we corrected the data by choosing for these cases the most likely interpretation (Figure 1B). Additionally, we assumed that a minimum of PCR products is missed, and that the chance of false-negative results is larger for Vκ-Jκ and Vκ-Kde rearrangements than for Intron-Kde and Kde germline, because of improper Vκ primer annealing.

With these provisions in mind, the results of the normal Igκ+ cells and Igκ+ MCL were largely comparable (no statistically significant differences). However, there was a statistically significant difference in the distribution of D/R and D/D cases in normal Igλ+ B cells and Igλ+ MCL. Normal Igλ+ cells showed an equal distribution between cells with monoallelic *IGK* deletions and cells with biallelic *IGK* deletions, whereas in Igλ+ MCL this distribution was skewed towards D/D cases. For comparison, our earlier data of the *IGK* gene

A *IGK* gene configuration



B

Vκ-Jκ	Kde	Possible interpretations	Assumed false negative PCR
0	IntronRSS-Kde 5'Kde-Kde	**a. Di/G** b. Di/R	a. Vκ-Jκ b. 2x Vκ-Jκ
0	IntronRSS-Kde -	**a. Di/D** b. Di/G c. Di/R	a. Vκ-Jκ and Vκ-Kde b. Vκ-Jκ and 5'Kde-Kde c. 2x Vκ-Jκ and 5'Kde-Kde
0	Vκ/Kde -	**a. D/R** b. D/G	a. Vκ-Jκ b. 5'Kde-Kde
0	5'Kde-Kde -	**a. G/G** b. R/G c. R/R d. R/D	a. – b. Vκ-Jκ c. 2x Vκ-Jκ d. Vκ-Jκ and Vκ-Kde
0	Vκ/Kde 5'Kde-Kde	**a. D/G** b. D/R	a. – b. Vκ-Jκ
1	IntronRSS-Kde 5'Kde-Kde	**c. Di/G** d. Di/R	c. – d. Vκ-Jκ
1	IntronRSS-Kde -	**a. Di/R** b. Di/G	a. Vκ-Jκ b. 5'Kde-Kde
1	Vκ/KDE -	**a. D/Di** b. D/R	a. IntronRSS-Kde b. 5'Kde-Kde
2	Vκ/KDE -	**a. D/Di** b. D/R	a. IntronRSS-Kde b. 5'Kde-Kde

Figure 1A. Single cell PCR approach for the detection of *IGK* deletions, as performed by Bräuniger *et al.*[16] The used primers are indicated as arrows and the obtained PCR products as bars. Four PCR reactions were performed on every cell, i.e. Vκ-Jκ, 5'Kde-Kde (for germline Kde), IntronRSS-Kde, and Vκ-Kde. The *IGK* gene configuration and the expected PCR products can be summarized as follows: G, germline; R, Vκ-Jκ rearrangement; Di, intronRSS deletion, i.e. Vκ-Jκ rearrangement followed by intronRSS-Kde rearrangement; D, Vκ-Kde deletion. **B.** Interpretation of single cell PCR results for inconclusive *IGK* PCR data. The disavantage of single cell PCR is the chance of false-negative PCR results. For cases we summarized the possible configurations. The most likely interpretation is mentioned first (a), followed by less interpretations (b, c, d). For interpretation we assumed that a minimum of PCR products is missed, and that the chance of false-negative results is larger for Vκ-Jκ and Vκ-Kde rearrangements than for IntronRSS-Kde and Kde germline, because of improper Vκ primer annealing.

configurations in Igκ$^+$ and Igλ$^+$ CBL were added in Figure 2 and showed for both Igκ$^+$ and Igλ$^+$ cases similar patterns as found in MCL. This suggests that the observed differences between normal and naive B cells and MCL do not result from the relatively small number of MCL.

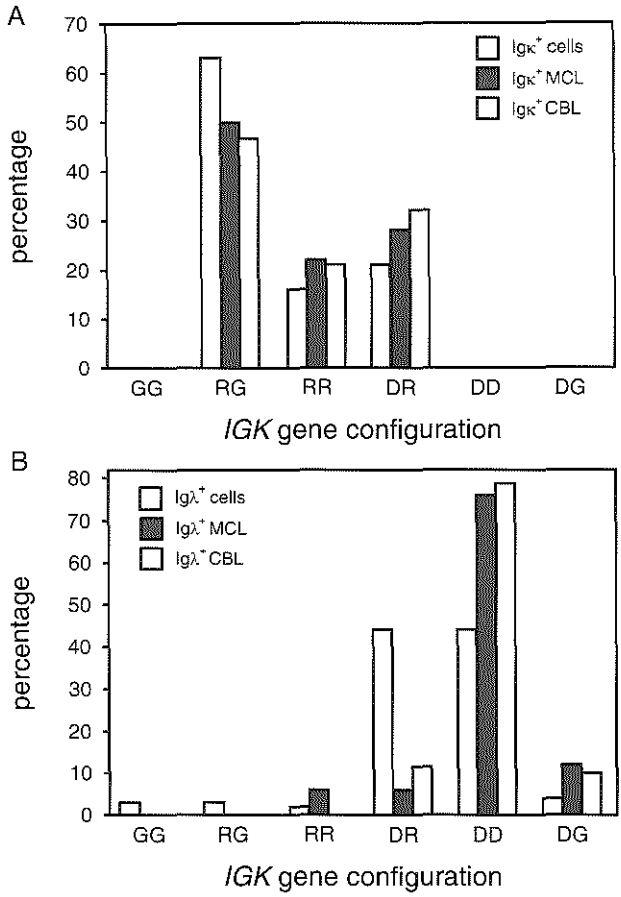


Figure 2A. and B. *IGK* gene configurations of normal Igκ+ naive B cells, Igκ+ MCL, and Igκ+ CBL (**A**) and normal naive Igλ+ B cells, Igλ+ MCL and Igλ+ CBL (**B**) with correction for the most likely interpretation in naïve B cells with inconclusive *IGK* PCR data.

Despite this difference in relative distribution, the vast majority of both normal naive Igλ+ B cells and Igλ+ MCL had at least one deletion in the *IGK* locus, suggesting that the Igλ+ B cells arose from hierarchical recombination processes. In these cells, the functional in-frame *IGL* gene rearrangements have thus been preceded by *IGK* gene rearrangements and deletions. Based on these findings, we conclude that MCL can be used as a model to study Ig gene recombination patterns in naive B cells.

Comparison of degree of hierarchy in *IGK* and *IGL* light chain gene rearrangements between MCL and CBL

To evaluate to what extent Ig light chain gene recombination patterns in naive B cells (MCL) are comparable or different from mature, partly somatically mutated B cells (CBL),

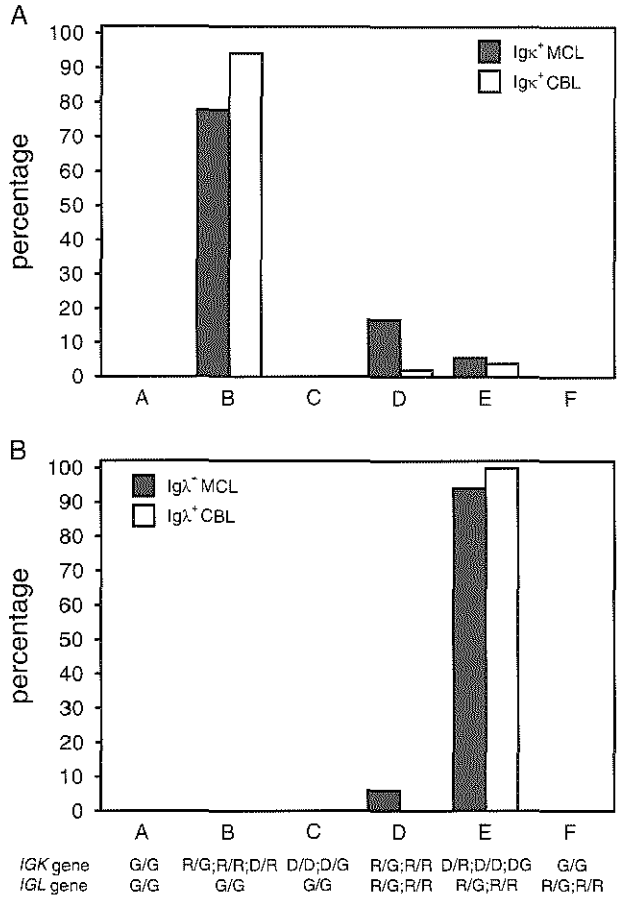


Figure 3A. and B. Distribution of the percentages of cases in different categories for Igκ+ MCL and CBL (**A**) and Igλ+ MCL and CBL (**B**). The *IGK* and *IGL* gene configurations of the different categories are indicated in the table.

the *IGK/IGL* recombination patterns were assigned to six categories (Figure 3).[18] Consecutive steps in a strictly ordered recombination process would be described by the subsequent categories: A (*IGK* and *IGL* germline), B (*IGK* rearranged, *IGL* germline), and E (*IGK* at least one allele deleted, *IGL* rearranged). Category D (*IGK* and *IGL* rearranged without *IGK* deletions) was added, because the ordered model appeared to be not absolutely strict, as *IGL* rearrangements did occur without prior *IGK* deletions. Category A and C (*IGK* deletions, no *IGL* gene rearrangements) are not present in surface Ig positive B cells, including MCL and CBL, because these cells have at least one Ig light chain rearrangement (*IGK* or *IGL*), but were found in precursor-B-ALL. Category F was introduced for cases with *IGL* gene rearrangements without *IGK* gene rearrangements, which therefore do not fit in the

ordered model. Such cases were not found in this MCL series, or in the previously described CBL series, but were observed in precursor-B-ALL (5%).[18]

Figure 3 shows the percentages of cases in the different categories of the Igκ+ and Igλ+ MCL and CBL. These data reveal a difference in the percentages of cases in category D (*IGK* and *IGL* gene rearrangements without *IGK* deletions) in Igκ+ cells between MCL and CBL (Figure 3A). with a larger percentage in MCL. However, probably due to the low number of analyzed MCL cases, the tendency in Igκ+ MCL towards the presence of *IGL* gene rearrangements, even without *IGK* deletions is not significant (p=0.057, χ^2 test). It will be of interest to determine whether the *IGL* recombinations in these four Igκ+ MCL represent true Vλ-Jλ gene rearrangements, or whether they represent illegitimate recombinations such as chromosomal translocations. If these recombinations indeed represent Vλ-Jλ rearrangements, they do not completely fit in the model for hierarchical accessibility of Ig light chain genes for recombination, because this model proposes that *IGL* gene rearrangements only occur if no functional *IGK* light chain rearrangement is present that can be expressed together with the Ig heavy chain. However, these *IGL* gene rearrangements might theoretically have taken place in parallel or after *IGK* gene rearrangements, but before Igκ expression. Alternatively, these *IGL* rearrangements occurred before *IGK* gene rearrangements, but are not functional. PCR amplification and sequence analysis should give more insight in this aspect.

CONCLUSIONS

The Ig light chain gene configuration patterns of MCL as a clonal model system for naive B cells showed that *IGK* and *IGL* gene recombinations occur in a hierarchical order, starting with *IGK* gene rearrangements usually followed by *IGK* deletions before subsequent *IGL* gene rearrangements. The *IGK* gene configuration data generated via Southern blot analysis of MCL were compared to data from single cell PCR analysis of sorted naive B cells. Based on both model systems it could be concluded that the Ig light chain gene rearrangements in naive B cells display hierarchy.[16] The only observed difference between normal and malignant naive B cells is that in Igλ+ MCL and also in Igλ+ the frequency of cases with monoallelic and biallelic *IGK* deletions is different from that of normal naive B cells. However, the total frequency of Igλ+ cells with at least one *IGK* deletion was similar in MCL and naive B cells. Despite the difference in relative distribution of cases with one or two *IGK* deletions, both strategies seem to describe *IGK* and *IGL* light chain gene recombination in a largely comparable way. Unfortunately, no *IGL* data were available from these single cell assays to allow complete comparison of *IGK* and *IGL* patterns in naive B cells and MCL.

The Ig light chain gene configuration patterns of MCL were also largely comparable to CBL, which can be considered as clonal model system for mature B cells. The only difference was the presence of slightly more *IGL* gene rearrangements in Igκ+ MCL. To further investigate this difference, the *IGL* gene rearrangements of the Igκ+ MCL will be analyzed by PCR and sequencing analysis. Moreover, it will be of interest to determine the frame of

the *IGK* and/or *IGL* gene rearrangements in all cases with more than one rearrangement (R). This will give insight whether allelic or isotypic exclusion are regulated in a similar way as found in CBL.[15] Overall, the similarity in Ig light chain gene recombination patterns between MCL and CBL suggests that these patterns are not influenced by somatic mutations that might have induced secondary rearrangements in the periphery (receptor revision). Ig light chain recombination patterns of multiple myeloma might clarify these observation, since multiple myeloma can be regarded as a model system for the end-stage of B-cell differentiation, i.e. after multiple rounds of somatic hypermutation and potential receptor revision.

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Chapter 3.5

BIASED Igλ EXPRESSION IN HYPERMUTATED IgD MULTIPLE MYELOMAS DOES NOT RESULT FROM RECEPTOR REVISION

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ABSTRACT

Normal IgM$^-$IgD$^+$ CD38$^+$ B-cells and IgM$^-$IgD$^+$ multiple myelomas (MM) are characterized by Cμ deletion, biased Igλ expression and hypermutated IgV regions. The predominant Igλ usage was proposed to result from secondary Ig gene rearrangements during extensive clonal expansion in the germinal center environment. Here, four cases of IgDλ MM were studied to address the question of light chain receptor revision in a "single cell" model. Detailed analyses of both *IGK* and *IGL* alleles of each case were performed by Southern blotting, (RT-) PCR, and sequencing. The expressed IgV genes were extensively mutated and Cμ deletion was confirmed in two cases. In addition, in the four MM a total of six non-functional deletional *IGK* rearrangements were identified, which proved to be unmutated. We conclude that IgD myelomas indeed originate from (post) germinal center B-cells in which, in spite of the fact that they are hypermutated, there is no evidence receptor revision.

INTRODUCTION

A minority of normal human B cells and rare B-cell malignancies express IgD in the absence of IgM. It has been reported that in the human tonsil, 2-5% of the CD38$^+$ germinal center B cells and 6-20 % of the plasmacells are IgM$^-$IgD$^+$.[1,2] In the peripheral blood, sIgM$^-$IgD$^+$ CD27$^+$ memory B cells have been detected with a frequency of less than 1%.[3] Among the B-cell malignancies, approximately 1% of multiple myelomas (MM) and 10% of hairy cell leukemias display the IgD-only phenotype.[4] The lack of IgM in all these cell populations is explained by unusual Cμ-Cδ class 'switch' recombination mediated by sequences in the J_H-Cμ and Cμ-Cδ introns, i.e. either homologous recombination between two direct 442 bp repeats (σ_μ and Σ_μ)[5,6] or non-homologous, so-called Sμ-σδ recombination,[4] both with deletion of the Sμ-Cμ region and precluding further isotype switching. The IgM$^-$IgD$^+$ B-cell populations mentioned share two additional molecular features. First, IgM$^-$IgD$^+$ B cells generally carry a very high load of somatic mutations, most likely as a result of extensive clonal expansion: whereas the frequency of nucleotide substitutions in the V_H genes of normal (post)germinal center B cells ranges between 2-6%,[3,7] the reported frequencies for both the normal and neoplastic single-IgD positive B cells are on average two- to threefold higher.[1-3,5,6,8] Second, the various normal IgM$^-$IgD$^+$ B cells almost exclusively express Igλ (>99%).[1] Also in IgM$^-$IgD$^+$ MM, high frequencies of Igλ light chain expression have been reported, ranging between 60 and 90%, illustrating the bias towards Igλ.[9-14]

It has been hypothesized that the biased Igλ expression of IgM$^-$IgD$^+$ B cells results from secondary Ig gene rearrangements at the light chain loci in the germinal center environment (i.e. receptor revision), elicited by unfavorable somatic mutations that cause loss of Ig expression or disturbed pairing of Ig heavy and light chains.[2] Ig light chain gene rearrangements generally occur in an ordered sequence, starting at the *IGK* locus followed by *IGK*

deletion and subsequent *IGL* rearrangements.[15] Therefore, secondary rearrangements are expected to result in skewing towards *IGL* gene usage.

Evidence for receptor revision as a rescue mechanism in mature human B cells is as yet scarce. Interestingly, occurrence of V_H replacements has recently been claimed in IgM$^-$ IgD$^+$ CD38$^+$ tonsillar B cells and in synovial-tissue B cells.[16,17] Previous reports however could not draw firm conclusions on the issue of receptor revision of Ig light chain genes since this requires analysis of all four Ig light chain alleles, i.e. of both *IGK* and both *IGL* alleles, of individual cells. To address the question of Ig light chain receptor revision in a 'single cell' model, detailed studies on the *IGH*, *IGK*, and *IGL* loci of four cases of IgDλ^+ MMs were performed using Southern blot (SB), PCR and sequence analyses.

MATERIAL AND METHODS

IGH, *IGK* and *IGL* genes were analysed by SB, PCR and sequencing. Fifteen μg of genomic DNA was digested with BamHI and BglII, size fractionated in a 0.7% agarose gel and transferred to a nylon membrane. The *Bam*HI filter was successively hybridized with the IGHMU and IGHJ6 probes for detection of *IGH* rearrangements and Cμ deletion. For detection of the IgM-IgD recombination by PCR primers upstream of σ_μ and downstream of Σ_μ were used (adapted from Arpin *et al.*[2]). The BglII filter was hybridized with *IGK* and *IGL* probes for determination of the *IGK*, and *IGL* gene configurations.[15,18] Family specific V_H, Vκ, and Vλ primers were used in combination with J_H, Jκ and Jλ primers (genomic PCR) or in combination with Cδ, Cκ or Cλ primers (RT-PCR).[15,19] Clonal (RT-)PCR products were either directly sequenced or cloned in pGEM-T Easy vector followed by sequencing of 4-6 individual clones.

RESULTS AND DISCUSSION

The configuration of the *IGH*, *IGK*, and *IGL* genes in four IgDλ MMs were studied by SB, PCR and sequencing (Table 1). MM-1 contained a single in-frame *IGH* gene rearrangement with deletion of the Cμ region caused by a σμ to Σμ recombination. In MM-2 and MM-4, no clonal bands could be identified at the DNA level by SB, most likely due to a limited tumor load in the bone marrow samples. However, by RT-PCR and sequencing, single clonal in-frame V_H-Cδ rearrangements were identified. MM-3 contained a biallelic *IGH* gene rearrangement with a deletion of the Cμ region on one allele, which was in accordance with identification of clonal in-frame V_H-Cδ transcripts, but no V_H-Cμ transcripts by RT-PCR. No second complete V_H-J_H rearrangement was identified by PCR analysis in MM-3 (Table 1). In myelomas 1, 2, and 3 in-frame *IGL* gene rearrangements were identified as well.

All the functional V_H and Vλ genes were indeed exceptionally hypermutated. The

Table 1. Summarized data of Ig gene configurations, gene segment usage, and somatic mutations in four IgDλ MM.

MM	Cμ deletion	Ig locus	SB analysis[a]	(RT-)PCR and sequencing (frame)[b]	Observed somatic mutations							
					Total	%	CDR			FR			
							R	S	R/S	R	S	R/S	p[f]
1	Yes	*IGH*	R/G	V_H3-23/D_H3-16/J_H2 (in)	31	10.5%	7	3	2.3	13	8	1.6	0.032
		IGK	D/G	Vκ1-33/Jκ2(out)+intronRSS-Kde	0	0%	-	-	-	-	-		-
		IGL	R/G	Vλ2-14/Jλ2 (in)	8	4.7%	1	1	1	5	1	5	nd
2	ni[e]	*IGH*	-[d]	V_H3-11/D_H5-24/J_H6 (in)	89	31.5%	16	5	3.2	48	20	2.4	0.075
		IGK	-	Vκ4-1/Kde	0	0%	-	-	-	-	-	-	-
		IGL	-	Vλ2-18/Jλ1 (in)	18	5.8%	6	3	2.0	9	0	-	nd
3	Yes	*IGH*	R/R	V_H4-31/D_H5-18/J_H2 (in) ni[e]	60	20.4%	15	2	7.5	27	16	1.7	0.026
		IGK	D/D	Vκ2-29/Jκ4(out)+intronRSS-Kde	0	0%	-	-	-	-	-	-	-
				Vκ1-43/Jκ5(out)+intronRSS-Kde	0	0%	-	-	-	-	-	-	-
		IGL	R/G	Vλ2-23/Jλ3 (in)	23	12%	4	1	4.0	12	6	2.0	nd
4	ni[e]	*IGH*	-[d]	V_H5-51/D_H5-12/J_H6(in)	31	10.5%	7	4	1.8	12	8	1.5	0.010
		IGK	-	Vκ3D-20/Jκ4(out)+intronRSS-Kde	0	0%	-	-	-	-	-	-	-
				Vκ2-24/Kde	0	0%	-	-	-	-	-	-	-
		IGL	-	ni[e]	-	-	-	-	-	-	-	-	-

a. G, germline configuration; R, rearranged allele; D, deletion of Jκ and/or Cκ region via a recombination involving the kappa deleting element (Kde).

b. IMGT nomenclature (http://imgt.cines.fr:8104) was used for assigning the V (D) and J gene segments.

c. R, replacement mutation; S, silent mutation.

d. Due to a low tumor load, no rearranged bands could be detected via Southern blot analysis.

e. ni, not identified; nd, not determined.

f. *p*, probability that the scarcity of R mutations in the FR resulted from chance only (see Chang and Casali).[22]

highest number of somatic mutations was found in the V_H region of MM-2, i.e. 89 mutations. MM-1, MM-3 and MM-4 carried 31, 60 and 31 mutations in their V_H genes, respectively (Figure 1). The average mutation frequency in the V_H regions of the four MM was thus 18%, whereas the reported average mutation frequency of the V_H region in non-IgD MM is 8%.[20,21] Also the Vλ regions showed a slightly higher average mutation load (7.5%) than reported for the light chains of non-IgD MM (6%).[21] In MM-1, 3, and 4, the ratios of replacement (R) versus silent (S) mutations in the framework regions (FR) was approximately 1.5, which is significantly lower than would be expected if the mutations had occurred randomly in the absence of selection.[22] However, in MM-2, with the extremely high mutation load of 31.5%, the R/S ratio was 2.4, suggesting that selectional forces to protect the FR regions and thus to maintain the integrity of the overall Ig structure had been less stringent. Still, this MM, like the other three cases, was found to express the IgDλ protein. Interestingly, a relative high number of replacement mutations in the FRs has been noted previously in the hypermutated IgD-only germinal center cells as well.[1]

The *IGH* sequences of the four cases were analyzed for the presence of hybrid V_H gene segments, indicative of V_H replacement processes.[16] However, we obtained no evidence for this type of receptor revision at the *IGH* locus.

Analysis of the *IGK* genes showed that all rearranged *IGK* alleles involved the kappa deleting element (Kde). This concerned four intronRSS-Kde rearrangements with preserved out-of-frame Vκ-Jκ rearrangements upstream (MM-1, 3, and 4). We detected no somatic mutations in the Vκ gene segments of any of these four non-functional Vκ-Jκ rearrangements. In MM-2 and the second allele of MM-4, Vκ-Kde rearrangements were found which deleted the complete Jκ-Cκ region. Also IgDκ MM, although not present in our limited panel, may contain, next to a functional *IGK* gene rearrangement, a Vκ-Jκ rearrangement at the second allele either or not followed by an intronRSS-Kde rearrangement. Such an Ig gene configuration may in a similar fashion be informative with respect to the topic of receptor revision.

Analysis of the *IGL* genes showed that the three identified *IGL* gene rearrangements used three different Jλ gene segments. This implies that the single IgD expression was not associated with a specific Igλ isotype.

The detailed information obtained on the *IGK* and *IGL* loci indicated that in at least two cases (MM-1 and MM-3) the cells had not expressed another Ig light chain prior to the identified in-frame Igλ chains, since all Vκ-Jκ rearrangements found upstream of the intronRSS-Kde recombinations were out-of-frame and not somatically mutated. This is formally not proven for MM-2 and MM-4 as the Vκ-Kde rearrangements precluded analysis of the possibly pre-existing Vκ-Jκ rearrangements.

