Lymphoid-Specific Gene- Rearrangement and –Mutation Mechanisms in Chronic Myeloid Leukemia Blast Crisis

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How excellent is thy loving kindness. O God!
Therefore the children of men put their thrust
under the shadow of thy wings.
They shall be abundantly satisfied with the fatness
of thy house; and thou shalt make them drink of
the rivers of thy pleasures.
For with thee is the fountain of life:
In thy light shall we see light.

PSALM 36:7-9

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Introduction

1.1 Hematopoiesis

Hematopoiesis is a development process that leads to the production of blood cells throughout normal life (Ginsburg et al., 1963; Sachs, 1996). This process occurs prenatally in organs like the placenta (embryonic yolk sac), fetal liver, and bone marrow and postnatally in the bone marrow, primarily, and other lymphatic tissues (Moore et al., 1970; Peault, 1996). Blood cells arise from a common stem cell population, which is pluripotent and self-renewing, and can successively differentiate into hematopoietic lineages (Osawa et al., 1996). Hematopoietic lineages are generated through intermediate progenitors including the common lymphoid progenitors (CLP), which generate B, T and natural killer (NK) cells and the common myeloid progenitors (CMP), which generate erythrocytes, granulocytes, monocytes and platelets (Kondo et al., 1997). Further downstream of the CLP and CMP are progenitors that are restricted in the number and type of lineages they can generate (Akashi et al., 2000; Figure 1)

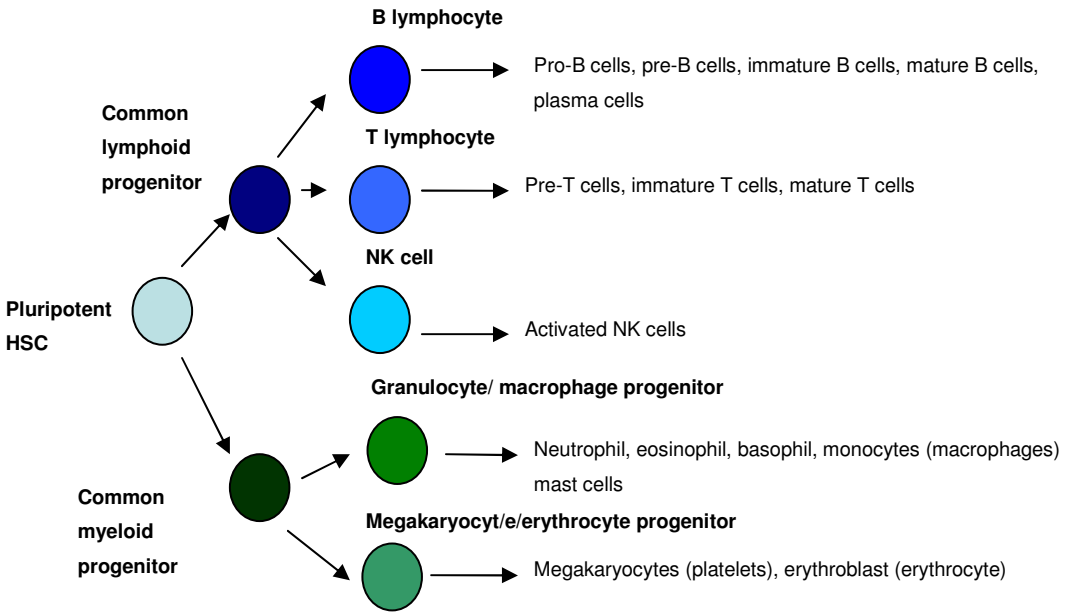


Figure 1: Hematopoiesis

The hematopoietic development of blood cells from a pluripotential stem cell, which is committed to several hematopoietic differentiation pathways.

Phenotypically, human hematopoietic stem cells (HSC) and primitive progenitors are suggested to be small quiescent cells that express the surface glycoprotein CD34 and lack the expression of lineage specific markers (Miller et al., 1999). In the course of hematopoiesis, cells develop other surface markers that characterize their lineage identity.

An interaction between the intrinsic genetic processes of blood cells and their environment, sustains the process of hematopoiesis and determines whether HSCs, progenitors and mature blood cells remain quiescent, self-renew, proliferate, differentiate, or become apoptotic (Domen et al., 2000; Orkin et al., 2002). Cytokines and chemokines are two of the many environmental regulators of hematopoiesis. Cytokines belong to protein families that function by engaging a specific receptor and activating signaling pathways, thus influencing proliferation, differentiation etc., while chemokines are molecules that regulate blood cell trafficking and homing cites and their proteins mediate processes like inflammation, leukocyte development, tumor cell growth etc (Wright et al., 2002).

1.1.1 Myeloid cells

The common myeloid progenitors are cells that give rise to granulocytes and macrophages of the immune system. Macrophages are large phagocytic cells, which enter the blood stream as monocytes, then migrate to tissues, where they further develop (Gordon, 2003; Gordon, 1982; Gordon, 1973). By processing and presenting antigens to T cells, as well as phagocytizing pathogens and cellular debris, they play an important role in innate immunity, inducts the activation of the adaptive immune system (Aderem et al., 1999).