In conclusion, our data indicate that IgDλ MM are most likely derived from IgM^-IgD^+ Igλ-positive B cells that were switched to IgD-only by Cμ deletion. In spite of the fact that the IgV regions were clearly hypermutated, proving that the progenitor cells must have undergone extensive clonal expansion in the germinal centers, we find no evidence of receptor revision at the Ig heavy nor Ig light chain loci. It thus remains to be established why IgD,

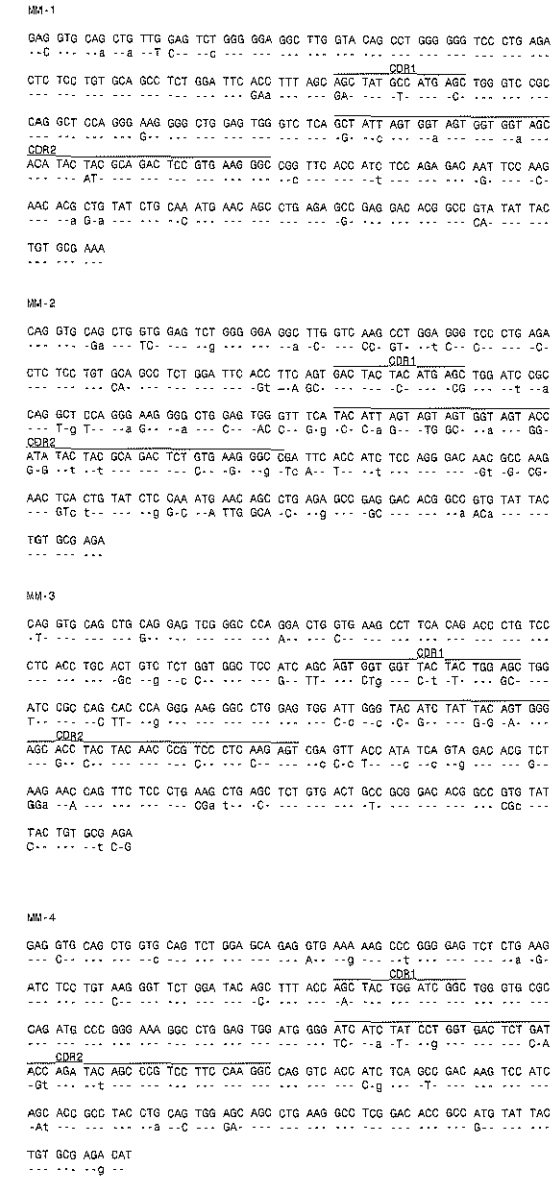

Figure 1. Sequences of the V_H regions of the four IgDλ MM patients (lower line) compared to the most homologous germline V_H gene segments (upper line) according to VBASE (http://www.dnaplot.de). The most homologous sequences were V3-23/DP-47 (V_H3-23, according to IMGT, see Table 1) for MM-1, V3-11/DP-35 (V_H3-11) for MM-2, V4.33/DP-65 (V_H4-31) for MM-3, and V5-51/ DP-73 (V_H5-51) for MM-4. Replacement mutations are indicated with capitals; silent mutations are indicated with lower cases.

in the absence of IgM, is mainly found in association with Igλ or alternatively why Cμ deletion occurs preferentially in Igλ+ B cells and whether the extraordinary level of somatic hypermutation has a causative role in the induction of this unusual Cμ deletion.

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Chapter 4

ONCOGENIC RECOMBINATIONS

4.1 Considerations for development of split-signal FISH for chromosome translocations in acute lymphoblastic leukemia.
Will be published as part of a review.

4.2 Rapid and sensitive detection of all types of *MLL* gene translocations with a single FISH probe set.
Leukemia 1999(13)2107-2113.

4.3 Effects of *E2A* translocations on Ig and TCR gene configuration patterns in pre-B-ALL.
Revised version of this chapter will be submitted.

4.4 Split-signal FISH for the detection of chromosome translocations in acute lymphoblastic leukemia.
Will be published as part of a review.

Chapter 4.1

CONSIDERATIONS FOR DEVELOPMENT OF SPLIT-SIGNAL FISH FOR CHROMOSOME TRANSLOCATIONS IN ACUTE LYMPHOBLASTIC LEUKEMIA

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Will be published as part of a review

Chromosome aberrations in acute lymphoblastic leukemia

Chromosome aberrations play an important role in the development of hematological malignancies.[1] In acute lymphoblastic leukemia (ALL), most of these aberrations concern balanced translocations involving genes that play key roles in the development and function of lymphoid cells, such as transcription factors, cell cycle regulators, and signal transduction molecules. Balanced translocations can result in fusion of two genes that encode leukemia-specific chimeric (fusion) proteins. The fusion proteins have different functional features than the corresponding wild type proteins and most likely play a role in leukemogenesis. In addition to the new features of the fusion protein, loss of wild type activity due to the translocation might contribute to oncogenesis. Alternatively, a chromosome translocation can result in deregulated expression of (onco) genes as a direct consequence of a translocation to a regulatory element, e.g. an immunoglobulin (Ig) or T-cell receptor (TCR) enhancer.

The most frequent translocations in precursor-B-ALL are t(1;19)(q23;p13) t(4;11)(q21;q23), t(12;21)(p13;q22), and t(9;22)(q34;q11), which all result in fusion genes. The t(1;19)(q23;p13) fuses the transcription factor-encoding gene *E2A* (*TCF3*)with the transcription factor *PBX1*. In t(4;11)(q21;q23), the *MLL* gene at 11q23, which encodes a putative DNA-binding protein, is translocated to the *AF4* (*MLLT2*) gene. The *MLL* gene is also involved in many other translocations in ALL and acute myeloid leukemia (AML). Until now, more than 30 partner genes have been identified.[2] The t(12;21)(p13;q22) involves the *TEL* (*ETV6*) gene at 12p13 and the transcription factor-encoding gene *AML1* (*RUNX1*). Finally, t(9;22)(q34;q11) results in fusion of the *BCR* gene at 22q11 with the cytoplasmic tyrosine kinase gene *ABL1*. In T-ALL, gene aberrations involving the *TAL1* gene are frequently observed aberration. The most frequent *TAL1* aberration concerns a submicroscopic deletion del(1)(p32p32) involving the *SIL* and *TAL1* genes. As a result of this ~90 kb deletion all coding exons of the *SIL* gene and the 5' untranslated region of the *TAL1* gene are lost, placing the *TAL1* coding region under direct control of the *SIL* promoter.

Several clinical studies have demonstrated that chromosomal translocations are useful markers for risk group classification. With current treatment protocols, t(12;21) is correlated with a good prognosis, whereas t(9;22) and translocations involving 11q23 such as t(4;11) are especially correlated with a poor prognosis. The t(1;19) is generally correlated with a poor prognosis, although this can be overcome with more intensive chemotherapy.[3,4] For chromosome aberrations involving *TAL1* the correlation with prognosis is less clear.[5,6] Detection of chromosome aberrations at diagnosis, but also at relapse, is therefore an important factor in risk classification. Other risk factors are age, white blood cell count, and treatment response to early remission-induction therapy.

Detection of chromosome aberrations

Several techniques can be used for the detection of chromosome aberrations, each having its inherent advantages and disadvantages. An advantage of conventional cytogenetics is that it is highly informative as all abnormalities can theoretically be detected. This includes not only translocations, but also numerical abnormalities such as hypo-, hyper- or pseudo-

diploidy. However, the interpretation may be difficult if the karyotype is complex. Another disadvantage is that for some samples no reliable results can be obtained because of a low mitotic index or poor chromosome morphology. In addition, some chromosome abnormalities are cryptic, i.e. they cannot be identified via conventional cytogenetics, because changes in chromosome banding patterns are too marginal to be detected, e.g. t(12;21) and *SIL-TAL1* gene fusions.[7]

Fluorescence *in situ* hybridization (FISH) is a molecular cytogenetic technique, which uses fluorescent labeled probes for detection of certain chromosome aberrations. The advantage of this technique is that besides dividing cells (metaphase nuclei) also non-dividing cells (interphase nuclei) can be analyzed, which allows a rapid screening of a large number of cells. In addition, also cryptic aberrations can be detected.[8] A disadvantage of FISH analysis compared to cytogenetics is that this technique is focused on a specific type of aberration, determined by the applied probe set. There are two approaches of FISH probe design, i.e. fusion-signal FISH and split-signal FISH. The advantages and disadvantages of both approaches are discussed in more detail below.

Chromosome translocations can also be identified at the DNA level via Southern blot or PCR analysis. These techniques are more laborious and their applicability for detection of chromosome translocations is limited, because the breakpoints are generally scattered over large regions. This is especially a limiting factor for PCR analysis. Southern blot analysis has been proven to be useful for detection of *MLL* translocations. As the *MLL* gene can have many translocation partner genes and the breakpoint region is relatively small (~ 8.4 kb), Southern blot analysis is suitable for the detection of *MLL* rearrangements, independent of the partner gene.[9,10] PCR analysis has been used for detection of *SIL-TAL1* fusion genes at the genomic level.[11]

An alternative approach, which is suitable for detection of chromosome translocations, resulting in the formation of fusion genes, is detection of fusion transcripts via (nested) RT-PCR analysis. The advantage of this approach is that it reaches sensitivities of 10^{-3} to 10^{-6}, which enables detection of minimal residual disease (MRD). A disadvantage of a PCR-based method is that variant translocations can be missed, if these variants are not covered by the primers.

Detection of chromosome translocations can also be performed at the protein level via detection of the fusion proteins. This technique, however, has not yet been implemented in diagnostics, due to lack of appropriate antibodies that specifically detect the fusion proteins.

Split-signal FISH versus fusion signal FISH

There are two different strategies of dual color FISH analysis for the detection of chromosome translocations. The classical fusion-signal FISH approach uses two differentially labeled probes, red and green, which flank the breakpoint regions of the two genes, which are involved in the translocation (Figure 1A). In normal situations, i.e. without chromosome aberration, two red and two green signals will be detectable. In case of a translocation, a red and a green signal will be juxtaposed giving rise to a colocalized green/red signal, which will

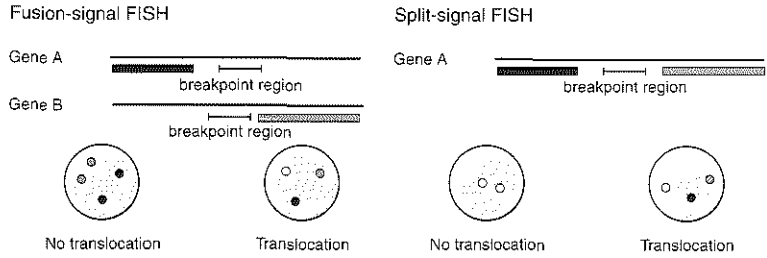

Figure 1A. Fusion-signal FISH with two probes located in the two genes, which are involved in the chromosome translocation. In normal cells, two green and two red signals will be present. In case of a translocation a green and a red signal colocalize, giving rise to a yellow signal together with the separate green and red signals of the unaffected genes. **B.** Spit-signal FISH with two probes positioned at opposite slides of the breakpoint region of the target gene, which is involved in the chromosome translocation. In normal cells, two yellow signals will be present, while in case of a translocation separate green and red signals will be present together with the colocalized signal of the unaffected gene.

in principle appear as a yellow signal, in addition to separate green and red signals of the unaffected chromosomes.

The split-signal FISH approach also uses of two differentially labeled probes, but these probes are located in one gene, hereafter called the target gene, and flank both sides of the breakpoint region in that gene (Figure 1B). In normal situations, two colocalized green/red signals will be visible. A translocation will give rise to a split of one of the colocalized signals, giving rise to separate green and red signals together with a fused signal of the unaffected chromosome.

The split-signal FISH approach has several advantages over the classical fusion-signal FISH. First, the detection of a translocation is independent of the involved partner gene. This is especially of great interest for detection of *MLL* gene translocation, because of the high number of partner genes that might be involved, but also for other genes with multiple partners. Although the detection is independent of the involved partner gene or partner chromosome, split-signal FISH in principle allows the identification of the partner chromosome if metaphase spreads are present on the slide. As a result of the translocation, one of the probes moves to the partner chromosome, i.e. derivative (der)(partner) , while the other probe remains on the der(target) chromosome. The split-signal approach therefore also allows the detection of new partner chromosomes or chromosome regions. Further molecular analysis can then be performed to identify the new partner gene, e.g. using panhandle PCR or long distance inversed PCR.[12,13]

Another advantage of split-signal FISH is absence of the relatively high levels of false-positivity as observed via the fusion-signal FISH approach, which range between 5 and 10%. This false-positivity is caused by the seemingly colocalization of two signals, which represent two separate signals in a three-dimensional nucleus, but are visible as one colocalized signal in the two-dimensional microscopic analysis of the nucleus. One could argue that split-signal FISH can give rise to low frequencies of false-negativity due to the coincidental

colocalization of split signals in leukemic cells, thereby making these cells indistinguishable from normal nuclei.

Outline of Chapter 4

In Chapter 4.2, the development of probes for the *MLL* gene is described, illustrating the general principle and the advantages of split-signal FISH. Chapter 4.3 describes a study on the effect of translocations involving the *E2A* gene on Ig and cross-lineage TCR gene configuration patterns in pre-B-ALL. The *E2A* gene codes for a transcription factor that plays an important role in regulation of V(D)J recombination. Chapter 4.4 summarizes split-signal FISH for the four most frequent chromosome aberrations in precursor-B-ALL as well as for *TAL1* gene aberrations in T-ALL.

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Chapter 4.2

RAPID AND SENSITIVE DETECTION OF ALL TYPES OF *MLL* GENE TRANSLOCATIONS WITH A SINGLE FISH PROBE SET

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ABSTRACT

The *MLL* gene on chromosome 11 band q23 is frequently involved in chromosome translocations in acute lymphoblastic leukemia and acute myeloid leukemia. The translocation results in the formation of a fusion gene on the derivative 11 chromosome consisting of the 5′ part of the *MLL* gene and the 3′ part of another gene; already more than 30 different partner chromosome regions have been described. *MLL* gene rearrangements are generally correlated with a poor prognosis. Therefore the presence of an 11q23 aberration has direct implications for treatment stratification, making early and rapid detection of utmost importance. In this study, we developed a FISH probe set for detection of *MLL* gene rearrangements according to strict design criteria. The cosmid probes are derived from the flanking regions of the *MLL* breakpoint region on chromosome 11 and when used in dual colored FISH experiments give rise to a split of the normally colocalizing (fused) signals in case of a translocation. This split signal was observed in seven out of ten cases with an 11q23 translocation with various partner chromosomes. In the three other cases, a deletion of the 3′ part of the *MLL* gene, downstream of the breakpoint region was also found. A low false positive value of only 1.7% was obtained for interphase cells in contrast to conventional dual colored FISH where the creation of a fusion signal has cut off values of at least 5-10%. A major advantage of our type of probe set is the application of a single FISH experiment to detect all types of *MLL* translocations. Moreover, since this cosmid probe set can be used for either interphase or metaphase studies, metaphases are no longer a prerequisite for detecting the presence of an 11q23 translocation. Nevertheless, metaphase FISH with the new probe set is helpful in determining the partner chromosome and therefore may lead to the identification of new partner genes.

INTRODUCTION

Chromosomal translocations involving the *MLL* gene on chromosome band 11q23 are found in de novo acute leukemias. It is the most common genetic aberration in both infant acute lymphoblastic leukemia (ALL) (70-80%) and infant acute myeloid leukemia (AML) (60%),[1,2] while the incidence in childhood and adult ALL and AML is much lower (5-8%).[3,4] 11q23 aberrations are also frequently found in patients with secondary AML after therapy including topoisomerase II inhibitors.[5]

The *MLL* gene, also named *ALL-1*, *Htrx* and *HRX*, is the human homologue of the *Drosophila* trithorax gene.[6] The human gene consists of 37 exons and spans a region of approximately 100 kb.[7] The MLL protein contains a transcription repression domain, an activation domain and two types of DNA binding domains (minor groove DNA binding 'AT-hook' motifs and major groove binding zinc fingers).[8] MLL functions as a transcriptional maintenance factor in morphogenesis.[9,10] Absence of the *MLL* gene blocks hematopoi-

etic differentiation *in vitro* and is possibly important in leukemogenesis.[11] Translocations involving *MLL* disrupt the gene between the two types of DNA binding motifs; the breaks are clustered within a 8.3 kb major break point region (mbr).[6,12] The 'AT-hook' motifs and the repression domain remain on the der (11), while the zinc fingers and the activation domain are either lost or translocated to the der(partner) chromosome.[8] In most translocations the fusion protein of the derivative 11 chromosome, with the 5′ part of the *MLL* gene fused to the 3′ part of the partner gene, contributes to the oncogenic process.[13] More than 30 different partner chromosome regions have been identified of which 18 partner genes have been cloned.[14] The t(4;11)(q21;q23) and t(11;19)(q23;p13.3) are the most common translocations in ALL, whereas t(6;11)(q27;q23), t(9;11)(p21-p22;q23), t(10;11)(p12;q23) and t(11;19)(q23;p13.1) are most frequent in AML.[15-20]

Rapid detection of an *MLL* gene translocation at diagnosis is of high clinical relevance, because the presence of this aberration has direct consequences for treatment stratification due to the fact that *MLL* gene rearrangements are generally correlated with poor prognosis.[4]

Translocations involving 11q23 can be detected by conventional cytogenetics, Southern blotting, RT-PCR and FISH. Each technique has its advantages and disadvantages concerning time, technical complexity, required laboratory facilities and level of experience. Conventional cytogenetics would allow, in principle, detection of all chromosome aberrations; however, metaphases are a prerequisite and a translocation can only be detected and identified if it causes a clear difference in the chromosome banding pattern. Southern blotting enables the detection of all 11q23 rearrangements, but DNA of high quality is required and distinction between duplication and rearrangement of the *MLL* gene is not possible. Moreover, the partner gene cannot be identified. RT-PCR can only detect *MLL* gene translocations with well-defined (sequenced) partner genes. FISH is a rapid technique, which has advantages above those previously mentioned.

In this study we designed a FISH probe set based on the concept that a translocation would result in separation of the fusion signal found under normal circumstances. This involved the selection of two probes flanking the mbr of the *MLL* gene on chromosome 11. For this purpose, PCR-generated inclusion and exclusion probes were used in order to screen a chromosome 11 specific cosmid library. The carefully selected FISH probes could be used both in interphase and in metaphase FISH. The advantage of this probe design strategy is that all types of *MLL* gene translocations can be detected in a sensitive way in a single FISH test, without the necessity for metaphases. Furthermore, this strategy avoids high levels of false-positivity, as is frequently seen with FISH probes, which give separate signals in case of normal cells, but a fusion signal in case of the chromosome aberration.

MATERIALS AND METHODS

Patient samples and cell line

For the present study, cytogenetic preparations of four ALL and six AML patients were selected based on the presence of an 11q23 translocation, together with five ALL and five AML patients with either a normal karyotype or no 11q23 aberration. Cytogenetic analysis at diagnosis was carried out on freshly obtained bone marrow or peripheral blood cells, which were cultured and harvested according to routine cytogenetic procedures. Chromosomes were described according to the convention of the International System for Human Cytogenetic Nomenclature (ISCN 95). To further characterize the samples, Southern blot analysis was performed to detect *MLL* gene rearrangements as described by Hernandez *et al.*[21] RT-PCR was done in a limited number of cases to confirm the presence of a particular *MLL* gene translocation.[22,23] The precursor-B-cell line RS4;11 characterized by t(4;11) was used as positive control for interphase and metaphase FISH.[24]

Inclusion and exclusion probes

Five primer sets were developed in exons 4, 8, 17, 28, and 37 of the *MLL* gene using the OLIGO 6.2 software program (Dr. W. Rychlik; Molecular Biology Insights, Inc., Cascade, CO) . DNA of a healthy individual was used to generate the PCR products of these exons. The PCR conditions were: 10 min at 94°C to activate the AmpliTaq Gold™ polymerase (PE Biosystems, Foster City, CA, USA) followed by 1 min. at 94°C, 1 min. at 60°C, and 2 min. at 72°C for 35 cycles. The PCR products were analyzed on a 1% agarose gel. The PCR products from exon 4 (MLL4) and exon 37 (MLL37) were used as screening probes for the chromosome 11 specific cosmid library and probes of exon 8, exon 17 and exon 28 (MLL8, MLL17 and MLL28) were used for further selection.

Library screening and cosmid isolation

Chromosome 11 specific cosmid library 107:L4/FS11 (Resourcezentrum im Deutschen Humangenomprojekt RZPD, Berlin, Germany) was hybridized with the ^{32}P-dATP and ^{32}P-dCTP labeled MLL4 and MLL37 inclusion probes. Bacterial colonies with positive cosmids were ordered and cultured according to the manufacturer's instructions. The cosmid DNA was isolated using an alkaline lysis protocol.[25]

Fluorescent in situ hybridization (FISH)

Dual colored FISH was carried out with FISH probes, which were labeled by nick-translation with either biotin-16-dUTP or digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany). For FISH analysis, leukemic metaphase preparations stored at -20°C for up to several years or freshly prepared preparations from –20°C stored methanol/acetic acid fixed cell suspensions were used. FISH was carried out according to standard procedures.[26] Briefly, slides were pre-treated with RNAse and pepsin, and subsequently fixed with

formaldehyde. Hybridization of 100 ng of each probe to the slides was performed overnight at 37°C. Biotinylated probes were detected via subsequent incubation with FITC-labeled avidin d, biotinylated goat-anti-avidin and avidin d-FITC (Vector, Burlingame, CA, USA), whereas digoxigenin-labeled probes were detected via sheep anti-digoxigenin TRITC-labeled antibodies (Boehringer Mannheim), followed by Texas-red-conjugated donkey-anti-sheep antibodies (Jackson ImmunoResearch, West Grove, PA, USA).

The slides were counterstained with DAPI and embedded in Vectashield/DABCO. Per sample a minimum of 200-300 interphase cells was blindly scored by either of two independent observers, as well as 5-10 metaphase cells whenever possible.

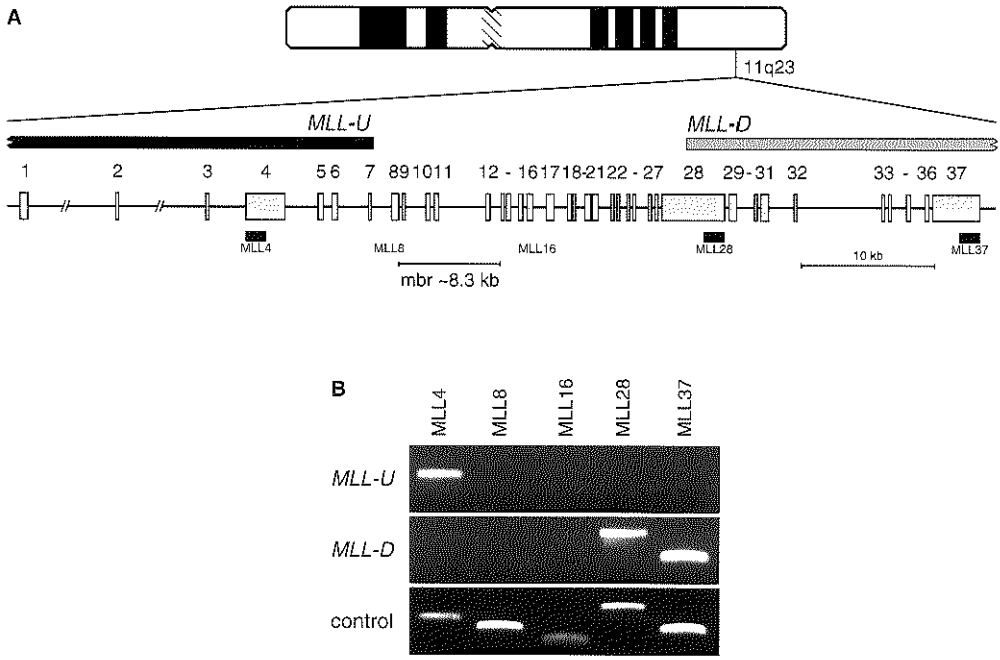

Figure 1A. Chromosome 11 with the *MLL* gene on band q23. The *MLL* FISH probes flanking the breakpoint region (mbr) are indicated. *MLL-U* is the upstream FISH probe and *MLL-D* is the downstream FISH probe. PCR-generated inclusion probes are depicted as black bars (MLL4, MLL28, and MLL37) and the exclusion probes are shown as gray bars (MLL8 and MLL16). **B.** PCR analysis of the FISH probes *MLL-U* and *MLL-D* with inclusion and exclusion primer sets. *MLL-U* is positive for the inclusion region MLL4 and *MLL-D* is positive for MLL28 and MLL 37. Both FISH probes are negative for the exclusion regions MLL 8 and MLL 16.

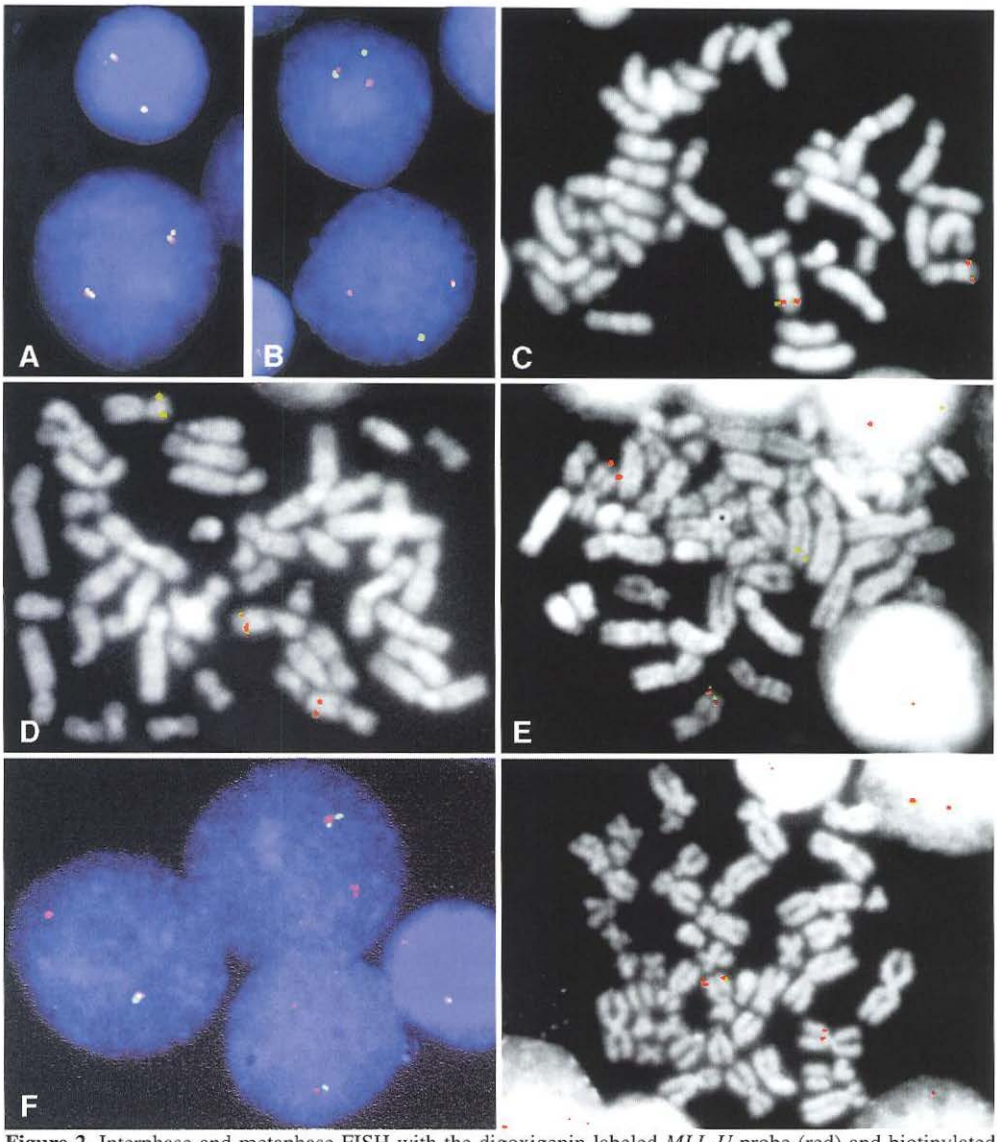

Figure 2. Interphase and metaphase FISH with the digoxigenin labeled *MLL-U* probe (red) and biotinylated *MLL-D* probe (green) on leukemic cells. **A.** Interphase nuclei of AML patient 12 without a 11q23 aberration showing two colocalized (fused) signals on the normal chromosomes 11.**B.** Interphase nuclei of ALL patient 2 with t(9;11)(p21-22;q23) showing a colocalized signal denoting the normal chromosome 11 and a separate green and red signal due to the translocation. **C.** Metaphase spread of AML patient 18 without a 11q23 aberration depicted in inverted DAPI staining with the colocalized signals on chromosomes 11. **D.** Inverted DAPI staining of a metaphase spread of ALL patient 1 with t(9;11)(p21-22;q23). The red *MLL-U* probe hybridized to der(11) and *MLL-D* (green) to der(9). A colocalized signal is visible on the normal chromosome 11. **E.** Metaphase spread of AML-M4 patient 4 with t(6;11)(q27;q23). The *MLL-D* probe moved to der(6) while *MLL-U* is located on der(11). **F. and G.** Interphase and metaphase cells of AML-M5 patient 8 with ins(10;11)(p12;q23q13). The colocalized signal and one red signal are visible, while the green signal is lost demonstrating the deletion of the 3' part of the *MLL* gene. Since the inserted region of chromosome 11(q23q13) into the short arm of chromosome 10 also includes the 5' end of the *MLL* gene, *MLL-U* is located on der(10).

RESULTS

Design of inclusion and exclusion probes

To select FISH probes that were positioned around the *MLL* mbr without overlapping the region, inclusion and exclusion regions were defined. As the FISH probes needed to be positive for either exon 4 or exon 37, two PCR-based inclusion probes were designed in these regions: MLL4 and MLL37 (Figure 1A). To exclude FISH probes that overlap with the breakpoint region, which could give rise to additional signals, two probes (MLL8 and MLL16) were designed in the exons juxtaposed to the breakpoint region. Probe MLL28 was used for further positioning of FISH probes in the downstream *MLL* region.

Library screening

The chromosome 11 specific cosmid library was screened with the inclusion probes to select the cosmids that could potentially be used as FISH probes. One positive colony was found with MLL4 and six with MLL37. Positivity of the cosmid clones was confirmed by PCR analysis (Figure 1B). All cosmids were negative for the exclusion probes MLL8 and MLL16, and therefore not overlapping with the breakpoint region. The cosmid, which was positive for MLL4 was used as upstream FISH probe (*MLL-U*). Although the six MLL37 positive cosmids were largely overlapping, as determined by PCR and restriction fragment analysis, only one of them was positive for MLL28 (Figure 1B).

Testing of FISH probes

The selected *MLL* upstream FISH probe (*MLL-U*) was tested in combination with the six *MLL* downstream probes in interphase FISH on slides of PHA-stimulated peripheral blood mononuclear cells of an healthy donor, on cell line RS4;11, and on one ALL and one AML sample, without 11q23 translocation. As downstream *MLL* FISH probe (*MLL-D*) the cosmid was selected, which gave the best colocalized (fused) signal in combination with *MLL-U* (Figure 2A). This was the cosmid positive for MLL28 (Figure 1B). When tested on the RS4;11 cell line, the presence of the *MLL* translocation resulted in the splitting of one of the two colocalizing signals, giving rise to separate green and red signals (Figure 2B). In metaphases, *MLL-U* remained on the der(11), whilst *MLL-D* moved to the der(4). Hybridization of *MLL-U* and *MLL-D* to metaphase spreads of AML patient without an 11q23 aberration clearly shows the positioning of the probes on chromosome 11 (Figure 2C).

Detection of *MLL* translocations and deletions

A series of ten ALL and AML samples with different 11q23 aberrations was selected (patients 1 to 10, Table 1). By conventional karyotyping seven different *MLL* translocations had been identified, involving six partner chromosomes 4, 6, 9, 10, 18, and 19. The presence of an *MLL* gene rearrangement was confirmed by Southern blotting in nine patients (2 to 10) and specific *MLL* translocations were demonstrated by RT-PCR in five patients (1 to 5).

In patients 1 to 7 a fused red/green signal, and separate red and green signals were

observed in 90.4-97.7 % of the interphase nuclei, demonstrating the presence of an *MLL* translocation (Table 1). The partner chromosome could be identified in those cases where metaphase spreads were present on the slide. Figure 2D and 2E show two metaphase spreads of patients 1 and 4 with t(9;11)(p21-22;q23) and t(6;11)(q27;q23), respectively. In both cases the *MLL-D* probe is located on the derivative partner chromosome: der(9) and der(6).

In patients 8 to 10 one fusion signal and one separate signal of the *MLL-U* probe were observed in 88.3-97.1% of the nuclei, whereas the signal of *MLL-D* was lost. Interphase nuclei of patient 8 are shown in Figure 2F. Loss of the *MLL-D* signal implies a deletion of the region telomeric from the breakpoint region with a minimum size of 40 kb i.e. the size of the cosmid. Deletion of the 3′ end of the *MLL* gene was observed in three different translocations: ins(10;11), t(6;11) and t(9;11). These translocations were also present in our series without a deletion. Figure 2G shows a metaphase spread of patient 8 with ins(10;11). The colocalizing (fused) signal is located on the normal chromosome 11 homologue. Since the inserted region of chromosome 11(q23q13) on the short arm of chromosome 10 also includes the 5' end of the *MLL* gene, the red signal of the *MLL-U* probe is located on the der(10). This type of translocation has been previously described by Beverloo *et.al.*[26] The green signal (*MLL-D*) was lost in this patient indicating the deletion of the 3′ part of the *MLL* gene.

Determining of cut-off values

To validate the *MLL* FISH probe set, the cut off value was determined. For this purpose ALL and AML samples were selected which did not have 11q23 aberrations based on karyotyping and Southern blotting in order to evaluate the performance of this *MLL* FISH probe set in a diagnostic setting. Two independent observers counted in total 500-800 cells per sample. The results are summarized in Table 1.

Although we did not expect to find split signals in the AML and ALL samples without 11q23 aberration, a few (false) positive cells were detected e.g. 1/700 (0.1%) in patient 12 and 8/500 (1.6%) in patient 19 (Table 1). The cut-off value for the translocation (RG/R/G, Table 1), defined as the mean value of the ten leukemia samples without 11q23 aberration plus 3 times the standard deviation, was 1.7%. The cut-off value for the translocations with *MLL* 3′ end deletions (RG/R or G) was 1.8%. All the percentages of the patterns RG/R/G and RG/R or G in the leukemia samples without 11q23 translocation appeared to be lower than the cut-off value.

We also evaluated the FISH probe set on material of 7 AML/ALL patients in complete remission (<2% blasts). The cut-off values were 1.0% for the translocation and 1.6% for the 3′ deletion, respectively (data not shown). This did not differ from the values observed in material from patients at diagnosis.

Table 1. Results of split-signal FISH using the probes *MLL-U* and *MLL-D* on acute leukemia patients with and without 11q23 translocation.

Nr.	Diagnosis	Type of 11q23 translocation	Southern blotting	Results of FISH studies			
				RG/RG[a]	RG/R/G	RG/R or G[b]	Rest[c]
1	AML-M5	t(9;11)(p21-22;q23)[d]	not tested	9.2	90.3	0.1	0.4
2	common ALL	t(9;11)(p21-22;q23)[d]	rearranged	4.5	94.5	0.5	0.5
3	AML-M5	t(10;11)(p12;q23)inv(11)(q14q23)[d]	rearranged	8.3	91.7	0	0
4	AML-M4	t(6;11)(q27;q23)[d]	rearranged	2.2	95.4	1.0	1.4
5	pro-B-ALL	t(4;11)(q27;q23)[d]	rearranged	7.7	91.7	0.3	0.3
6	AML-M4/5	t(11;19)(q23;p13)	rearranged	0.6	97.7	1.6	0.1
7	AML-M5b	t(11;18)(q23;p23)	rearranged	2	92.9	0.9	4.2
8	AML-M5	ins(10;11)(p12;q23q13)	rearranged	6.6	1.7	90.7	1
9	AML-M4	t(6;11)(q27;q23)	rearranged	0.5	0.8	97.1	1.6
10	pre B-ALL	t(9;11)(p21-22;q23)	rearranged	10.1	0.4	88.3	1.2
11	common ALL	-	germline	97.3	0	0.3	2.4
12	AML-M1	-	germline	95.1	0.1	0.3	4.5
13	AML-M6	-	germline	92.2	0	0.4	7.4
14	AML-M2	-	germline	95.8	0	0.1	4.1
15	common ALL	-	germline	94.5	0	1	4.5
16	null ALL	-	germline	96.5	0	0.7	2.8
17	common ALL	-	germline	97.5	0	0.4	2.1
18	AML-M4	-	germline	97.4	0	0.7	1.9
19	common ALL	-	not tested	95.8	1.6	0	2.6
20	common ALL	-	not tested	98	0	1.4	0.6

a. RG= red-green fused signal, R=red, G=green signal.
b. Separate signal of *MLL-U*, which could be red or green depending on the labeling, with loss of the *MLL-D* signal, probably due to loss of the 3'part of the *MLL* gene.
c. Polyploid variations of the other three patterns.
d Confirmed by RT-PCR.

DISCUSSION

Traditionally, in dual colored FISH experiments the presence of a translocation is detected by the formation of a fusion signal.[27] The inherent disadvantage of this approach of probe selection is the rather high risk of false-positive results, due to coincidental colocalization, which results in background levels of at least 5 to 10%. This makes the classical FISH probe design unsuitable for detection of low frequencies of malignant cells. Moreover, both partner genes need to be known to select FISH probes for the detection of the translocation, which is difficult since the *MLL* gene is involved in more than 30 different translocations.

We developed a different approach for FISH probe design to circumvent these disadvantages. Our method is based on the split-signal FISH concept, which means that two colocalized (fusion) signals are present on normal chromosomes and that the presence of an *MLL*

gene translocation results in a split of one of the fused signals, i.e. giving rise to separate red and green signals. For this purpose FISH probes were selected, which flanked both sides of the *MLL* breakpoint region without overlap. This approach has two major advantages: it prevents the traditional high levels of false-positivity and it allows the detection of all types of *MLL* gene translocations, independent of the partner gene. Split-signal FISH can give rise to low frequencies of false-negativity due to coincidental colocalization of signals; these cells are not distinguishable from normal nuclei. However, 5-10% false-negativity (as expected from conventional FISH) within the leukemic cell population will not alter the result in diagnostic material where the blast percentage is over 30%.

We have shown that the selected FISH probe set allows the detection of 11q23 translocations by interphase FISH, independent of the partner chromosome, with a cut-off value of only 1.7%. In our series of ten patients with different 11q23 translocations, three patients had a deletion of at least 40 kb of the region telomeric from the *MLL* breakpoint region. This phenomenon has also been observed by other investigators and the incidence was estimated to be 25-30%.[28-30] The occurrence of 3′ deletions of the *MLL* gene supports the molecular observations that the fusion of the 5′ part of the *MLL* gene carries the critical part for leukemogenesis.[13]

MLL rearrangements are generally correlated with a poor prognosis, especially in infants. However, several studies showed that adult primary AML and childhood AML patients with t(9;11)(p21-p22;q23) have a significantly better clinical outcome than patients with other translocations involving 11q23.[31,32] For this reason simultaneous identification of the partner chromosome is needed for predicting clinical behavior in patients with *MLL* translocations.[33] With this split-signal probe set, the partner chromosome can be identified in the leukemic metaphase spreads present on the slide. As a result of the translocation, the one probe (*MLL-D*) moves to the partner chromosome of chromosome 11, i.e. der(partner), while the other (*MLL-U*) probe remains on the der(11) chromosome. However, in cases with a deletion of the 3′ part of the *MLL* gene, the *MLL-D* probe cannot be used for partner chromosome identification, unless the 5′ part of the *MLL* gene translocated to the partner chromosome, as was observed in the rare case with ins(10;11).[26] Several techniques such as (multiplex) RT-PCR analysis and a RACE based PCR have been described for the identification of translocations involving known partner genes;[34,35] whilst Felix *et al.* have applied pan-handle PCR analysis for the identification of unknown partner genes.[36,37]

Our split-signal FISH methodology will facilitate the identification of known and new translocations involving *MLL*. Together with the application of molecular techniques such as pan-handle PCR analysis, this may lead to the identification of new partner genes.

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Chapter 4.3

EFFECTS OF *E2A* TRANSLOCATIONS ON IG AND TCR GENE CONFIGURATION PATTERNS IN PRE-B-ACUTE LYMPHOBLASTIC LEUKEMIA

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Revised version of this chapter will be submitted

ABSTRACT

The basic helix-loop-helix transcription factor E2A plays an important role during B-cell differentiation, especially in the regulation of V(D)J recombination processes of Ig genes. Similarly, E2A plays a role in regulation of TCR gene recombination during T-cell differentiation by induction of accessibility of TCR loci. In this study, we determined the effect of chromosome aberrations involving the *E2A* gene on Ig and TCR gene rearrangement patterns in pre-B-acute lymphoblastic leukemia (pre-B-ALL). For this purpose ten pre-B-ALL with a t(1;19)(q23;p13) resulting in fusion of the *E2A* gene to the *PBX1* gene were studied and compared to ten *E2A-PBX1*-negative pre-B-ALL. No differences were found in *IGH* gene rearrangement patterns, but the frequency of *IGK* gene rearrangements was reduced in the *E2A-PBX1*-positive pre-B-ALL. Differences in cross-lineage TCR gene rearrangements were more apparent. The overall frequency of TCR gene rearrangements was lower in *E2A-PBX1*-positive pre-B-ALL; the frequency of *TCRD* gene rearrangements was reduced, whereas *TCRG* gene rearrangements were completely absent. However, analysis of cross-lineage TCR gene rearrangements in cell lines with either an *E2A-PBX1* fusion or with an *E2A-HLF* gene fusion resulting from a t(17;19)(q22;p13) revealed the presence of *TCRG* gene rearrangements on five of the six alleles in *E2A-HLF*–positive cell lines, but none of the alleles in *E2A-PBX1* positive cell lines. In conclusion, the presence of E2A-PBX1 fusion proteins and the parallel reduction of levels of wild type *E2A* seems to influence cross-lineage TCR gene rearrangements and to a lesser extent rearrangements of the Ig loci, whereas these effects were not observed in the presence of *E2A-HLF* fusion genes. This suggests that E2A-induced accessibility of antigen receptor loci is influenced by the type of fusion partner in the involved *E2A* aberration.

INTRODUCTION

Transcription factors play an important role during B-cell differentiation.[1,2] Especially E2A plays a central role in the regulation of B-cell differentiation, including the development of uncommitted progenitor cells into pro-B cells, progression to pre-B and immature B-cells, and also in class switch recombination taking place in activated B-lymphocytes (reviewed by Kee *et al*).[3] E2A is not only essential for B-cell differentiation, as absence of E2A also results in disturbed T-cell differentiation.

The *E2A* gene encodes two proteins, i.e. E12 and E47, which arise by alternative splicing.[4,5] These proteins are members of the basic helix-loop-helix (bHLH) family of transcription factors and belong to the class I subgroup of bHLH proteins also referred to as E-proteins. The HLH domain allows dimerization with other HLH proteins, whereas the basic domain is responsible for DNA binding. E2A also contains two activation domains (AD1 and AD2). E2A is widely expressed, but tissue specificity is, in part, determined by the dimer-

ization partner. In B cells, E2A exists only as homodimer. E2A has originally been characterized by binding to E-box motifs (5'-CANNTG-3') that are present in the immunoglobulin kappa (*IGK*) enhancer as well as in the *IGH* enhancer. In T cells, heterodimers of E2A and HEB are able to bind E-box motifs. In addition to binding of E2A to E-box motifs in Ig enhancers, E2A has been shown to induce expression of B-cell specific genes such as the transcription factor *EBF* (early B-cell factor) gene, the surrogate light chain *λ5* gene, but also recombination activating gene 1 (*RAG1*), which mediates V(D)J recombination.[6] Deletion of the *E2A* gene results in a B-cell differentiation block before the onset of D_H-J_H rearrangements.[7]

These combined data are in line with the observation that E2A plays a direct role in the V(D)J recombination process as shown by the induction of particular Ig and T-cell receptor (TCR) gene rearrangements in non-lymphoid cells upon ectopic expression of E2A in combination with RAG1 and RAG2.[8-11] Transcription factors EBF and HEB were also shown to be involved in V(D)J recombination of certain Ig and TCR gene segments, respectively.[9,10] It is assumed that these transcription factors induce accessibility of the Ig and/or TCR loci via chromatin remodeling and thereby target the V(D)J recombination machinery to these loci.

In precursor-B-ALL three types of translocations involving the *E2A* gene are found. The most important *E2A* translocation is t(1;19)(q23;p13) which results in fusion of the *E2A* gene to the *PBX1* gene in 90-95% of cases, leading to expression of the chimeric E2A-PBX1 protein.[12-14] The t(1;19)(q23;p13) is found in approximately 25% of childhood pre-B-acute lymphoblastic leukemia (pre-B-ALL). In the resulting E2A-PBX1 fusion protein the two activation domains of E2A are fused to the dimerization domain and the homeodomain of PBX1, which normally binds DNA cooperatively with HOX proteins. E2A-PBX1 has been shown to be involved in leukemogenesis, most probably due to its new properties such as altered association with regulatory protein complexes as compared to either wild-type E2A or PBX1.[15] In addition, the leukemogenic effect of t(1;19)(q23;p13) might also result from reduced level of wild-type E2A, which has recently been shown to have antiproliferative capacity in B-cell progenitors.[15,16]

In t(17;19) (q22;p13), which is found in ~1% of precursor-B-ALL, *E2A* is fused to the hepatic leukemia factor (*HLF*) gene.[17-19] The E2A-HLF fusion protein contains both E2A activation domains and the basic region and the leucine zipper domain of HLF, which are responsible for dimerization and DNA binding.[19]

A third *E2A* gene alteration is a cryptic inversion inv(19)(p13;q13), which results in an *E2A-FB1* fusion gene.[20] The function of FB-1 is as yet unknown, but analogous to the fusion proteins E2A-PBX1 and E2A-HLF, a role for E2A-FB1 in development and/or progression of leukemogenesis has been suggested.[20]

In a large study on precursor-B-ALL, the effect of various chromosome aberrations and the effect of age on Ig and TCR gene rearrangements was described and this study showed effects of the presence of *E2A-PBX1* fusions.[21] As E2A plays an important role during B-cell differentiation, and especially because of its role in regulation of V(D)J recombi-

nation, the aim of our study was to determine the effect of different *E2A* translocations on Ig and cross-lineage TCR gene configuration patterns in pre-B-ALL. For this purpose pre-B-ALL with and pre-B-ALL without a cytogenetically defined t(1;19) was selected. In addition, three t(1;19) positive cell lines and three t(17;19) positive cell lines were analyzed. The involvement of the *E2A* gene in these chromosome aberrations was confirmed via *E2A* split-signal FISH analysis and via RT-PCR analysis of *E2A-PBX1* fusion transcripts.

MATERIALS AND METHODS

Patient samples and cell lines

Twenty pre-B-ALL bone marrow or peripheral blood samples were collected. Cytogenetic data with respect to chromosome region 19p13 are summarized in Table 1. In addition, three pre-B-ALL cell lines with translocation t(1;19)(q23;p13) were used: RCH-ACV, SUP-B27, and 697.[22] Common-ALL cell lines HAL-01, UoC-B1, and YCUB-2 carrying a t(17;19)(q22;p13) were also included in this study.[22,23]

Table 1. Summarized data of 20 pre-B-ALL, regarding cytogenetics with respect to chromosome region 19p13, *E2A* split-signal FISH analysis, and the presence or absence of *E2A-PBX1* fusion transcripts.

Pre-B-ALL	Patient code	Cytogenetics with respect to 19p13	FISH analysis[a,b]	*E2A-PBX1* fusion transcripts
1	3162	-	nd	-
2	3559	-	rg/rg	-
3	3697	-	rg/rg	-
4	4526	-	nd	-
5	4976	-	nd	-
6	5189	-	nd	-
7	5529	-	nd	-
8	5565	-	nd	-
9	5582	-	nd	-
10	6198	t(1;19)(q23;p13)	rg/rg	-
11	3510	t(1;19)(q23;p13)	rg/r/g	+
12	4618	t(1;19)(q23;p13)	nd	+
13	4659	t(1;19)(q23;p13)	rg/r/g	+
14	5208	t(1;19)(q23;p13)	rg/r/g	+
15	5570	t(1;19)(q23;p13)	rg/r/g	+
16	5851	t(1;19)(q23;p13)	rg/r/g	+
17	5868	t(1;19)(q23;p13)	rg/r/g	+
18	6157	t(1;19)(q23;p13)	rg/g	+
19	6199	t(1;19)(q23;p13)	rg/g	+
20	6592	t(1;19)(q23;p13)	rg/g	+

a. Abbreviations: nd, not determined; r, red signal from *E2A* probe downstream of breakpoint region; g, green signal from *E2A* probe upstream of breakpoint region.

b. Described by *Boomer et al.*[24]

DNA extraction was performed using a phenol/chlorophorm extraction method. RNA was isolated with an RNA extraction kit (Sigma, St. Louis, MO, USA). In addition, methanol/acetic acid fixed cell suspensions were prepared for FISH analysis, if enough cell material was available.

Fluorescent in situ hybridization (FISH)

Dual colored FISH was performed with split-signal *E2A* FISH probes described by Boomer *et al.*[24] The *E2A* probes were labeled by nick-translation with either biotin-16-dUTP or digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany). For FISH analysis, methanol/acetic acid fixed cell suspensions were used. FISH was carried out as previously described.[25] The upstream *E2A* probe was biotinylated and detected with FITC, the downstream *E2A* probe was labeled with digoxigenin and detected with Texas Red/TRITC.

RT-PCR analysis of *E2A-PBX1* fusion transcripts

cDNA was prepared from RNA using Superscript RT enzyme (Life Technologies, Paisley, UK) according to the manufacturer's instructions. RT-PCR analysis was performed using BIOMED-1 Concerted Action primers to determine the presence of *E2A-PBX1* fusion transcripts.[26]

Southern blot analysis

Fifteen µg of DNA was digested with the appropriate restriction enzymes (Life Technologies, Rockville, MD, USA), separated in 0.7% agarose gels and transferred by vacuum blotting to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany).[27] *Bgl*II and *Bam*HI/*Hin*dIII digests were used for analysis of Ig gene rearrangements. For analysis of the *IGH* locus the IGHJ6 probe was used, analysis of the *IGK* locus was carried out with the IGKJ5, IGKC, and IGKDE probes, and the *IGL* locus was analyzed using the IGLJ2 and IGLC1D probes.[28-30] For analysis of TCR genes *Eco*RI and *Hin*dIII restriction enzyme digests were used in combination with TCRBJ1, TCRJB2, TCRG13, and TCRDJ1 to determine the configurations of the *TCRB, TCRG,* and *TCRD* loci, respectively.[31-33]

PCR analysis and sequence analysis of Ig and TCR gene rearrangements

The Ig and TCR gene rearrangements were further analyzed by PCR analysis according to protocols of the BIOMED-2 Concerted Action (Van Dongen *et al.*, manuscript in preparation). Resulting PCR products were subsequently analyzed via heteroduplex analysis or GeneScan analysis to determine whether the PCR products were derived from clonal rearrangements. Clonal PCR products were directly sequenced on an ABI 377 fluorescent cycle sequencer (Applied Biosystems, Foster City, CA, USA) with Dye Terminator mix or Big Dyes (Applied Biosystems) according to the manufacturer's instructions. V, (D), and J segments as well as the frame of the rearrangement were identified using DNAPLOT software (W. Müller, H-H. Althaus, University of Cologne, Germany) via VBASE and IMGT databases (http://imgt.cnusc.fr:8104).[34,35]

RT-PCR analysis of *E2A-PBX1* fusion transcripts

The wild type *E2A* alleles of the six cell lines were amplified using Expand™ Long Template PCR System (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. The resulting PCR products were subsequently sequenced as described above.

RESULTS

Detection of *E2A* abnormalities in pre-B-ALL patients

In nine pre-B-ALL cases no aberrations in chromosome region 19p13 were detected via conventional karyotyping (Table 1). In the remaining eleven cases a t(1;19)(q23;p13) was present.

To determine whether the t(1;19) in the pre-B-ALL cases involved *E2A* and *PBX1*, split-signal FISH analysis as well as RT-PCR analysis was performed. *E2A* split-signal FISH analysis showed that *E2A* was involved in ten of the eleven analyzed pre-B-ALL cases. In pre-B-ALL-10, only two fusion signals (red/green) were observed, representing two intact *E2A* alleles. RT-PCR confirmed that in pre-B-ALL-10 *E2A* is not involved in the t(1;19) as shown by absence of *E2A-PBX1* fusion transcripts. In 5-10% of cases with a cytogenetically detectable t(1;19) neither *E2A* nor *PBX1* is involved.[36]

Six pre-B-ALL cases (pre-B-ALL-11, 13, 14, 15, 16, 17) showed a fusion signal together with a separate green and red signal, representing the balanced form of the reciprocal translocation t(1;19). By RT-PCR, *E2A-PBX1* fusion signals were identified in these 6 pre-B-ALL, as well as in pre-B-ALL-12, which was not studied by FISH. In three pre-B-ALL (pre-B-ALL-18, 19, and 20) the unbalanced type of t(1;19) was identified based on the presence of one fusion signal together with a green signal, but without the red signal of the downstream part of the *E2A* gene. The unbalanced translocation t(1;19) might result from non-disjunction leading to loss of the der(1) and replacement with a second copy of the remaining normal chromosome 1.[37,38] In these three cases *E2A-PBX1* fusion transcripts were detected as well.

Comparison of Ig gene configuration patterns between *E2A-PBX1* positive and *E2A* negative pre-B-ALL

In all analyzed pre-B-ALL cases biallelic *IGH* gene rearrangements were observed. As can be expected from the presence of cytoplasmic Igμ expression in pre-B-ALL, in-frame rearrangements were found in seventeen of the twenty analyzed pre-B-ALL. In three pre-B-ALL without *E2A-PBX1*, the in-frame *IGH* could not be identified. There was no statistically significant difference in the V_H gene segment usage between pre-B-ALL with or without *E2A-PBX1* gene fusion. V_H gene segments of the V_H3 family were most frequently used.

However, differences were observed in the *IGK* gene configurations. In six of the ten pre-B-ALL without an *E2A* gene aberration, *IGK* rearrangements or deletions were found,

involving a total of seven of the 20 alleles, whereas in pre-B-ALL with *E2A* gene aberrations only two out of ten cases had monoallelic *IGK* gene rearrangements (2/20 alleles). In the first mentioned category, three rearranged alleles (Vκ-Jκ rearrangements) and four deleted alleles (rearrangements involving the kappa deleting element) were found. Monoallelic *IGL* gene rearrangements were detected in a single pre-B-ALL case with *E2A* gene aberration as well as in a single pre-B-ALL case without *E2A* gene aberration.

Comparison of TCR gene configuration patterns between *E2A-PBX1* positive and *E2A-PBX1* negative pre-B-ALL

Presence of cross-lineage TCR gene rearrangements is a common phenomenon in precursor B-ALL. From a large study on 59 pre-B-ALL it is known that cross lineage TCR gene rearrangements (involving the *TCRD, TCRG,* and *TCRB* locus) are present in 85% of cases (Figure 1).[39] In our small series of twenty pre-B-ALL cases, cross-lineage *TCR* gene rearrangements were present in 70% of cases without *E2A-PBX1*, whereas only 40% of pre-B-ALL with *E2A-PBX1* had cross-lineage *TCR* gene rearrangements.

If we focus in more detail on the three studied *TCR* loci, it becomes clear that the most prominent difference concerns the configuration of the *TCRG* loci (Figure 2). No *TCRG* gene rearrangements were observed in pre-B-ALL with *E2A-PBX1* in contrast to 35% of rearranged alleles in *E2A-PBX1* negative pre-B-ALL. Also in the *TCRD* gene configuration differences are observed in the frequencies of rearrangements and deletions (Figure 2). Seventy percent of *TCRD* alleles were rearranged or deleted in pre-B-ALL without an *E2A* gene aberration rearrangements, whereas only 25% of the *TCRD* alleles were rearranged or deleted in pre-B-ALL with an *E2A* gene aberration (Figure 1). In the *E2A-PBX1* negative pre-B-ALL, the frequency of *TCRD* rearrangements was 30% and the frequency of *TCRD* dele-

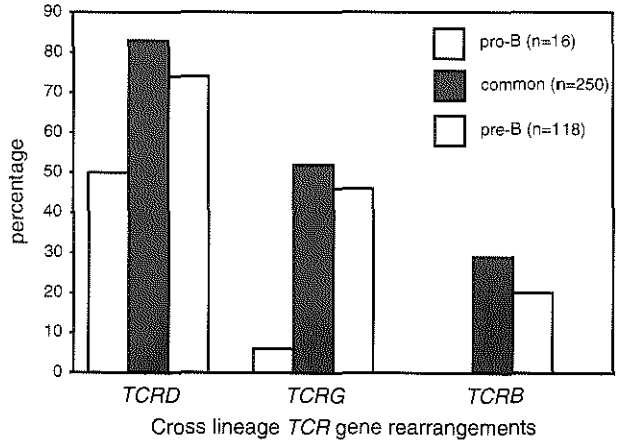

Figure 1. Frequencies of cross-lineage *TCRD, TCRG,* and *TCRB* gene rearrangements in pro-B-ALL, common ALL, and pre-B-ALL given in percentages of rearranged alleles as described by Szczepański *et al.*[39]

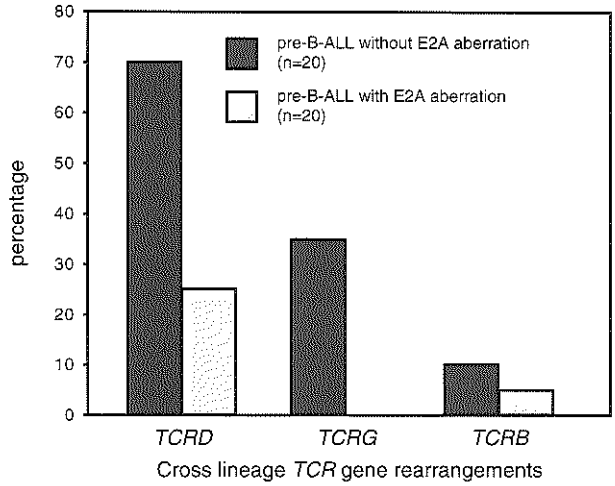

Figure 2. Frequencies of cross-lineage *TCRD, TCRG,* and *TCRB* gene rearrangements in *E2A-PBX1*-positive and *E2A-PBX1*-negative pre-B-ALL given in percentages of rearranged alleles.

tions was 40%. In the *E2A-PBX1*-positive pre-B-ALL, slightly more *TCRD* rearrangements than *TCRD* deletions were observed (15% and 10%, respectively). Sequence analysis revealed that in both categories *TCRD* gene rearrangements concerned Dδ2-Dδ3 couplings, and *TCRD* deletions were Vδ2-Jα couplings. No apparent differences were present in the rearrangements of the *TCRB* locus being rearranged in 5% and 10% of *E2A-PBX1*-positive and *E2A-PBX1*-negative subgroups, respectively (Figure 2).

Ig and TCR gene configuration patterns of *E2A-PBX1* and *E2A-HLF* positive cell lines

To investigate whether the type of *E2A* gene aberration influences the frequency or type of cross lineage *TCR* gene rearrangements, the patterns of three cell lines with an *E2A-PBX1* gene fusion were compared to the patterns of three cell lines with an *E2A-HLF* gene fusion (Table 2). Although the number of analyzed cases is rather low, it is remarkable that five of the six *TCRG* alleles in the cell lines with the *E2A-HLF* gene fusion are rearranged, while *TCRG* gene rearrangements are lacking in the *E2A-PBX1*-positive cell lines, as well as in primary pre-B-ALL cases with the *E2A-PBX1* gene fusion. In both types of cell lines *TCRD* rearrangements were present in high frequencies, whereas *TCRB* rearrangements were absent.

Table 2. Analysis of cross lineage *TCR* gene rearrangements of three cell lines with *E2A-PBX1* fusion and three cell lines with *E2A-HLF* gene fusion.

Cross lineage *TCR* gene rearrangements	*E2A-PBX1* (6 alleles)	*E2A-HLF* (6 alleles)
TCRD	6	6
TCRG	0	5
TCRB	0	0

Sequence analysis of E2A wild-type alleles

In addition to the oncogenic potential of *E2A* fusion proteins, reduction of wild type (wt) E2A function has also been shown to play a role in cells with *E2A* aberrations.[15] To determine whether reduced E2A activity only results from the *E2A* translocation and not from additional mutations in the unaffected *E2A* allele, full length cDNA of the wt *E2A* allele was analyzed of the six cell lines with *E2A* translocations. Cell lines were analyzed, because unlike pre-B-ALL, cell lines do not have a background of normal cells, which might hamper reliable analysis. Preliminary results show that there are no mutations in the wt *E2A* alleles, which might further contribute to oncogenic transformation or which would further reduce wt E2A activity.

DISCUSSION

E2A plays an important role during V(D)J recombination. In this study we focused on Ig and cross lineage TCR gene rearrangements in pre-B-ALL with *E2A-PBX1* fusions and compared the patterns to those of pre-B-ALL without *E2A-PBX1* fusions. The *IGH* gene configuration patterns were largely comparable, suggesting that the *E2A-PBX1* aberration and the subsequent reduced level of wild type E2A did not have a large impact on the *IGH* gene rearrangement process. The *E2A* aberration did however seem to influence the frequency of *IGK* gene rearrangements. In pre-B-ALL without *E2A-PBX1* the frequency of *IGK* gene rearrangements and/or deletions was higher (7/20 alleles) than in pre-B-ALL with *E2A-PBX1* (2/20 alleles). Although the number of analyzed cases is relatively low, these findings suggest a more dominant role of E2A in *IGK* gene rearrangements as compared to its role in *IGH* gene rearrangements.

As cross-lineage TCR gene rearrangements are a common phenomenon in precursor-B-ALL,[39] we analyzed the *TCRD, TCRG,* and *TCRB* gene configuration patterns in the two groups of pre-B-ALL. The total number of cross-lineage TCR gene rearrangements in pre-B-ALL with *E2A-PBX1* fusion was essentially lower, which was due to a reduced frequency of *TCRD* gene rearrangements and especially due to a complete absence of *TCRG* recombinations. These observations are in line with the study of Brumpt *et al.*[21] Interestingly, *E2A* is known to be important for proper regulation of *TCRG* and *TCRD*, as was shown in E2A$^{-/-}$ mice, which have a reduced ability to perform recombinations involving certain gene segments.[40] Similarly, using a non-lymphoid transfection model it was shown that *E2A* can induce endogenous *TCRG* and *TCRD* gene rearrangements when co-transfected with recombinase activating genes *RAG1* and *RAG2*.[10,11] Control of *TCRD* gene rearrangements was also suggested to be dependent on the level of *E2A* based on analysis of rearrangement patterns in *E2A*-deficient mice and *E2A*-heterozygous mice.[40] This parallels the reduced frequency of *TCRD* gene rearrangements as observed in *E2A-PBX1*-positive pre-B-ALL, which can also be regarded to be heterozygous for *E2A*, as sequence analysis of wt *E2A* alleles in *E2A-PBX1*-positive cell lines did not show mutations. So far, no effect of *E2A* on *TCRB*

recombination was shown,[10] which also fits with our current data where no clear differences in cross-lineage *TCRB* rearrangements were found.

To investigate whether the observed effect is dependent on the type of fusion partner, cross-lineage TCR gene rearrangements were also studied in cell lines with either an *E2A-PBX1* fusion or an *E2A-HLF* fusion. Surprisingly, the three cell lines with an *E2A-HLF* gene fusion all had rearrangements in the *TCRG* locus (5/6 alleles), whereas *TCRG* gene rearrangements were absent in *E2A-PBX1*-positive cell lines. This suggests that disruption of one *E2A* allele due to a translocation is not the only factor, which is responsible for the regulation of *TCRG* gene rearrangements. It should be noted that *E2A-HLF*-positive cell lines have a common ALL immunophenotype, which is more immature than the phenotype of *E2A-PBX1*-positive cell lines, which are of pre-B-ALL origin. However, the frequencies of cross-lineage TCR gene rearrangements in common ALL do not statistically differ from the frequencies in pre-B-ALL (Figure 1),[39] and therefore the differentiation stage of the cell lines could not explain the observed differences in *E2A-PBX1*-positive and *E2A-HLF*-positive cell lines.

In conclusion, high frequencies of cross-lineage TCR gene rearrangements are present in precursor-B-ALL, whereas the types of rearrangements, which are found are similar to those that can be induced in a non-lymphoid model system by E2A in the presence of RAG1 and RAG2.[10] The presence of E2A-PBX1 fusion proteins, resulting from t(1;19)(q23;p13) inhibit these cross-lineage rearrangements at the *TCRD* locus, and particularly at the *TCRG* locus. This can be explained by limited accessibility of these loci due to reduced levels of wild type E2A. However, remarkable differences in cross-lineage *TCR* gene rearrangements were observed between *E2A-PBX1*-positive cell lines and *E2A-HLF*-positive cell lines; in the latter high frequencies of *TCRG* rearrangements were found that were completely absent in the *E2A-PBX1*-positive cell lines. This suggests that E2A-induced accessibility of antigen receptor loci is influenced by the type of fusion partner in the involved *E2A* aberration. It might be that dimerization of E2A-PBX1 to wild-type E2A has a more dominant negative effect than dimerization of E2A-HLF to wild-type E2A. Alternatively, E2A-HLF or the reciprocal fusion protein HLF-E2A, if expressed at all, could somehow contribute to accessibility of TCR loci. In theory, E2A-HLF might not at all interfere with E2A-induced accessibility. To further investigate the observed differential effects of E2A-PBX1 and E2A-HLF, these fusion proteins should be cloned and tested together with wild-type E2A in the earlier mentioned non-lymphoid cell system. Analysis of the induced rearrangements should then give more insight in understanding of E2A-induced accessibility.

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Chapter 4.4

SPLIT-SIGNAL FISH FOR DETECTION OF CHROMOSOME TRANSLOCATIONS IN ACUTE LYMPHOBLASTIC LEUKEMIA

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INTRODUCTION

Chromosome translocations play an important role in hematological malignancies. In acute lymphoblastic leukemia (ALL), most of these aberrations concern balanced translocations involving genes that play key roles in the development and function of lymphoid cells, such as transcription factors, cell cycle regulators, and signal transduction molecules. Balanced translocations can result in fusion of two genes that encode leukemia-specific chimeric (fusion) proteins. The most frequent translocations in precursor-B-ALL are t(1;19)(q23;p13), involving *E2A* (*TCF3*) and *PBX1*, t(4;11)(q21;q23) with an *MLL-AF4* (*MLL-MLLT2*) fusion, t(12;21)(p13;q22) with a *TEL-AML1* (*ETV6-RUNX1*) fusion and t(9;22)(p13;q22) with fusion of *BCR* and *ABL*. In T-ALL, gene aberrations involving the *TAL1* gene are frequently observed. The most frequent *TAL1* gene aberration concerns a submicroscopic deletion del(1)(p32) involving the *SIL* and *TAL1* genes, but also *TAL1* gene translocations do occur in T-ALL.

Several clinical studies have demonstrated that chromosomal translocations are useful markers for risk group classification. With current treatment protocols, t(12;21) is correlated with a good prognosis, whereas t(9;22) and translocations involving 11q23 such as t(4;11) are especially correlated with a poor prognosis. The t(1;19) is generally correlated with a poor prognosis, although this can be overcome with more intensive chemotherapy. Split-signal FISH probes have been designed for five frequent translocations, thereby enabling rapid and reliable detection of translocations.

PROBE DESIGN FOR SPLIT-SIGNAL FISH

Aberrations involving the *E2A* gene (19p13.2-p13.3)

Three different types of *E2A* gene aberrations are found in precursor-B-ALL (Table 1). The t(1;19)(q23;p13) results in 90-95% of cases in fusion of the *E2A* gene to the *PBX1* gene, leading to expression of the chimeric E2A-PBX1 protein.[1-3] In the remaining cases, the *E2A* and *PBX1* genes are not involved and therefore this translocation is referred to as an *E2A-PBX1*-negative t(1;19). The t(1;19)(q23;p13) is found in approximately 25% of childhood pre-B-ALL and can occur as balanced or as unbalanced form, i.e. der(19)t(1;19) with loss of der(1).[4,5] The unbalanced type may arise by non-disjunction leading to loss of the der(1) and replacement with a second copy of the unaffected chromosome 1. Loss of

Table 1. Chromosome aberrations involving the *E2A* gene (19p13).

Chromosome aberration	Involved genes	Ref.
t(1;19)(q23;p13)	*E2A-PBX1*	1
t(17;19)(q22;p13)	*E2A-HLF*	12
inv(19)(p13;q13)	*E2A-FB1*	15

der(1) can arise during clonal evolution, as both types can be detected within one patient sample.[5-7] Translocation t(1;19) is generally correlated with a poor prognosis, which can be overcome with more intensive chemotherapy, except for cases with the balanced t(1;19).[8,9] The E2A-PBX1 fusion protein is able to transform cells by constitutive activation of genes, which are normally regulated by PBX1 or by other members of the PBX1 protein family. In addition, the leukemogenic effect of t(1;19)(q23;p13) might also result from a reduced level of wild-type E2A, which has recently been shown to have antiproliferative capacity in B-cell progenitors.[10,11]

In the second type of *E2A* gene aberration t(17;19)(q22;p13), *E2A* is fused to the transcription factor *HLF* (hepatic leukemia factor). This *E2A-HLF* fusion is found in ~1% of precursor-B-ALL, especially in a rare form of high risk pro-B-ALL in adolescents.[12-14] E2A-HLF proteins influence an evolutionary conserved anti-apoptotic pathway via activation of SLUG, which is the mammalian homologue of the cell death specification protein CES-1 in *C. elegans* (reviewed by Seidel *et al.*).[14]

The third *E2A* gene alteration concerns the cryptic inversion inv(19)(p13;q13), which results in the *E2A-FB-1* fusion gene.[15] The function of FB-1 is as yet unknown, but analogous to the fusion proteins E2A-PBX1 and E2A-HLF, a role for E2A-FB1 in development and/or progression of leukemogenesis has been suggested.[15]

Split-signal FISH analysis for aberrations involving the E2A gene

Most translocation breakpoints in the *E2A* gene occur in a 3.5 kb intron region between exon 13 and 14 (Figure 1).[2,16] Two probes were designed, which flank the breakpoint region without overlapping it (adapted from the probes described by Boomer *et al.*).[17]

In cases without a translocation, two colocalized signals will be present (Figure 2A and 3B). A balanced translocation involving the *E2A* gene results in split of one of the colocalized signals, giving rise to one separate green signal and one separate red signal, together with a fusion signal of the unaffected *E2A* gene (Figure 2C). An unbalanced t(1;19) involving *E2A* leads to the presence of a separate red signal of the der(19) and a fusion signal of the unaffected chromosome (Figure 2D). The separate green signal is absent due to loss of der(1).

This *E2A* probe set detects both types of translocations t(1;19) and t(17;19) and should theoretically also detect inv(19), although ALL samples samples bearing such aberrations have not yet been analyzed.

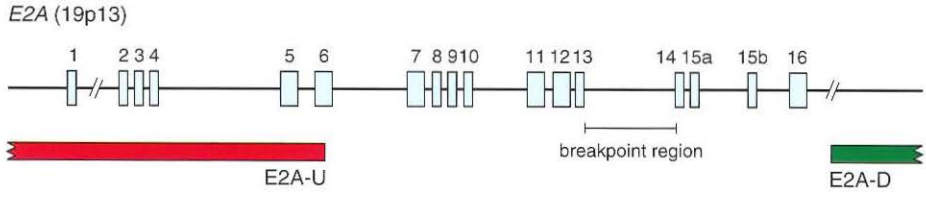

Figure 1. *E2A* gene (19p13) with the position of the breakpoint region and the positions of the E2A-U and E2A-D probes.

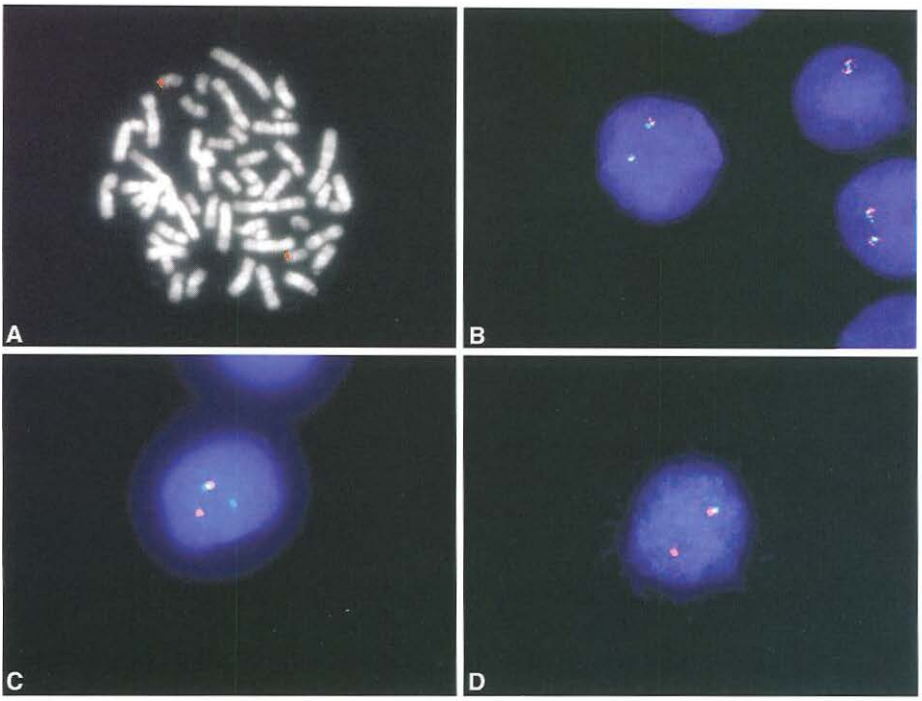

Figure 2. FISH analysis with E2A-U and E2A-D probes. **A.** Metaphase spread of a healthy donor. **B.** Interphase FISH analysis on material from an ALL without *E2A* gene aberration. **C.** ALL with balanced t(1;19)(q23;p13). **D.** ALL with unbalanced t(1;19)(q23;p13) with loss of der(1).

Translocations involving the *MLL* gene (11q23)

Chromosomal translocations involving the *MLL* gene (Mixed Lineage Leukemia) on chromosome band 11q23 are found in 70%-80% of infant acute lymphoblastic leukemia (ALL) and in 60% of infant acute myeloid leukemia (60%), whereas the frequency in pediatric and adult ALL is only 5%.[18-20] *MLL* gene aberrations are also frequently found in patients with secondary AML following therapy including topoisomerase II inhibitors.[21,22]

The *MLL* gene, also named *ALL-1*, *Htrx* and *HRX*, is the human homologue of the *Drosophila* trithorax gene.[23] The MLL protein contains a transcription repression domain, an activation domain and two types of DNA binding domains.[24] Absence of the *MLL* gene blocks hematopoietic differentiation *in vitro* and is therefore possibly important in leukemogenesis.[25] Translocations involving *MLL* disrupt the gene between the two types of DNA binding domains.[23,26] The 'AT-hook' motifs and the repression domain remain on the der (11), while the zinc fingers and the activation domain are either lost or translocated to the der(partner) chromosome.[24] In most translocations the fusion protein of the derivative 11 chromosome, with the 5′ part of the *MLL* gene fused to the 3′ part of the partner gene, contributes to the oncogenic process.[27,28]

More than 54 different partner chromosome regions have now been identified, of which 31 partner genes are known (Table 2).[29,30] The t(4;11)(q21;q23), t(11;19)(q23;p13.3), and t(9;11)(p23;q23) are the most common translocations in ALL, whereas t(6;11)(q27;q23), t(9;11)(p21-p22;q23), t(10;11)(p12;q23) and t(11;19)(q23;p13.1) are most frequent in AML.[31-36]

Table 2. Summary of identified *MLL* partner genes with chromosome region as reviewed by Huret, *et al.*[30]

Partner genes with chromosome region					
AFX1	Xp13	AF6q21	6q21	Not *AF15*	15q15
Septin2	Xq22	*AF6*	6q27	CBP	16p13
AF1P	1p32	*AF9*	9p23	*GAS7*	17p13
AF1q	1q21	*FBP17*	9q34	*AF17*	17q21
LAF4	2q11	*AF10*	10p12	*RARA*	17q21
AF3p21	3p21	*ABI1*	10p11	*MSF*	17q25
GMPS	3q25	*LARG*	11q23	*ELL*	19p13.1
AF4	4q21	*Gepherin*	14q24	*ENL*	19p13.3
GRAF	5q31	*AF15q14*	15q14	*HCD Crel*	21q11
AF5q31	5q31	*AF15*	15q15	*P300*	22q13

Split-signal FISH analysis for MLL gene translocations

The human gene consists of 37 exons and spans a region of approximately 100 kb (Figure 3).[37] The breaks are clustered within a 6.5 kb major break point region (mbr) located on an 8.3 kb *Bam*HI fragment (Figure 3). A FISH probe set was designed in such a way that the two probes flanked both sides of the *MLL* breakpoint region without overlap (Figure 3 and Chapter 4.2).[38]

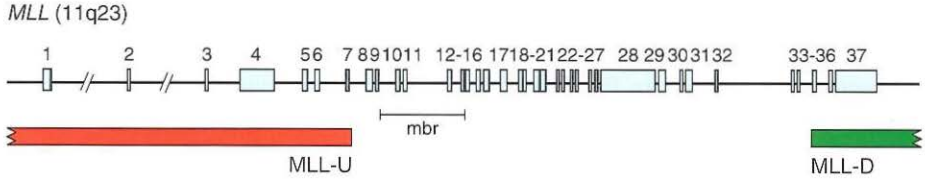

Figure 3. *MLL* gene (11q23) with the position of the major breakpoint region (mbr) and the positions of the MLL-U and MLL-D probes.

In normal cells without *MLL* gene translocations, two gresen/red (yellow) colocalized signals will be present (Figure 4A and 4B), whereas the presence of an *MLL* translocation results in a split-signal giving rise to separate green and red signals of the MLL-U and MLL-D probes, respectively (Figure 4C). In approximately 30% of cases with an *MLL* translocation a region telomeric from the mbr is lost, which results in loss of the MLL-D probe signal (Figure 4D).[38,39]

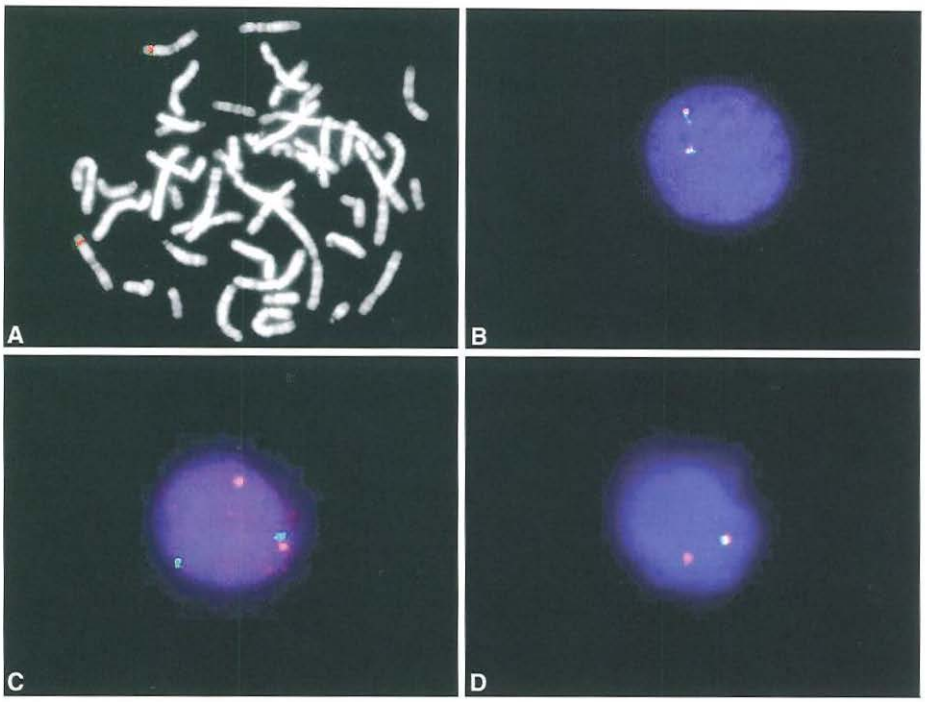

Figure 4. FISH analysis with MLL-U and MLL-D probes. **A.** Metaphase spread of a healthy donor. **B.** Interphase FISH analysis on material from an ALL without *MLL* gene aberration. **C.** ALL with *MLL* gene translocation. **D.** ALL with *MLL* gene translocation with loss of 3' part of *MLL* gene.

Translocations involving the *TEL* gene (12p13)

The *TEL* gene (*t*ranslocation, *e*ts, *l*eukemia), also known as *ETV6* (*ETS*-Variant Gene 6), is a member of the ETS family of transcription factors, which is characterized by a helix-turn-helix DNA-binding domain (ETS domain) with a transcriptional regulatory function. TEL also contains a HLH (PNT) domain that mediates homotypic oligomerization.[40,41]

Translocations involving the *TEL* gene are found in a broad range of leukemias, including ALL, acute myeloid leukemia (AML), chronic myelomonocytic leukemia (CMML), and myelodysplastic syndrome (MDS).[40,42] In the various types of leukemias, different partner genes are involved and it is noteworthy that in these translocations different exons and functional domains of the *TEL* gene are involved (Table 3).

The t(12;21)(p13;q22) involving the *AML1* gene is the most frequent translocation in childhood ALL. This translocation is present in ~ 25% of childhood precursor-B-ALL and is correlated with a favorable prognosis. The t(12;21) concerns a cryptic translocation that can not be detected via standard cytogenetics and that is frequently accompanied by aberrations in the 12p region of the non-translocated *TEL* allele (39% identified by cytogenetics).[43] The cytogenetically detectable 12p aberrations represent mainly deletions, dicentric and unbal-

Table 3. Summary of *TEL* translocations in different disease categories and position of the breakpoint area.

Chromosme translocations	Fusion transcript	*TEL* breakpoint area	Disease	Ref.
t(12;21)(p13;q22)	*TEL-AML1*	Intron 5	ALL	80,81
t(5;12)(q33;p13)	*TEL-PDGFRB*	Intron 4	CMML	82
t(9;12)(q34;p13)	*TEL-ABL*	Intron 4	ALL, AML, atypical CML	83
t(9;12)(p24;p13)	*TEL-JAK2*	Intron 4-5	ALL, atypical CML	84
t(12;22)(p13;q11)	*MN1-TEL*	Intron 2-3	MDS, AML	85
t(3;12)(q26;p13)	*TEL-ELI1*	Intron 2	MDS	86
t(4;12)(q11-q12;p13)	*BTL-TEL*	Intron 2	AML-L0	87
t(1;12)(q25;p13)	*TEL-ARG*	Intron 5	AML-M4	88
t(12;15)(p13;q25)	*TEL-TRKC*	Intron 4	AML	89
t(12;13)(p13;q12)	*TEL-CDX2*	Intron 2	AML	90

anced translocations. Loss of heterozygosity studies and FISH analyses report high frequencies of 72% of cases with loss of the wild type *TEL* gene.[43] Loss of the non-translocated *TEL* allele is assumed to contribute to the oncogenic process as wild type TEL proteins might interfere with the oncogenic properties of the TEL-containing fusion proteins.[44,45] These deletions are considered to be a secondary event.

Split-signal FISH analysis for TEL gene translocations

Our split-signal FISH *TEL* probe set was designed for detection of translocations in precursor-B-ALL with breakpoints in intron 4 or 5 (See Table 3 and Figure 5). Two *TEL* gene probes were designed flanking this breakpoint region.

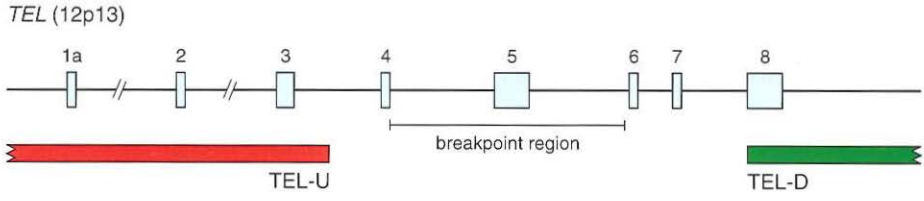

Figure 5. *TEL* gene (12p13) with the position of the breakpoint region and the positions of the TEL-U and TEL-D probes.

In normal cells two colocalized signals are present in the nucleus (Figure 6 A, B). In case of a translocation two separate signals will be visible (Figure 6C). Because of the frequent loss of the non-translocated *TEL* allele, the colocalized signal of this allele will be frequently absent (Figure 6D).

Translocations involving the *BCR* gene (22q11)

The Philadelphia (Ph) chromosome, which results from the reciprocal translocation t(9;22)(q34;q11) is the hallmark of chronic myeloid leukemia (CML), occurring in >95% of

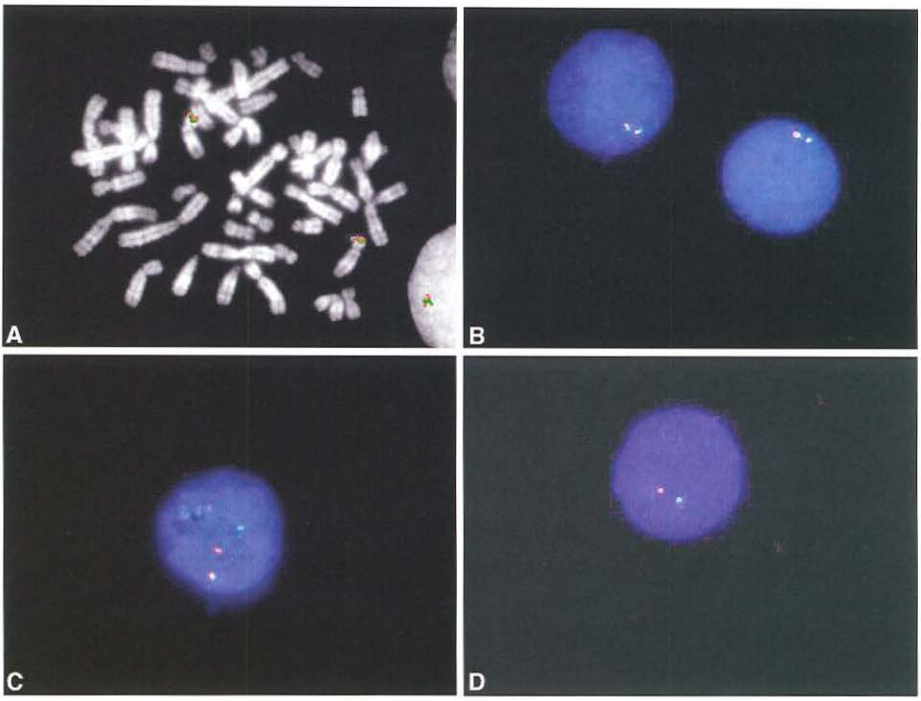

Figure 6. FISH analysis with TEL-U and TEL-D probes. **A.** Metaphase spread of a healthy donor. **B.** Interphase FISH analysis on material from an ALL without *TEL* gene aberration. **C.** ALL with t(12;21). **D.** ALL with t(12;21) with loss of second *TEL* allele.

CML cases.[46] This translocation is also found in 5% of childhood ALL and in 20-50% of adult ALL.[46-48] The translocation results in the joining of the 3' end of the *ABL* gene (9q34), creating the *BCR-ABL* fusion gene to the 5' part of the *BCR* gene (22q11). The *ABL* gene encodes a tyrosine kinase, which has an important role in signal transduction and regulation of cell growth. In the BCR-ABL fusion protein the N-terminal part of BCR interferes with the ABL regulatory domain making ABL constitutively active.[46]

The breakpoints in the *ABL* gene on chromosome 9 almost exclusively occur in a 200 kb breakpoint region upstream of exon a2. Breakpoints in the *BCR* gene are clustered in three breakpoint cluster regions (bcr). The breakpoints of CML cases occur almost exclusively in the major bcr (M-bcr) (Figure 7 and Table 4), which can lead to two types of mRNA molecules, i.e. b2a2 or b3a2, dependent on occurrence of the breakpoints in intron 13 or intron 14, respectively.[46,49,50] Both transcripts encode a BCR-ABL fusion protein of 210 kDa ($p210^{BCR-ABL}$). In Ph^+ ALL not only the M-bcr is involved, but breakpoints are also found in the minor bcr (m-bcr) between the two alternative exons and exon 2 (Figure 7 and Table 4).[46,51,52] The resulting e1a2 fusion transcript encodes the $p190^{BCR-ABL}$ fusion protein. Although the $p190^{BCR-ABL}$ fusion protein is mainly found in ALL, it is linked to 3% of atyp-

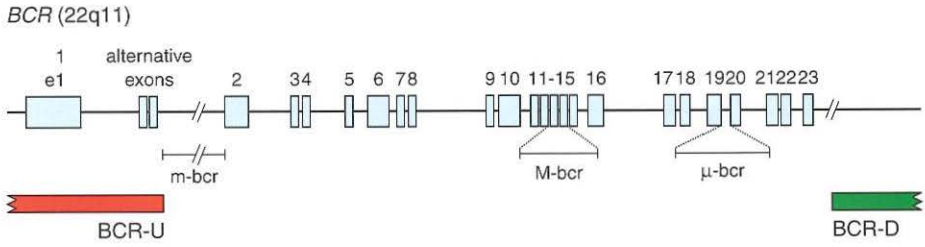

Figure 7. *BCR* gene (22q11) with the position of the minor, major and micro breakpoint cluster regions (m-bcr, M-bcr, μ-bcr) and the positions of the BCR-U and BCR-D probes with probe position.

ical CML cases.[53,54] A third bcr in the *BCR* gene located between exons 19 and 20 was identified in a small proportion of CML (Figure 7 and Table 4).[55] Breakpoints in this micro bcr (μ-bcr) result in the generation of a fusion transcript named e19a2 encoding a 230 kDa fusion protein ($p230^{BCR-ABL}$).[55]

Recently, it was suggested that large deletions adjacent to the breakpoint on the der(9) might be associated with a subgroup of CML patients with less favorable prognosis.[56] This finding could not be explained by lack of *ABL-BCR* expression, because absence of this expression did not correlate with deletions on the der(9) or with a shorter survival in CML patients.[57] So, the molecular basis of the negative effect of large deletions on prognosis has not been identified, but the survival disadvantage might be explained by loss of another gene within the deleted region.[57]

Table 4. Relative frequencies of chromosome aberrations involving different breakpoint regions of the *BCR* gene (22q11) in CML, childhood ALL, and adult ALL.

BCR breakpoint region	BCR-ABL fusion protein	CML	Childhood ALL	Adult ALL
m-bcr	p190	3%	3%	13-25%
M-bcr	p210	95%	1-2%	8-25%
μ-bcr	p230	rare	Not reported	Not reported

Split-signal FISH analysis for translocation involving BCR

Two large probes were developed located upstream of the m-bcr and downstream of the μ-bcr (Figure 7). This allows detection of *BCR* translocations involving any of the three breakpoint cluster regions, all giving rise to the presence of a separate green and red signal and one colocalized signal of the unaffected allele (Figure 8C) as compared to two fusion signals if no translocation is present (Figure 8A and B).

Aberrations involving the *TAL1* gene (1p32)

Up to 30% of T-cell acute lymphoblastic leukemias (T-ALL) have aberrations in the *TAL1* gene (T cell acute leukemia gene 1) in chromosome region 1p32, also called *SCL* or *TCL5* (Table 5).[58,59] These aberrations result in ectopic TAL1 protein expression. In another

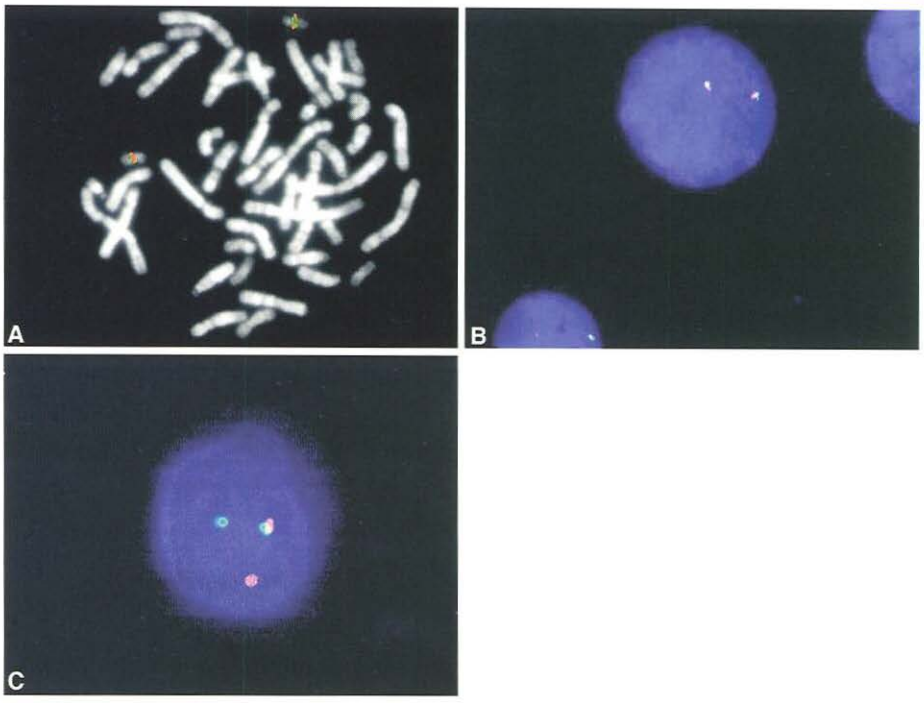

Figure 8. FISH analysis with BCR-U and BCR-D probes. **A.** Metaphase. **B.** Interphase FISH analysis on material from an ALL without *BCR* gene aberration. **C.** ALL with t(9;22).

group of T-ALL (~30%), TAL1 protein is also ectopically expressed in the blasts, but in these T-ALL no *TAL1* gene abnormalities have been found.[59,60] TAL1 is required for embryonic and adult hematopoiesis, as well as for terminal erythroid differentiation.[61-63] TAL1 belongs to the basic helix-loop-helix (bHLH) family of transcription factors and can form heterodimers with more widely expressed bHLH class I proteins referred to as E proteins.[64]

The majority of *TAL1* gene aberrations comprise intrachromosomal submicroscopic deletions of ~90 kb in region 1p32 with an incidence of 12-26% in childhood T-ALL.[65-67] This deletion removes all coding exons of the *SIL* (*SCL* interrupting locus) gene and the 5' untranslated region of the *TAL1* gene, placing the *TAL1* coding region under direct control of

Table 5. Chromosome aberrations involving the *TAL1* gene (1p32).

Chromosome aberration	Partner genes	Ref.
del(1)(p322)	*SIL* (*SIL-TAL1* gene fusion)	65
t(1;14)(p32;q11)	*TCRD*	73
t(1;7)(p32;p35)	*TCRB*	75
t(1;3)(p32;p21)	*TCTA*	58
t(1;5)(p32;q31)	?	77

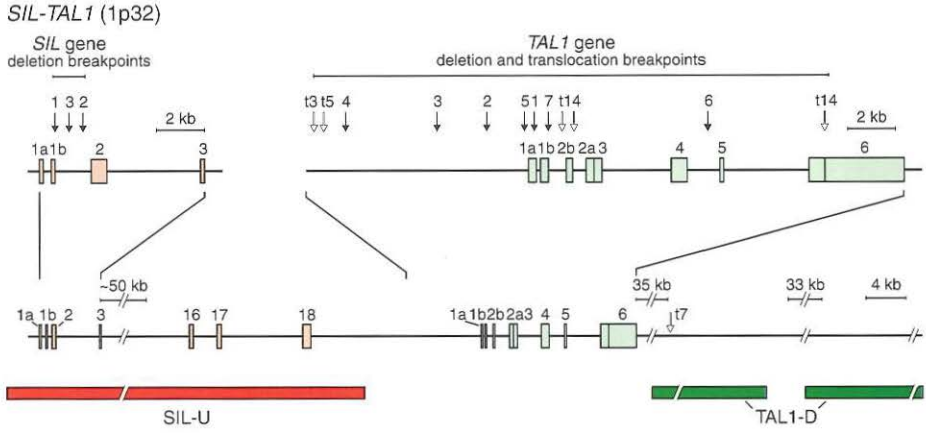

Figure 9. *SIL-TAL1* gene region with probe position.

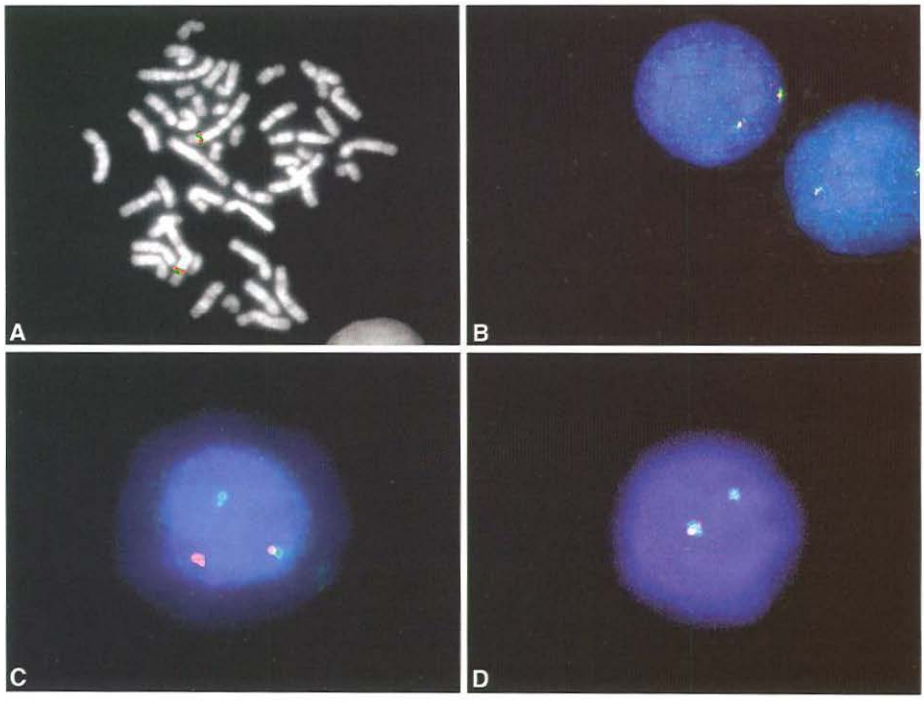

Figure 10. FISH analysis with SIL-U and TAL1-D probes. **A.** Metaphase. **B.** Interphase FISH analysis on material from a T-ALL without *TAL1* gene aberration. **C.** T-ALL with translocation. **D.** T-ALL with *SIL-TAL1* gene fusion.

the *SIL* promoter. As the *SIL* gene is ubiquitously expressed, this so-called *SIL-TAL1* fusion gene transcript results in ectopic TAL1 expression in T cells.[68] In the *SIL* gene three deletion breakpoints (sildb1 to 3) have been identified, of which sildb1 is most frequently used (95%) (Figure 9).[65,67,69] The *TAL1* gene contains seven deletion breakpoints (taldb1 to 7), with two being involved in 98% of cases (taldb1 and 2) (Figure 9).[48,66,67,69-72]

In 3% of T-ALL, ectopic TAL1 expression is caused by t(1;14)(p32;q11).[73] This translocation involves the T-cell receptor delta (*TCRD*) locus at chromosome 14q11, which replaces the non-coding 5' part of the *TAL1* gene.[74] As a result of t(1;14), the *TAL1* gene is controlled by the regulatory elements of the *TCRD* gene, resulting in ectopic TAL1 expression. Three additional rare *TAL1* translocations have been reported: t(1;7)(p32;p35) involving the *TCRB* locus, with a breakpoint 35 kb downstream of the *TAL1* coding sequences;[75] t(1;3)(p32;p21) involving the *TCTA* gene;[58,76] and t(1;5)(p32;q31) of which the exact partner gene has not yet been identified.[77] Like in t(1;14), the breakpoints in t(1;3)(p32;p21) and t(1;5)(p32;q31) are located in the 5' untranslated region of the *TAL1* gene. Although aberrations in the *TAL1* gene are the most common defect in T-ALL, no clear correlation was found between *TAL1* gene aberrations and clinical outcome in a large series of 182 children with newly diagnosed T-ALL.[72] Kikuchi *et al.*, however, suggested that the presence of *SIL-TAL1* fusion genes is correlated with a good prognosis.[78]

Split-signal FISH analysis for TAL1 gene aberrations

Two probes are required for the detection of both types of *TAL1* gene aberrations i.e. the *SIL-TAL1* fusion gene (microscopic deletion) and *TAL1* gene translocations in a single FISH test. The upstream FISH probe is positioned in the ~90 kb region that is deleted during a *SIL-TAL1* fusion, whereas the downstream probe is positioned downstream of the *TAL1* breakpoints (Figure 9).[79]

Two colocalized signals are present in normal situations without a *TAL1* aberration (Figure 10 A, B). A *TAL1* translocation will result in a split-signal (one red (R) and one green (G) signal) together with a colocalized signal (Figure 10C). In cases with *SIL-TAL1* fusion genes, the upstream probe will be lost giving rise to one separate green signal of the downstream probe and one colocalized (green/red) signal of the unaffected *SIL-TAL1* region (Figure 10D).

DISCUSSION AND CONCLUSION

Split-signal FISH probe sets have been designed for the five most frequent translocations in ALL. Each split-signal FISH probe set consists of two differentially labeled probes, which are located in the target gene flanking the breakpoint region. All five probe sets are directly labeled and work smoothly in combination with our newly developed PNA-blocking system, which allows combined blocking and hybridization in a single step. This single-step overnight hybridization procedure makes split-signal FISH an easy, rapid, and sensitive tool

for molecular cytogenetics.

The major advantages of the split-signal FISH approach over fusion-signal FISH is that translocations involving the target gene can be detected independent of the involved partner gene. This is especially of interest for the *MLL* gene, because already 54 partner chromosome regions have been described. The second advantage is absence of high levels of false-positivity due to coincidental colocalization, as observed via the classical fusion-signal FISH approach. On the other hand, one could argue that split-signal FISH can give rise to similar frequencies of false-negativity due to the same coincidental colocalization.

Here, we focused on chromosome translocations in ALL, but more probe sets might be developed according to this split-signal FISH approach for other translocations, which frequently occur in other disease categories, e.g. for translocations in mature B-cell malignancies, but also for translocations occuring in solid tumors.

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Chapter 5

GENERAL DISCUSSION

Recombination processes underlying the generation of antibody diversity and anitgen-specificity during human B-cell differentiation, form the central theme of this thesis. There are three main processes, which are involved in the generation of diversity and specificity, i.e. V(D)J recombination of Ig genes, somatic hypermutation (SHM), and class switch recombination (CSR), which is the recombination process that, determines the effector function of the Ig molecule via switching to various C domains. Oncogenic recombinations can be a direct consequence of aberrations during one of the physiological recombination processes, V(D)J recombination, SHM, and CSR, but might also occur through different processes, which are as yet not fully understood.

Analysis of gene segment usage in V(D)J rearrangements has shown that the position of a gene segment within the Ig locus and the composition of the RSS are main determinants for V(D)J recombination. We studied these phenomena in more detail for the *IGL* locus, i.e. heterogeneity of *IGL* rearrangements including usage of different J-Cλ isotypes and the two human *IGL* polymorphisms (Chapter 2). One of the human polymorphisms concerns an amplification resulting in the presence of additional functional J-Cλ gene regions, which seemed to influence the frequency of Igλ protein expression on peripheral B cells.

Proper regulation of recombination processes is crucial for normal B-cell differentiation. For example, strict regulation of V(D)J recombination is required to guarantee the generation of monospecific B cells. Allelic exclusion, i.e. expression of one *IGH* allele plus one *IGK* or *IGL* allele plays an important role in monospecific Ig expression. In our studies, B-cell malignancies were used as model system to study regulation of recombination and allelic exclusion processes during several stages of B-cell differentiation. These studies mainly focused on *IGK* and *IGL* gene recombination patterns, and the influence of SHM on these patterns (Chapter 3).

IGL LOCUS: POLYMORPHISMS AND REARRANGEMENT PATTERNS

In Chapter 2.2, two polymorphisms in the human *IGL* locus were unraveled. The first concerns the J-Cλ2 polymorphism $Ke^{+}Oz^{-}$ and the second is a polymorphic amplification in the Cλ2-Cλ3 region. As the $Ke^{+}Oz^{-}$ polymorphism is not involved in antigen recognition, but concerns an amino acid substitution in the constant region, it is questionable whether this polymorphism contributes to the generation of diversity or to repertoire development. The $Ke^{+}Oz^{-}$ polymorphism might theoretically influence the repertoire, if the polymorphism would result in selective pairing with a specific Ig heavy chain. However, there is no indication for such effect.

The second polymorphism, however, does have an effect on repertoire development. The presence of additional functional J-Cλ gene regions as a result of the polymorphic amplification gives rise to a higher relative frequency of $Ig\lambda^{+}$ B cells in the peripheral blood. This

is probably caused by the possibility of extra consecutive rearrangements in the *IGL* locus, resulting in a chance for a successful (functional) *IGL* rearrangement.

Southern blot analysis of *IGL* gene rearrangements in various types of B-cell malignancies is described in detail in Chapter 2.3. The data show that more than 99% of the *IGL* rearrangements in Igλ+ B-cell malignancies involve the J-Cλ1, J-Cλ2, and J-Cλ3 regions. The frequencies of J-Cλ usage in Igλ+ B-cell malignancies appeared to be similar to frequencies reported in normal Igλ+ B cells. Due to lack of essential nucleotides in the Jλ4 and Jλ5 RSS elements, no rearrangements can occur to these regions and consequently were not found. Although the RSS sequences of Jλ6 and Jλ7 segments contain all essential nucleotides, rearrangements to these segments were found to be rare. Rearrangements to the J-Cλ6 region, which only encodes a truncated Igλ protein, were even completely absent in Igλ+ B-cell malignancies. However, they were detected in 19% of precursor-B-ALL with *IGL* gene rearrangements, which do not express Ig molecules. Collectively, these data suggest that proximity to the Vλ gene segments plays a role in J-Cλ usage and thereby influences repertoire development. Based on the analysis of J-Cλ usage in B-cell malignancies a strategy for reliable identification via Southern blot analysis was developed, which was essential for further studies on allelic exclusion of Ig light chain genes, as described in Chapter 3.

REGULATION OF ALLELIC EXCLUSION OF IG LIGHT CHAIN GENES

Regulation of recombination processes is of utmost importance for normal B-cell differentiation. In our studies, B-cell malignancies were used as single-cell model system to study regulation of Ig gene recombination processes and allelic exclusion, during several stages of B-cell differentiation (Chapter 3). These studies were mainly based on analysis of *IGK* and *IGL* gene configuration patterns and on the influence of SHM on these patterns.

Value of B-cell malignancies as clonal 'single-cell' model system

The major advantage of B-cell malignancies as model system is the possibility of complete and exact analysis of all Ig loci (*IGH, IGK,* and *IGL*) by Southern blot analysis and sequencing with a low risk of missing rearrangements. Although, it cannot be completely ruled out that Ig recombination patterns are influenced by oncogenic processes, B-cell malignancies can nevertheless provide insight in Ig gene rearrangement processes as long as the specific features of the various types of B-cell malignancies are taken into consideration. One such important feature is whether malignant transformation occurred during or after the Ig gene recombination process.

An alternative approach to study Ig light chain gene configuration patterns would be single-cell PCR analysis of sorted B cells. The obvious advantage of single-cell analysis is that normal B cells can be studied. However, the disadvantage of the single-cell PCR approach is the risk of missing rearrangements due to false-negative PCR results, because it

will not be possible to achieve reliable PCR amplification of each Ig gene rearrangement of each B cell under study.

Precursor-B-ALL (pro-B-ALL, common ALL, and pre-B-ALL) were used as clonal model system for precursor-B-cells at three stages of differentiation (pro-B, pre-B-I, and pre-B-II). In precursor-B-ALL, malignant transformation occurs during the recombination process given the continuous presence of the recombinase enzymes and the occurrence of ongoing rearrangements. For this reason precursor-B-ALL constitutes a model system that can give insight in the recombination process itself, without any form of selection of the functionality of the rearrangement, which may be important for understanding how allelic exclusion is achieved.

Mantle cell lymphomas (MCL) were used as clonal model system for naive B cells without somatic mutations whereas chronic B-cell leukemias (CBL) were used as clonal model system for mature B cells. Malignant transformation in MCL and CBL is assumed to occur independent of Ig light chain gene recombination. Approximately half of CBL cases are derived from B cells that have not been selected by antigen (Ag) based on the absence of somatic mutations, whereas the other half carry somatic mutations.[1,2] MCL and CBL could give insight in how allelic exclusion of Ig light chain genes is regulated in naive and mature B cells. In addition, the influence of SHM on Ig gene configuration patterns can be evaluated by comparing of MCL and CBL cases.

Ordered Ig light chain gene recombination patterns

Analysis of Ig light chain gene configuration patterns in three types of B-cell malignancies, i.e. precursor-B-ALL, MCL, and CBL representing different stages of B-cell differentiation demonstrated a hierarchical order in Ig light chain gene rearrangements. For the Ig expressing malignancies (MCL and CBL) most of the Igκ$^+$ cases had both *IGL* alleles in germline configuration, whereas in almost all Igλ$^+$ cases at least one of the *IGK* alleles was deleted.

Six categories were defined to describe and visualize the degree of order of Ig light chain gene rearrangements. The six categories and the distribution of the three types of B-cell malignancies over these categories are depicted in Figure 1. Strict order of Ig light chain gene combinations would be reflected by categories A→B→E, i.e. *IGK* and *IGL* loci germline (A) → *IGK* rearranged, *IGL* germline (B) → *IGK* deleted (at least one allele), *IGL* rearranged (E). However, the Ig light chain gene recombination process is not fully strict, given the presence of cases belonging to the extra categories C, D, and F. Category C is exceptional, because cases in this category do not contain Vκ-Jκ or Vλ-Jλ rearrangements, but only *IGK* deletions. Therefore, no MCL or CBL cases, but only precursor-B-ALL cases, which do not express Ig light chains, are found in this category. Cases with *IGK* and *IGL* gene rearrangements, but without *IGK* deletions are ascribed to category D. The final category F does not fit in the ordered model of Ig light chain gene recombinations, because the leukemias in this category contain *IGL* rearrangements in the absence of *IGK* rearrangements or deletions; category F only contained precursor-B-ALL, no MCL or CBL.

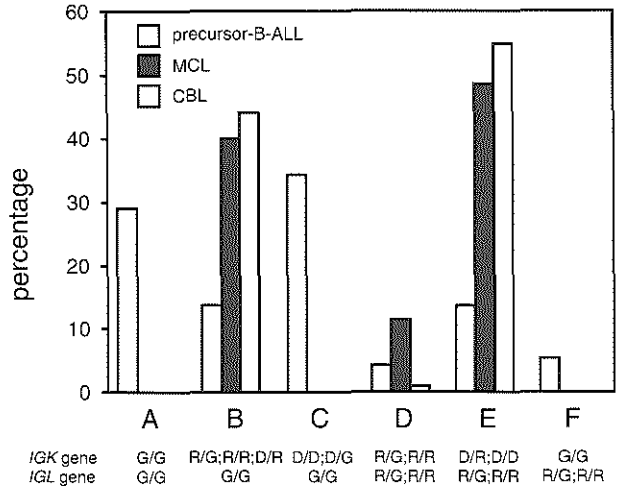

Figure 1. Six categories of *IGK* and *IGL* gene configurations and the distribution of the three types of B-cell malignancies (precursor-B-ALL, MCL, and CBL) over these categories.

Proposed model for regulation of allelic exclusion

The combined results of studies on regulation of allelic exclusion in various B-cell differentiation model systems confirm that Ig gene recombinations are ordered: *IGH* → *IGK* → *IGL*. It is known from literature that recombination is induced at one of the two alleles, and that this induction does not occur randomly. Recently, it has been shown for the murine *IGK* locus that asynchronous replication determines at which allele the recombination process is initiated.[3] *IGL* gene rearrangements are generally preceded by deletions at the *IGK* locus, given the observation that in most, if not all, cases with *IGL* gene rearrangements, *IGK* deletions are observed. A remaining question is whether *IGK* deletions are actively involved in the induction of *IGL* gene rearrangements, or whether other regulating mechanisms induce the *IGK* deletion and *IGL* recombination to occur consecutively or even simultaneously. Until now, there is no firm proof for an active role of *IGK* deletions in the induction of *IGL* gene rearrangements.

The proposed model for allelic exclusion is depicted in Figure 2A. Two major mechanisms seem to determine the regulation of Ig rearrangements and allelic exclusion. First of all, initiation of V(D)J recombination requires accessibility of Ig loci. Transcription factors, which are differently regulated during B-cell differentiation, play an important role in the induction of Ig gene accessibility and might probably even determine the order of accessibility of the three Ig loci. The second prerequisite for V(D)J recombination is the activity of the recombinase machinery itself, with the RAG1 and RAG2 proteins as key players. RAG expression is tightly regulated during B-cell differentiation. Part of this regulation comes from Ig protein expression. As soon as a functional *IGH* rearrangement results in pre-BCR

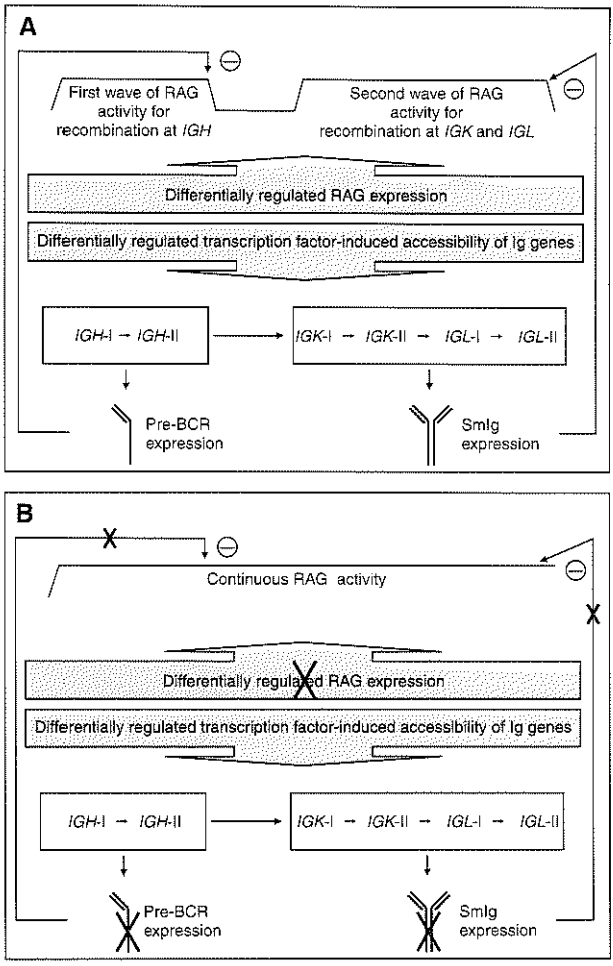

Figure 2A. Proposed model for allelic exclusion. For description, see text. **B.** Regulation of allelic exclusion in precursor-B-ALL with a deregulated differentiation. For description, see text.

expression, the *RAG* genes are down-regulated. Likewise, expression of a complete Ig molecule (BCR) is involved in down-regulation of the second wave of RAG expression. In this model multiple rearrangements can still occur at each allele, until a functional rearrangement results in protein expression. In conclusion, this model for regulation of allelic exclusion is based on two components, i.e. regulation of *RAG* gene expression and transcription-factor-induced accessibility of Ig genes, which are both influenced by the stage of B-cell differentiation. The model in Figure 2A is limited to expression of a complete Ig molecule on an immature B cell, and therefore does not include regulation of subsequent receptor editing of autoreactive (precursor-) B cells.[4]

Induction of allelic exclusion in a precursor-B-cell model

As previously discussed, precursor-B-ALL can be used as model system to study Ig recombination processes without selection of the functionality of the rearrangement, because precursor-B-ALL are characterized by continuous presence of recombinase activity and by a block in differentiation prohibiting expression of Ig light chains. *IGH* genes are only expressed in pre-B-ALL, which form the most mature subgroup of precursor-B-ALL. Analysis of Ig gene configuration patterns revealed two main observations: (a) induction of Ig light chain gene rearrangements seems not to require the presence and expression of a functional *IGH* gene rearrangement, and (b) Ig light chain gene recombination occurs in an hierarchical order, starting with *IGK* and followed by *IGL* rearrangements. These observations suggest that the hierarchical rearrangement process is independent of Ig protein expression. It is therefore tempting to speculate that Ig protein expression is not directly involved in regulation of accessibility of the Ig loci, but rather in regulation of the V(D)J recombinase enzyme system, i.e. expression of *RAG1* and *RAG2* (Figure 2B).

This illustrates once more that regulation of allelic exclusion concerns two different components, i.e. accessibility of Ig loci and recombinase activity. This is further confirmed by transfection studies in non-lymphoid cells, in which different transcription factors open different endogenous antigen receptor loci whereas RAG activity mediates the actual recombination process.[5-7]

Regulation and maintenance of monospecific Ig light chain expression in a mature B-cell model

Monospecific Ig expression can theoretically be regulated at three different levels, i.e. at the DNA, RNA, and protein level. One of the aims of the large CBL study was to determine which of the three levels is most prominant to regulate monospecific Ig light chain expression (see Chapter 3.2).

Regulation at the DNA level appeared to be responsible for monospecific Ig light chain gene expression in 90% of CBL, as demonstrated by the presence of only one functional allele. In addition, the Ig light chain recombination patterns showed an hierarchical order. These two combined findings suggest that the ordered recombination process is the main determinant for monospecific Ig expression.

A second level of regulation of monospecificity would be transcription of only one functional allele in cases with more than one functional allele. This type of regulation does not seem to play a role, because both in-frame and out-of-frame rearrangements appeared to be transcribed in the subset of CBL that was studied for this aspect (see Table 6 in Chapter 3.2). Regulation at the (post-)translational level is therefore more likely.

Assembly of one Ig heavy chain with one Ig light chain would be the third level of regulation. It might well be that preferential assembly of Ig heavy and Ig light chains contributes to the overall regulation of monospecific Ig light chain expression. A cell can theoretically express two Ig light chains in the cytoplasm, but if only one of them can assemble with the Ig heavy chain, monospecificity is still guaranteed. Unfortunately, CBL were not the

optimal model system to study this phenomenon in more detail, because the level of cytoplasmic Ig expression in CBL is too low to be reliably detected via flow cytometry or via cytocentrifuge preparations.

Is ordered recombination at the *IGL* locus less strict than at the *IGK* locus?

Remarkable observations came from detailed analysis of CBL with more than one Ig light chain gene rearrangement as detected via Southern blot analysis (Table 1). Six CBL showed biallelic *IGK* rearrangements, nine showed *IGK* as well as *IGL* rearrangements, and another eleven cases showed biallelic *IGL* gene rearrangements. In the six CBL with biallelic *IGK* rearrangements, only one *IGK* allele appeared to be functional. However, when *IGL* rearrangements were involved (either *IGK* and *IGL* or biallelic *IGL*), 50% of cases appeared to carry two in-frame rearrangements (Table 1). Furthermore, the frequency of somatic mutations was higher in cases with biallelic *IGL* rearrangements (5/5) as compared to cases with *IGK* and *IGL* rearrangements (3/5). Consequently, the occurrence of somatic mutations in CBL with two in-frame Ig light chain gene rearrangements (8/10) was higher than in the total group of CBL (~55%).[1] These data suggest that the presence of somatic mutations might affect the Ig light gene configuration patterns via induction of secondary rearrangements, i.e. receptor revision. Somatic mutations might then theoretically result in skewing towards *IGL* gene rearrangements. However, the existence of receptor revision is still highly disputable, which is discussed in more detail in the next section of this General Discussion.

Table 1. Characteristics of 26 CBL from a series of 113 CBL with two light chain gene rearrangements.

	IGK/IGK	*IGK/IGL*	*IGL/IGL*
CBL cases with 2 rearrangements[a]	6	9	11
Both in-frame	0	5 SHM in 3/5	5 SHM in 5/5

a. Rearrangements are here defined as Vκ-Jκ rearrangements, which is not followed by a IntronRSS-Kde rearrangement on the same allele, and Vλ-Jλ rearrangements.

Unfortunately, we could not determine whether all in-frame rearrangements are expressed as protein in the cytoplasm due to the low expression levels of Ig moleculres in CBL and whether preferential assembly of Ig heavy and Ig light chains plays a role. Another limitation is that no discrimination could be made between two different Igλ proteins on the membrane, due to lack of Vλ family-specific antibodies.

Our data indicate that regulation of ordered recombination is more strict at the *IGK* locus than at the *IGL* locus, particularly because of the presence of more in-frame rearrangements with somatic mutations, if the *IGL* locus is involved in the recombination process. Differential demethylation of the two *IGK* alleles have been shown to establish allelic exclusion at the *IGK* locus.[8] Similar studies might be performed at the *IGL* locus to determine whether or not allelic exclusion of the *IGL* locus is regulated in a similar way.

However, an alternative explanation for this observation might be that the *IGL* locus

does not have a deleting mechanism similar to the Kde mechanism. This might explain the high frequency of biallelic in-frame Vλ-Jλ rearrangements, whereas in case of similar biallelic in-frame Vκ-Jκ rearrangements, one Vκ-Jκ allele would have been deleted.

EVIDENCE FOR OR AGAINST RECEPTOR REVISION?

Influence of somatic mutations on Ig light chain gene rearrangement patterns

The process of V(D)J recombination occurs during antigen-independent B-cell differentiation in the bone marrow. Later on, during the SHM process in the antigen-dependent phase, mutations are introduced in the variable regions of the V(D)J rearrangements. If such mutations do not affect the encoding amino acid, they are called 'silent'. Alternatively, mutations can result in an amino acid substitution and the mutation will be referred to as a 'replacement' mutation. SHM is followed by selection of Ig molecules with higher antigen affinity due to favorable mutations. This SHM and selection process in the germinal center of the peripheral lymphoid organs, are the two components of affinity maturation. One or more cycles of germinal center reactions are required for the generation of high affinity antibodies.

So far, we analyzed CBL as model system for mature B cells, but they are only partly somatically mutated. Multiple myelomas (MM), which are the malignant counter part of the end stage of B-cell differentiation might be a more informative model system in this respect, because MM contain the highest frequency of somatic mutations. Therefore, MM might be the model system *par excellence* to study the influence of somatic mutations on Ig light chain gene rearrangements. Such studies are ongoing in our group

Effect of 'unfavorable' somatic mutations

SHM can also result in unfavorable changes, causing a lower affinity of the Ig molecule. SHM can theoretically even result in loss of assembly of Ig heavy and Ig light chains due to amino acid substitutions or in complete loss of Ig expression, if the mutation leads to the generation of a stop codon. If unfavorable somatic mutations occur, the B cell is thought to die via apoptosis. However, it was also postulated that B cells that gained 'unfavorable' somatic mutations might be rescued by the induction of secondary rearrangements, i.e. receptor revision. Receptor revision would require re-expression of the *RAG* genes in the periphery. During recent years, the phenomenon of receptor revision was highly under debate (see also Chapter 3.1). The main discussion topic in this respect is whether the re-circulating RAG-positive B cells represent Ag selected B cells in which *RAG* expression is re-induced or whether the cells are derived from the B-cell compartment in the bone marrow, and did not yet down-regulate expression of the *RAG* genes.

Our approach to address this question was to analyze Ig rearrangement patterns in MM. MM arise from plasma cells that are regarded as cells at the end-stage of B-cell differentiation. These cells contain the highest number of somatic mutations and should therefore

have the highest chance for SHM-induced receptor revision, if this process exists at all. For this purpose, two types of MM were studied, i.e. $IgM^{-}IgD^{+}Ig\lambda^{+}$ MM and MM with CyIgκ/CyIgλ double expression that only secrete IgGλ.

IgD MM with high level of somatic mutations and biased Igλ expression

Within MM, IgD positive MM represent a special subtype (only 1% of MM), characterized by an extremely high level of somatic mutations (higher than observed in other MM) and biased Igλ expression. Due to the high frequency of somatic mutations unfavorable somatic mutations are likely to occur and might result in induction of peripheral receptor revision.

Indications for receptor revision in IgDλ MM would have been provided by the presence of in-frame Vκ-Jκ rearrangements with somatic mutations that rendered the *IGK* rearrangement inactive by either the presence of a stop codon or through a rearrangement of IntronRSS to Kde. Alternatively, the mutated in-frame Vκ-Jκ rearrangement might not be expressed, because the expressed Igκ protein is not able (anymore) to assemble with the mutated Ig heavy chain. Both situations would have explained a skewing towards *IGL*, which is observed on IgD cells.

We analyzed four IgDλ MM in detail, but no indications were found for receptor revision (Chapter 3.5). The Vκ-Jκ rearrangements that were identified were all out-of-frame and did not contain somatic mutations. Theoretically, it might still be that the number of analyzed MM was too low to find evidence for receptor revision, but in a recent study on normal B cells by Goossens *et al.* similar observations were reported.[9] These two studies make the existence of receptor revision less likely.

MM showing cytoplasmic Igκ and Igλ expression, but secreting only IgGλ

A second approach to find evidence for or against receptor revision might come from an ongoing analysis of two MM with cytoplasmic Igκ *and* Igλ expression, but which only secrete IgGλ. This type of MM forms a minor part of the total group of MM and only two of such cases were identified in our laboratory during the past twenty years. First, some characteristics of the Ig protein expression and of Ig gene configurations and of these two MM (MM-1 and MM-2) will be discussed.

Characteristics of Ig gene configurations and Ig protein expression

Figure 3 shows that in bone marrow cells of MM-1 both cytoplasmic Igκ and Igλ expression was present. In the serum of both patients only IgGλ was identified. This finding was supported by ELISA on protein extracts of MM-2 showing that IgG was bound to Igλ, but not to Igκ (In collaboration with Richard Bende, Dept. of Pathology, AMC, Amsterdam, The Netherlands).

The Ig gene configurations of both MM were determined by Southern blot analysis followed by PCR and sequence analysis. The combined data are summarized in Table 2. Both MM had one *IGH* gene rearrangement, one out-of-frame and one in-frame *IGK* rearrange-

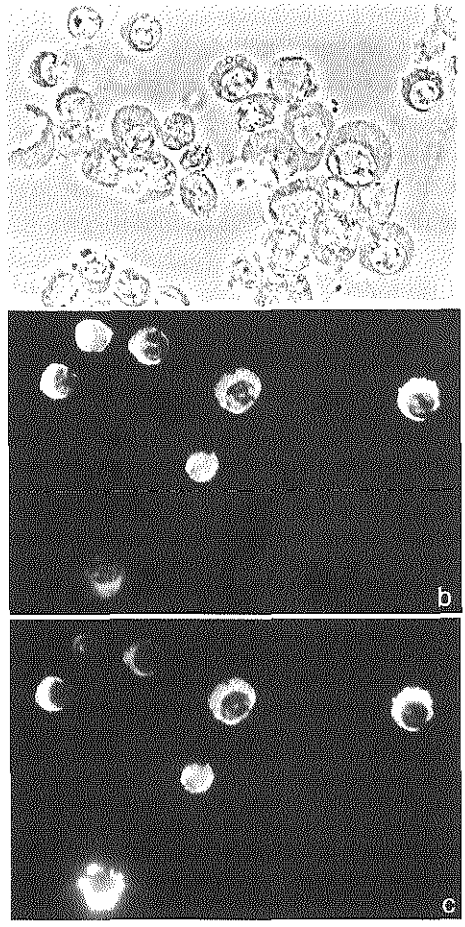

Figure 3. Double Ig light chain expression as determined by double immunofluorescence staining using fluorescence microscopy. **A.** Phase contrast morphology of MM-1, **B.** Igκ-positive cells (TRITC labeled, and **C.** Igλ-positive cells (FITC labeled).

ment, and one in-frame *IGL* gene rearrangement. All in-frame Ig gene rearrangements contained somatic mutations.

Receptor revision or receptor editing?

The results of the Ig gene configurations and Ig protein expression in our two CyIgκ/CyIgλ positive MM might be explained by two models: receptor editing (Figure 4A) or SHM-induced receptor revision (Figure 4B).

If SHM-induced receptor revision would have occurred, the following events would have taken place: during early B-cell differentiation in the bone marrow an in-frame *IGH* gene rearrangement resulted in CyIgμ expression and subsequent induction of Ig light chain

Table 2. Ig gene configurations of two IgDλ multiple myelomas (MM-1 and MM-2) as determined by Southern blot analysis, PCR and sequencing.

MM	Ig locus	SB	PCR and sequencing	Frame	Somatic mutations
1	*IGH*	R/G	V_H4-59/D_H4-11/J_H4	In	+
	IGK	R/R	Vκ3-11/Jκ4	In	+
			Vκ4-1/Jκ2	Out	-
	IGL	R/G	Vλ2-14/Jλ3	In	+
2	*IGH*	R/G	V_H3-21/D_H6-6/J_H4	In	+
	IGK	R/R	Vκ1-39/Jκ4	In	+
			Vκ3-11/Jκ5	Out	-
	IGL	R/G	Vλ3-21/Jλ3	In	+

gene recombination; the Ig light chain gene recombination process started at the *IGK* locus; no functional *IGK* gene rearrangement was generated at the first allele, but rearrangement of the second allele resulted in a functional rearrangement; the IgMκ positive B cell left the bone marrow and entered the periphery; after antigen encounter, a germinal centre reaction was initiated and somatic mutations were introduced in the V regions; in addition, CSR to IgG occurred; unfavorable somatic mutations in either *IGH* or *IGK* resulted in the loss of assembly of the Ig heavy chain and the Igκ light chain; in response to this loss of assembly, the recombinase machinery was re-induced, leading to an in-frame *IGL* gene rearrangement with Igλ protein expression; the mutated IgG chain was able to bind to the Igλ chain, which also gained somatic mutations, most probably during subsequent rounds of Ag selection, whereas Igκ expression was not lost due to inactivation by rearrangements to Kde; the IgGλ positive B cell finally further differentiated into an IgGλ secreting plasma cell.

Receptor editing in the bone marrow compartment is an alternative model to explain the results of these two MM (Figure 4A). Receptor editing is the occurrence of secondary rearrangements taking place in the bone marrow. If receptor editing would have played a dominant role, the order of recombination events would have been as follows: in CyIgμ positive pre-B-cells, Ig light chain gene recombinations were initiated in the bone marrow at the *IGK* locus; on one allele an out-of-frame Vκ-Jκ rearrangement occurred, and on the other allele an in-frame Vκ-Jκ rearrangement occurred; the expressed Igκ chain could not assemble with the CyIgμ chain; the Ig light chain gene recombination process continued at the *IGL* locus, without prior inactivation of the *IGK* alleles via rearrangement to Kde; the IgMλ positive B cell entered the periphery, was selected by antigen and somatic mutations were induced in all transcribed Ig alleles, including the in-frame *IGK* allele; after CSR to IgG the cell further differentiated into an IgGλ secreting plasma cell, but continued to express CyIgκ proteins.

Experimental approach

To distinguish between the two models, we are currently in the process of cloning all Ig rearrangements of the three Ig loci. Not only the rearrangements with the somatic mutations as they are found in the two MM, but also the Ig rearrangements without the somatic

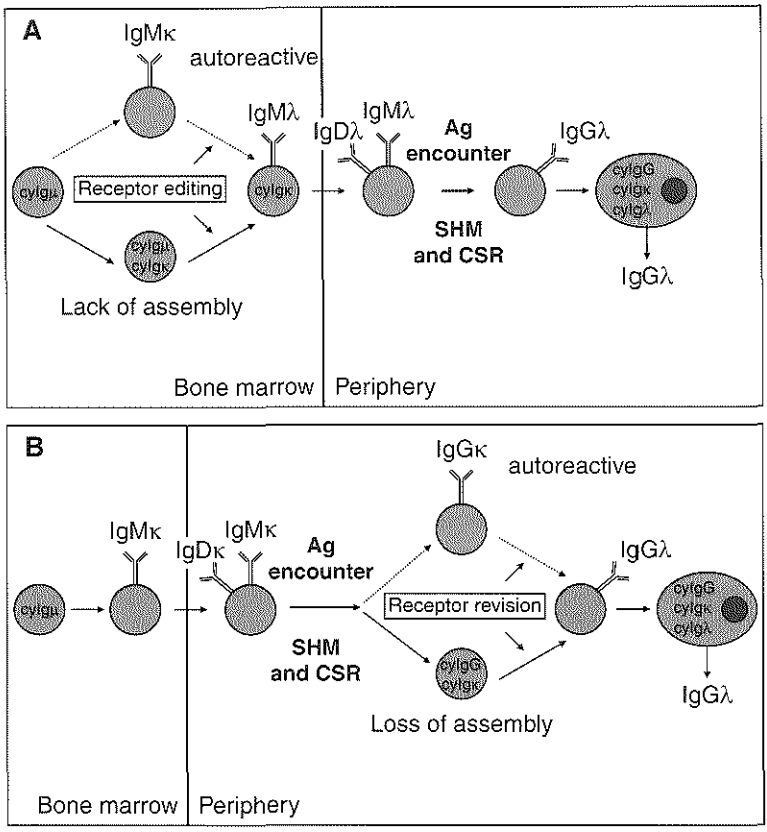

Figure 4. Two models explaining the observations in MM-1 and MM-2 regarding Ig gene configuration and expression patterns. **A.** Receptor editing model. **B.** Receptor revision model.

mutations, hereafter referred to as wild-type (wt) rearrangements, will be studied. The Ig rearrangements will be cloned into eukaryotic expression vectors and transfected in Ig-negative plasma cells in different combinations. ELISA will be used as read-out system to determine whether the transfected Ig heavy and light chains can assemble. The mutated Ig heavy chain should assemble with the mutated Igλ chain, but not with the mutated Igκ chain, which would be in line with the *in vivo* situation and is therefore the validation of the procedure (Table 3).

Table 3 also summarizes the most informative transfections of the unmutated (wt) Ig heavy chain with unmutated (wt) Ig light chains and their potential results. If the wt Ig heavy chain and the wt Igκ assemble, this is suggestive for receptor revision in the periphery, but if these chains do not assemble, receptor editing in the bone marrow is more likely to have occurred.

Transfections with wt IgH and wt Igλ chains alone are less informative. If the wt IgH

Table 3. Informative transfections of Ig heavy and Ig light chain with respect to receptor editing and receptor revision based on potential results of assembly.

Transfection	Potential assembly	Interpretation
Mutated IgH + mutated Igκ	-	Expected results to validate that approach reflects
Mutated IgH + mutated Igλ	+	*in vivo* situation in MM
wt IgH + wt Igκ	+	Suggestive for receptor revision
	-	Suggestive for receptor editing
wt IgH + wt Igλ	+	In combination with wt IgH and wt Igκ (-), suggestive for receptor editing
	-	Unlikely to be found, because somatic mutations would then induce assembly of Igλ with IgH
Mutated IgH + wt Igλ	+	In combination with mutated IgH and mutated Igκ (-) *and* wt IgH and wt Igκ (+), suggestive for receptor revision
	-	Unlikely to be found, because somatic mutations in Igλ would then induce assembly with IgH

and wt Igλ appear to assemble and in addition the wt IgH and wt Igκ appear not to assemble, this would collectively be suggestive for receptor editing. However, if the wt IgH and wt Igκ can assemble, the transfection of wt IgH with wt Igλ would not give additional information. It is nevertheless unlikely that the wt IgH does not assemble with wt Igλ, because this would imply that IgH and the Igλ chains can only assemble *after* somatic mutations.

As third combination, the mutated IgH and wt Igλ can be transfected. Similar to the wt IgH and wt Igλ transfection, absence of assembly is unlikely to be found, because somatic mutations in the Igλ chain are required for assembly with the mutated IgH. However, if these chains indeed appear to assemble and in addition the mutated IgH and mutated Igκ do not assemble and the wt IgH and wt Igκ can assemble, these three transfections are strongly suggestive that receptor revision has occurred.

If these transfection studies indicate that receptor editing appears to be the model for explaining the Ig gene configuration and expression in the two MM cases, this still does not completely rule out that receptor revision can occur, but makes it less likely. If receptor revision appears to be more likely, it suggests that this phenomenon can occur. In conclusion, these experiments will be highly informative, irrespective of the outcome.

Role of auto-reactive B cells in receptor editing and revision

Auto-reactivity (indicated via dashed lines in Figure 4A and 4B) was not discussed, although it is an important issue of B-cell differentiation. The fate of these B cells depends on the type and dose of the antigen, the degree of antigen receptor engagement and T cell help.[18] Auto-reactive B cells, which are generated in the bone marrow, might theoretically be rescued via receptor editing. However, in the situation of these two CyIgκ$^+$/CyIgλ$^+$ MM

autoreactivity is not likely to play a role in the receptor editing model, because if the IgMκ molecule would have been autoreactive, the encoding *IGK* allele would probably have been deleted to prevent further expression. Alternatively, the newly formed Igλ chain must have had a much better fit with the IgH chain resulting in preferentially assembly. This phenomenon might be tested by competition transfection experiments with wt IgH, wt Igκ and wt Igλ.

In the periphery, SHM can render B cells autoreactive. These B cells might be deleted via apoptosis or rescued via receptor revision. Autoreactivity-induced receptor revision is unlikely to have occurred in the here presented MM cases for the same reasons as were mentioned in the context of the receptor editing model.

Concluding remarks and future direction

In conclusion, until now there is no strong indication for the occurrence of receptor revision. Moreover, more studies fail to show evidence for receptor revision in B-cell populations that would theoretically be prone to undergo it. However, it still remains of interest to further investigate this phenomenon, for example in the two MM samples. If receptor revision appears to exist, its contribution to antibody diversity will be limited, but it might still contribute to B-cell memory. At a certain point the absence of proof for the presence of receptor revision becomes proof for the absence of that phenomenon.

CHROMOSOMAL TRANSLOCATIONS AS PROGNOSTIC MARKERS

In Chapter 2 and 3, B-cell malignancies were used as model system to study regulation of recombination events such as allelic exclusion and receptor revision. In Chapter 4, our studies in B-cell malignancies focused on oncogenic recombination events. Oncogenic recombinations play an important role in malignant transformation of B cells. Some oncogenic recombinations can be used for classification of B-cell malignancies, especially those that are (partly) mediated via 'normal' recombination processes involving one of the Ig loci. Other chromosomal translocations result in the formation of fusion genes that encode chimeric proteins with altered functions. These translocations are generally found in the more immature malignancies, especially in acute lymphoblastic leukemias (ALL). Genes that are involved in these translocations play key roles in development and function of lymphoid cells, such as transcription factors, cell cycle regulators, and signal transduction molecules. The t(1;19)(q23;p13) for example involves the *E2A* gene, which codes for a basis helix-loop-helix transcription factor that plays an important role in regulation of V(D)J recombination and other processes during B-cell differentiation. As a result of this translocation the *E2A* gene is fused to the *PBX1* gene. In Chapter 4.3, the effect of translocations involving the *E2A* gene on Ig and cross-lineage TCR gene rearrangement patterns in pre-B-ALL was studied. The disruption of one of the *E2A* alleles because of an *E2A-PBX1* gene fusion seems to result

in a mild reduction of *IGK* gene rearrangements, but a strong decrease of cross-lineage TCR gene rearrangements. The frequency of cross-lineage *TCRD* gene rearrangements was reduced, but *TCRG* gene rearrangements were completely absent. These observations confirm the role of *E2A* in the regulation of *TCRD* and *TCRG* gene rearrangements. In contrast, in cell lines with *E2A-HLF* gene fusions, resulting from t(17;19)(q22;p13), *TCRG* gene rearrangements were present on five of the six alleles. Further analyses are required to understand these opposite results. In line with this study, effects of other translocations involving genes, which play important roles in the regulation of V(D)J recombination such as *PAX5* or *EBF*, on Ig and/or TCR gene configuration patterns might be studied.

Importance of detection of chromosomal translocations

Development of split-signal FISH analysis

Some chromosomal translocations have high prognostic value and are therefore useful markers for risk group classification.[10] For this purpose, we developed a rapid and sensitive method for detection of the five most important translocations in acute lymphoblastic leukemia i.e. for translocations involving the *MLL* gene, the *E2A* gene, the *TEL* gene, *BCR* gene and the *TAL1* gene (Chapter 4.4). We developed split-signal FISH probe sets, which allow detection of these translocations independent of the partner gene with low cut-off values. These probe sets will become commercially available via DAKO A/S as a kit using a new hybridization and PNA-based blocking method. Early and rapid detection of these translocations at diagnosis is of utmost importance, because it can have implications for treatment strategies in involved patients.

Prognostic value of translocations

The t(9;22)(q34;q11) and translocations involving the *MLL* gene (11q23) are the two translocations with a clear poor prognostic value and presence of one of these translocation is used for classification in the high risk group. Although the t(12;21)(p13;q22) is correlated with a relatively good prognosis, this does not imply that ALL cases carrying this translocation do not relapse. At the moment, other parameters in addition to chromosome translocations are used for risk classification, such as white blood cell count, early treatment response, and central nervous system involvement.[10] However, it would be important to define additional parameters, which might be informative already at diagnosis with respect to the chance of relapse. DNA chip technology is a promising new technology with the potential to identify such parameters.

POTENTIAL ROLE OF DNA CHIP TECHNOLOGY IN IDENTIFICATION OF ADDITIONAL PROGNOSTIC FACTORS

DNA chip technology for classification of subgroups within diseases of clustering of different subgroups

During the last few years, DNA chip technology has developed in such a way that gene expression profiles of thousands of genes can be analyzed in one sample and compared between different samples. General aspects of this new type of technology are summarized in recent papers.[11-13]

DNA chip technology has been shown to be of high value in classification of subgroups within seemingly homogenous disease categories. The first example came from DLBCL, which is a clinically heterogeneous disease. Gene expression profiling allowed the identification of two molecular distinct forms of DLBCL.[14] The first type expressed genes that are characteristic for GC B cells, whereas the second type showed a gene expression profile as seen in activated peripheral blood B cells. This sub-classification of DLBCL appeared to correlate with clinical outcome, i.e. DLBCL of the GC-like expression pattern had a better overall survival than DLBCL with an activated B cell-like expression pattern. Gene expression profiling of B-CLL showed that B-CLL with somatic mutations as well as B-CLL without somatic mutations, which represent subgroups with different clinical outcome, have a common origin, being more related to memory B cells than to naive B cells or GC-derived B cells.[15,16] Nevertheless, a restricted number of genes (<30) appeared to be differentially expressed in the two B-CLL subgroups.[15]

Use of DNA chip technology in search for new prognostic factors

DNA chip technology might not only be applicable for definition of subgroups within one disease entity or clustering of different subgroups as belonging to one disease based on their common origin, but also for identification of new prognostic factors. One approach would be to compare precursor-B-ALL with the same chromosome translocation, but with a different clinical outcome, e.g. t(12;21)(p13;q22) positive cases with or without a clinical relapse.

A related question that might give insight in the development of a relapse can be: What are the differences in gene expression patterns between the diagnosis and the relapse samples of the same patient? Comparison of different diagnosis-relapse pairs may lead to the identification of common "relapse" genes. Our preliminary data show that not so much multidrug resistance genes were upregulated in the relapse samples, but rather genes involved in cell cycle regulation, DNA synthesis, signal transduction (protein kinases), and gene activation (transcription factors) (Figure 5).

Consideration of technical aspects

The abundance of information as obtained via DNA chip technology requires well-

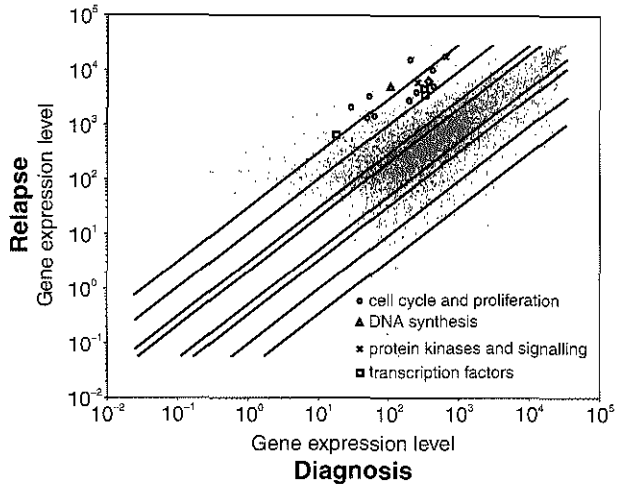

Figure 5. Gene expression profile of RNA from a precursor-B-ALL patient at diagnosis and relapse using microarray type U95A (Affymetrix).

defined cell material in order to get informative and valuable answers. This implies that not only the cell samples that are compared need to be clearly defined, but also the reference samples.

It should be kept in mind that the tumor load of the samples needs to be higher than 95%, as otherwise the presence of non-tumor cells will disturb the subsequent analyses. Gene expression differences between e.g. two ALL (groups) might be masked by expression level of the same genes in the non-tumor cells. For analysis of multiple patient samples, Affymetrix DNA microarray technology (oligonucleotide-based arrays covering 12,000 or preferably >39,000 nonlymphocyte-biased genes) is preferred over glass slide-based chip technology (cDNA-based array covering 18,000 lymphocyte-biased genes), because glass-slide cDNA based microarray technology is based on pair-wise comparisons of samples. This is in contrast to the Affymetrix approach, which is based on standardized analysis of individual samples, which can be compared, even at a later stage, to many different other samples.

CONCLUSIONS AND FUTURE PERSPECTIVES TO IDENTIFY MISSING LINKS IN RECOMBINATION PROCESSES DURING B-CELL DIFFERENTIATION

Molecular processes during human B-cell differentiation form the central theme of this thesis. Our studies mainly focused on V(D)J recombination processes of *IGK* and *IGL* light chain genes. First, the *IGL* locus was studied in detail. The two human *IGL* polymorphisms were unraveled and based on the frequencies of J-Cλ gene segment usage, a strategy

for Southern blot analysis of *IGL* gene rearrangements was developed. Subsequently, regulation of Ig light chain gene recombinations was studied using different types of B-cell malignancies as clonal "single-cell" model system for the various B-cell differentiation stages, varying from precursor-B cells to mature plasma cells. These studies showed hierarchical patterns of Ig light chain gene rearrangements, starting with *IGK* rearrangements followed by *IGK* deletions and subsequently *IGL* rearrangements. This order of Ig light chain gene rearrangements appeared to be the main determinant for allelic exclusion in order to generate monospecifc B-cells. Furthermore, these studies indicate that V(D)J recombination is regulated via two main determinants, i.e. differentially regulated RAG expression and differentially regulated transcription factor-induced Ig gene accessibility. In addition, the influence of somatic mutations on Ig light chain gene rearrangement patterns was investigated and studies on this topic are currently being extended to explore whether somatic mutation-induced receptor revision occurs, or whether this phenomenon does not exist at all.

Many steps in recombination processes during B-cell differentiation are known in detail. However, the complete processes, including its regulation, are not yet fully unraveled. Future *in vitro* studies or *in vivo* studies using normal B cells will further focus on the exact function of known factors in these recombination processes, e.g. the role of transcription factors in chromatin remodeling in Ig genes, the precise function of Artemis in V(D)J recombination and the role of AID in SHM and CSR. However, there are still missing links in the complete picture of these processes, such as a "RAG-like" activity in CSR, which is responsible for the introduction of the double stranded breaks in the switch regions. DNA chip technology for studying gene expression profiles might lead to the identification of new components. AID was identified by a subtraction of cDNAs derived from switch-induced and uninduced murine B cell lymphoma cells, an approach that can be regarded as a precursor method of DNA chip technology.[17] This example clearly demonstrates that comparison of gene expression profiles might provide clues for identification of new components. It is tempting to speculate that in addition to analysis of gene expression profiles of normal cells via DNA chip technology, samples of immunodeficient patients with an apparent defect in one of the recombination processes (not caused by a mutation in one of the currently known genes), might lead to the discovery of additional new components.

Integration of the results that will be obtained from studies on normal B cells, malignant B cells, and from B cells of immunodeficient patients is expected to contribute to the identification of the missing links in the intriguing recombination processes taking place at the DNA, which form the basis of antibody diversity.

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ABBREVIATIONS

Ag	Antigen
ALL	Acute lymphoblastic leukemia
APC	Antigen presenting cell
B-CLL	B-cell lymphocytic leukemia
BCR	B-cell receptor
BL	Burkitt's lymphoma
BM	Bone marrow
CBL	Chronic B-cell leukemia
CSR	Class switch recombination
D	Diversity gene segment
DLBCL	Diffuse large B-cell lymphoma
DSB	Double stranded break
FDC	Follicular dendritic cell
FISH	Fluorescence *in situ* hybridization
GC	Germinal center
Ig	Immunoglobulin
Igκ	Immunoglobulin kappa light chain
Igλ	Immunoglobulin lambda light chain
IgH	Immunoglobulin heavy chain
IGH	Immunoglobulin heavy chain gene
IGK	Immunoglobulin kappa light chain gene
IGL	Immunoglobulin lambda light chain gene
J	Joining gene segment
Kde	Kappa deleting element
MCL	Mantle cell lymphoma
MM	Multiple myeloma
NHEJ	Non-homologous end joining
PCR	Polymerase chain reaction
RAG	Recombination activating gene
RSS	Recombination signal sequence
SB	Southern blot
SHM	Somatic hyper mutation
SmIg	Surface membrane bound Ig molecule
TCR	T-cell receptor
TdT	Terminal deoxynucleotidyl transferase
V	Variable gene segment

SUMMARY

During normal differentiation, human B cells undergo a sequence of immunophenotypic and immunogenotypic changes, finally resulting in the formation of mature B cells with receptors specific for the antigen to be recognized. Recombination processes underlying the generation of the antigen-specificity of these receptors formed the central theme of this thesis. There are two main molecular processes, i.e. V(D)J recombination of immunoglobulin (Ig) genes and somatic hypermutation (SHM). Class switch recombination (CSR) is a third molecular process in human B cells; it determines the effector function of the Ig molecule via replacing of the constant domains at the DNA level by another one. Oncogenic recombinations can be a direct consequence of aberrations during one of the physiological recombination processes, i.e. V(D)J recombination, SHM, and CSR, but might also occur through different processes, which are as yet not fully understood.

The structure of an Ig locus, i.e. the position of a gene segment within the Ig locus and the composition of the recombination signal sequence (RSS), has been shown to influence gene segment usage. In Chapter 2, we therefore focused on two structural aspects of the human Ig lambda (*IGL*) locus. First, two human *IGL* polymorphisms were unraveled (Chapter 2.2). One concerns a single, two-fold or threefold amplification of a 5.4 kb DNA fragment in the Cλ2-Cλ3 region. The second polymorphism concerns the $Mcg^{-}Ke^{+}Oz^{-}$ isotype. So far, it remained unclear whether the $Mcg^{-}Ke^{+}Oz^{-}$ isotype is encoded by a Cλ gene segment on the amplified Cλ2-Cλ3 region. We now unraveled both *IGL* polymorphisms at the DNA level and found that they actually are not linked. Sequence analysis showed that the amplification polymorphism is likely to result from unequal crossing over of the J-Cλ2 and J-Cλ3 region via a 2.2 kb homologous repeat. The presence of additional functional J-Cλ gene regions appeared to result in higher numbers of $Ig\lambda^{+}$ peripheral B cells. The second aspect that was studied with respect to the *IGL* locus, was the usage of seven J-Cλ isotypes in various types of B-cell malignancies as determined via Southern blot analysis (Chapter 2.3). The data showed that more than 99% of the *IGL* rearrangements in $Ig\lambda^{+}$ B-cell malignancies involved the J-Cλ1, J-Cλ2, and J-Cλ3 regions, suggesting that proximity to the Vλ gene segments plays a role in J-Cλ usage. Furthermore, these frequencies appeared to be similar to those of normal $Ig\lambda^{+}$ B cells.

Regulation of the V(D)J recombination process is of utmost importance for normal B-cell differentiation. In Chapter 3, B-cell malignancies were used as single-cell model system to study regulation of Ig gene recombination processes and allelic exclusion, during several stages of B-cell differentiation. These studies focused on analysis of Ig kappa (*IGK*) and *IGL* gene configuration patterns and on the influence of SHM on these patterns. Analysis of Ig light chain gene configuration patterns in three types of B-cell malignancies, i.e. precursor-B acute lymphoblastic leukemia (precursor-B-ALL), mantle cell lymphoma (MCL), and chronic B-cell leukemia (CBL) representing immature, naive, and activated stages of B-cell differentiation, respectively. These analyses demonstrated a hierarchical order in Ig light

chain gene rearrangements (Chapters 3.3, 3.2, and 3.4). For the Ig expressing model systems (MCL and CBL) most of the Igκ$^+$ cases had both *IGL* alleles in germline configuration, whereas in almost all Igλ$^+$ cases at least one of the *IGK* alleles was deleted.

Precursor-B-ALL, which are characterized by absence of surface Ig expression and continuous recombinase activity, could be considered as model system for Ig recombination processes without selection of functionality of the rearrangement (Chapter 3.3). Analysis of the Ig gene configuration patterns in these different B-cell differentiation stages revealed two main observations: (a) induction of Ig light chain gene rearrangements does not seem to require the presence of a functional *IGH* gene rearrangement, and (b) Ig light chain gene recombination occurs in an hierarchical order starting with *IGK* and followed by *IGL* rearrangements. Collectively, these observations suggest that the hierarchical rearrangement process is independent of Ig protein expression. Ig protein expression might therefore not be directly involved in regulation of accessibility of Ig loci, but rather in regulation of the V(D)J recombinase enzyme system, i.e. expression of the *RAG1* and *RAG2* genes. This study illustrated that regulation of allelic exclusion is likely to concern two different components, i.e. accessibility of Ig loci and actual recombinase activity.

One of the aims of the study on CBL described in Chapter 3.2 was to determine at which level (DNA, RNA or protein) monospecific Ig light chain expression is regulated. The obtained data showed that monospecific Ig light chain expression is in about 90% of cases regulated at the DNA level, as demonstrated by the presence of only one functional allele. In combination with the observation that recombination patterns show an hierarchical order, this suggests that the ordered recombination process is the main determinant for monospecific Ig expression. Cases with more than one Ig light chain gene rearrangement were studied in more detail. In CBL with two *IGK* rearrangements, only one *IGK* allele appeared to be functional. However, when *IGL* rearrangements were involved (either *IGK* and *IGL* or biallelic *IGL*), 50% of cases appeared to carry two in-frame rearrangements. In cases with two functional Ig light chain rearrangements, both alleles were transcribed, but only one was expressed on the cell surface. This suggests that in these cases allelic exclusion is not regulated at the RNA level, but either at the level of translation, or protein stability or via preferential assembly of one of the Ig light chains with the Ig heavy chain. The occurrence of somatic mutations in CBL with two in-frame Ig light chain gene rearrangements was higher than in the total group of CBL, which suggests that the presence of (unfavorable) somatic mutations might affect assembly of Ig heavy and Ig light chains and might even result in the induction of secondary rearrangements in the periphery, i.e. receptor revision. The existence of receptor revision, however, is still highly under debate.

This phenomenon was further studied in IgDλ multiple myelomas (MM) (Chapter 3.5). IgD MM represent a special subtype of MM characterized by an extremely high level of somatic mutations and biased Igλ expression. Due to the high frequency of somatic mutations unfavorable somatic mutations are likely to occur and might result in induction of receptor revision, if it exists at all. In this study on four IgDλ MM, no indications were found for receptor revision. In the General Discussion (Chapter 5) an experimen-

tal approach is discussed to further address this topic.

In Chapter 4, our studies in B-cell malignancies were focused on oncogenic recombination events. Oncogenic recombinations play an important role in malignant transformation of B cells. Some chromosomal translocations have high prognostic value and are therefore useful markers for risk group classification. For this purpose, we developed a rapid and sensitive method for detection of the five most important translocations in ALL, i.e. t(1;19)(q23;p13), involving *E2A* and *PBX1*, t(4;11)(q21;q23) with a *MLL-AF4* fusion, t(12;21)(p13;q22) with a *TEL-AML1* fusion, t(9;22)(p13;q22) with fusion of *BCR* and *ABL*, and translocations and microscopic deletions involving the *TAL1* gene region, based on the split-signal FISH principle. Split-signal FISH makes use of two differentially labeled probes, which flank both sides of the breakpoint region. In normal situations, two colocalized yellow (green/red) signals will be visible (see also Chapter 4.1). A translocation will result in a split of one of the colocalized signals, giving rise to a separate green and red signal together with a fused signal of the unaffected chromosome. The advantage of this approach is that detection of a chromosome aberration involving the target gene is independent of the partner gene. This is especially of great interest for detection of *MLL* translocations, because of the high number of partner genes that might be involved as described in Chapter 4.2. Chapter 4.4 summarizes the development of split-signal FISH probes for the five most frequent chromosome aberrations in ALL.

Chapter 4.3 describes a study on the effect of translocations involving the *E2A* gene on Ig and T-cell receptor (TCR) gene rearrangement patterns in pre-B-ALL. E2A is a transcription factor that plays an important role in regulation of V(D)J recombination. The presence of E2A-PBX1 fusion proteins and the parallel reduction of wild type E2A levels did not seem to influence Ig gene rearrangements to a great extent, but resulted in lower frequencies cross-lineage TCR gene rearrangements and even complete absence of *TCRG* gene rearrangements. However, analysis of cell-lines with an *E2A-HLF* gene fusion did show the presence of *TCRG* gene rearrangements, which were not found in *E2A-PBX1*-positive cell lines, suggesting that E2A-induced accessibility of antigen receptor genes is influenced by the type of fusion partner in the involved *E2A* aberration.

Although many steps in recombination processes during B-cell differentiation are already known in detail, the complete mechanisms, including regulation of these processes, are not yet fully unraveled. Future *in vitro* studies and *ex vivo* studies using normal B cells, malignant B cells or B cells of immunodeficient patients, will focus on the precise function of known factors involved in recombination processes (discussed in the General Discussion; in Chapter 5). Recent developments in DNA chip technology might be useful for the identification of new factors, which could possibly be "missing-links" in the complete picture. Integration of the results might contribute to the a better understanding of the intriguing recombination processes taking place at the DNA level, which provide the basis for antibody diversity.

SAMENVATTING VOOR NIET-INGEWIJDEN

B cellen zijn witte bloedcellen, die verantwoordelijk zijn voor de productie van antistoffen. Antistoffen spelen een belangrijke rol in de afweer tegen ziekteverwekkers. Een antistofmolecuul wordt ook wel een immunoglobuline (Ig) molecuul genoemd en bestaat uit twee identieke zware ketens ("Ig heavy chains") en twee identieke lichte ketens ("Ig light chains"). Er bestaan twee typen lichte ketens te weten Ig kappa (Igκ) en Ig lambda (Igλ). Iedere Ig keten is opgebouwd uit een variabel en een constant deel. Met het variabele deel wordt een deel van de ziekteverwekker of ander lichaamsvreemd bestanddeel herkend. Deze Ig moleculen komen niet alleen in het bloed en andere lichaamsvloeistoffen voor, maar ook op het oppervlak van B cellen. Zij vormen dan de receptor waarmee de B cel een ziekteverwekker kan herkennen.

Gezien het feit dat er enorm veel verschillende ziekteverwekkers zijn, is het van belang dat er een grote diversiteit aan Ig moleculen is, zodat elke ziekteverwekker hekend kan worden door specifiek daar aan bindende antistoffen (Ig moleculen). Als ieder Ig molecuul gecodeerd zou worden door een eigen Ig gen (stukje DNA, deel van een chromosoom) dan zou een cel niet groot genoeg zijn om al het DNA in zich te dragen. Om antistofvormende cellen toch al die verschillende Ig moleculen te kunnen laten maken, bestaat er een mechanisme om de Ig moleculen met verschillende bouwstenen op te bouwen. Deze bouwstenen worden gensegmenten genoemd. Het Ig zware keten gen bijvoorbeeld is opgebouwd uit 74 Variable (V) gensegmenten, 37 Diversity (D) gensegmenten en 6 Joining (J) gensegmenten. Tijdens de vroege ontwikkeling van de B cel in het beenmerg wordt één D aan één J gekoppeld en vervolgens wordt één V aan de DJ gekoppeld; er vindt dus een Ig zware keten genherschikking plaats. Dit moleculaire proces wordt V(D)J recombinatie genoemd. Voor de Ig lichte keten genen vindt een vergelijkbaar proces plaats. Het enige verschil is dat Ig kappa en Ig lambda genen slechts V en J gensegmenten bezitten en geen D gensegmenten. Op basis van de combinatiemogelijkheden tussen de diverse gensegmenten zijn ongeveer één miljoen verschillende combinaties mogelijk. Tijdens de koppeling van gensegmenten worden daarnaast kleine stukjes DNA weggehaald en ingebouwd, waardoor uiteindelijk meer dan duizend miljard verschillende combinaties mogelijk zijn. Elke afzonderlijke B cel is specifiek voor één bepaald antigeen, dankzij het feit dat al de Ig moleculen op zijn oppervlak specifiek zijn voor dat antigeen. Er zijn dus net zoveel verschillende specifieke B cellen als nodig zijn om alle verschillende lichaamsvreemde stoffen te kunnen herkennen.

Als een Ig molecuul op het oppervlak van een B cel een ziekteverwekker herkent, krijgt de B cel een signaal en worden er veranderingen aangebracht in het DNA dat codeert voor het variabele deel van het Ig molecuul. Deze mutaties worden somatische mutaties genoemd en kunnen er voor zorgen dat een Ig molecuul de ziekteverwekker nog beter herkent. Somatische mutaties kunnen ook resulteren in een slechtere herkenning. Een selectieproces zorgt er dan voor dat deze cellen worden verwijderd en dat alleen de B cellen met de best passende Ig moleculen worden geselecteerd en verder kunnen uitgroeien tot een anti-

stofvormende B cel, die de Ig moleculen uitscheidt.

In hoofdstuk 2 van dit proefschrift staan de Ig lambda (*IGL*) genen centraal. Eerst zijn twee polymorfismes (d.w.z. variaties in DNA in de populatie) in het *IGL* gen ontrafeld op DNA niveau. Het eerste polymorfisme is een amplificatie polymorfisme welke resulteert in de aanwezigheid van extra J en constante (C) gensegmenten. Op basis van de DNA sequentie kon worden afgeleid hoe het amplificatie polymorfisme moet zijn ontstaan. Uit deze studie bleek dat personen met dit polymorfisme vaker een Ig lambda molecuul gebruikten op hun B cellen dan personen zonder dit polymorfisme. Het tweede polymorfisme is een verandering van één aminozuur, d.w.z. één bouwsteen van het Ig molecuul. Dit polymorfisme heeft zeer waarschijnlijk geen effect op de functie van Ig moleculen. Tevens is in dit hoofdstuk de frequentie van J en C gensegment gebruik in diverse B-cel maligniteiten bestudeerd.

De regulatie van Ig kappa (*IGK*) en *IGL* lichte keten genherschikkingen is het thema van Hoofdstuk 3. Strikte regulatie van het herschikkingsproces moet er voor zorgen dat een B cel Ig moleculen van slechts één type (of Igκ of Igλ) tot expressie brengt (dit heet "allelische exclusie"). Om deze processen goed te kunnen bestuderen zijn de *IGK* genherschikkingsprofielen bestudeerd in diverse B-cel maligniteiten. B-cel maligniteiten zijn gebruikt als zogeheten 'single-cell' model, omdat een B-cel maligniteit is ontstaan uit een enkele cel, tijdens een bepaald ontwikkelingsstadium en vervolgens is uitgegroeid tot een kloon van identieke cellen. In diverse B-cel maligniteiten hebben wij kunnen aantonen en bevestigen dat Ig genen in een bepaalde volgorde herschikken: het genherschikkingsproces start met Ig zware keten herschikkingen gevolgd door Ig lichte keten genherschikkingen. Van de lichte keten genen herschikken eerst de *IGK* genen en vervolgens de *IGL* genen. Zodra een functionele herschikking leidt tot de expressie van een Ig molecuul wordt het herschikkingsproces gestopt. Het genherschikkingsproces heeft twee onafhankelijke regulatie-componenten (1) de aanwezigheid van herschikkingsmachinerie, d.w.z. de eiwitten die het eigenlijke proces uitvoeren, (2) toegankelijkheid van de Ig genen ("accessibility").

In Hoofdstuk 4 staan chromosoomafwijkingen bij leukemie centraal. Chromosoomafwijkingen spelen een rol bij het ontstaan van een leukemie. Sommige chromosoomafwijkingen hebben een voorspellende waarde omdat gebleken is dat patiënten met een bepaalde chromosoomafwijking slecht of juist goed reageren op therapie. In dit hoofdstuk staat de ontwikkeling van de 'split-signal FISH' methode beschreven. Deze methode is ontwikkeld om snel en betrouwbaar de aanwezigheid of afwezigheid van een bepaalde chromosoomafwijking te kunnen vaststellen. Hierbij wordt gebruik gemaakt van twee verschillend fluorescerende (rood of groen) probes (stukken DNA), die hechten aan het DNA in de celkernen aan verschillende kanten van het breukpuntgebied. In normale cellen hechten beide probes zo dicht bij elkaar dat ze met een fluorescentiemicroscoop zichtbaar zijn als één geel of gecombineerd rood/groen signaal. In geval van een chromosoomafwijking gaan de rode en groene signalen uit elkaar en is er in de kern een los groen en los rood signaal te zien tengevolge van een breuk in één van de chromosomen waar die beide probes normalitair aan hechten. Daarnaast is er één geel signaal van het niet-aangedane chromosoom. Deze methode is ontwikkeld voor vijf van de meest voorkomende chromosoomafwijkingen bij acute

lymfatische leukemie (ALL).

In de General Discussion (Hoofdstuk 5) worden alle conclusies uit de verschillende hoofdstukken geïntegreerd besproken en wordt uiteengezet waar verder onderzoek zich in de toekomst op zou moeten richten. De recente opkomst van DNA chip technologie zal in de komende jaren wellicht een belangrijke rol gaan spelen bij het identificeren van nieuwe factoren die betrokken zijn bij de moleculaire processen, die aan de basis liggen van het ontstaan van antistoffen. DNA chip technologie zal tevens worden gebruikt om nieuwe factoren te identificeren, die mogelijk kunnen dienen als extra of zelfs betere voorspellende factoren bij leukemie.

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