Granulocytes are cells with a granular cytoplasm and an irregularly formed cell nucleus. There are three types of granulocyte cells, namely: basophile cells, eosinophile cells and neutrophile cells, which are vital to the innate immune response towards invading microorganisms (Greenberg et al., 2002). In order to eliminate pathogens, they migrate, phagocytize or produce reactive oxygen intermediates by being recruited to sites of inflammation (Cicchetti et al., 2002). The proliferation, survival, maturation and functional activation of cells from the granulocyte lineage are influenced by the granulocyte colony-stimulating factor (GC-SF) (Avalos, 1996). Another myeloid cell type is mast cells, which develop in the bone marrow, mature in body tissue and play a role in the initiation of allergic reactions (Mekori et al., 2000).

Further subsets of the myeloid progenitor cells are megakaryocytes and erythroblasts, which give rise to platelets and erythrocytes (red blood cells), respectively.

1.1.2 Development of B lymphocytes

B cells are generated from HSCs and develop in the bone marrow as immature B cells before they migrate into blood to reach peripheral lymphoid organs, where they give rise to heterogeneous populations of peripheral B cells including naïve, memory and plasma cells (Hardy et al., 1982; Melchers et al., 1989; Melchers et al., 1994). Of note, these end-stage B cells (plasma cells), which develop after the antigen activation of mature B cells, return and colonize the bone marrow (Manz et al., 1997).

The successive order of the B cell development can be defined by the immunoglobulin-gene (Ig) rearrangement status, cell surface phenotypic transition and differences in functional activity (Coffman et al., 1981; Whitlock et al., 1982; Hardy et al., 1991). Although pro-B cells do not express Ig on their surface, they can be identified using other B lineage surface markers and by the initiation of the Ig heavy chain (*IGH*) gene rearrangement process. Pre-B cells express a pre-B cell receptor (pre-BCR), which is an association of the surrogate light chain with a portion of the intracellular μ heavy chains expressed in pre-B cells. Prior to the Ig gene rearrangements, two transmembrane proteins (Igα and Igβ) are expressed within early B lineage cells. These proteins are critical for the delivery of the pre-BCR on the cell surface and in its signaling function. B cells express transmembrane Ig molecules in the BCR and plasma cells secrete the antibodies (Bradl, 2001; Manz, 1997).

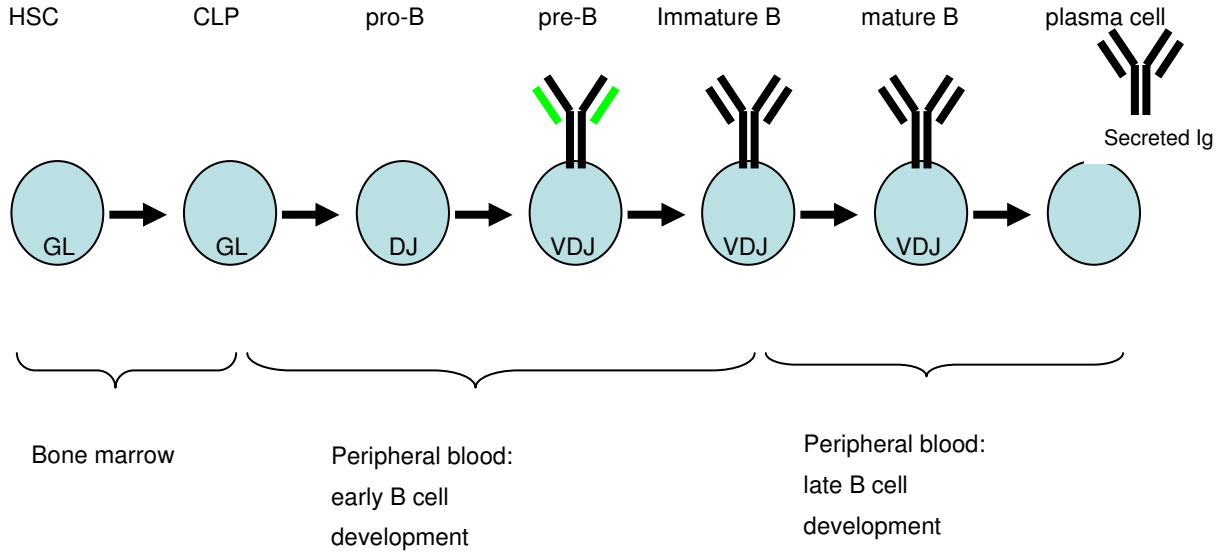


Figure 2: Schematic representation of B cell development

An illustration of the differentiation of a hematopoietic stem cell (HSC) to a common lymphoid progenitor (CLP), pro-B cell, pre-B cell, immature B cell, mature B cell and plasma cell, indicated by sequential rearrangements of the immunoglobulin gene loci, its expression on the cell surface as well as its secretion at the plasma cell level.

Figure 2 illustrates the differentiation of a hematopoietic stem cell (HSC) to a common lymphoid progenitor (CLP), pro-B cell, pre-B cell, immature B cell, mature B cell and plasma cell. This development is characterized by a sequence of somatic recombinations within the immunoglobulin heavy chain locus (*IGH*). In the transition from HSC to CLP, which takes place in the bone marrow, the *IGH* locus is in germline (GL) configuration. In the early B cell development, during the transition of the CLP to the pro-B cell stage, the rearrangement of a D_H to a J_H gene segment occurs. This is followed by the development of the pre-B cells in the peripheral blood, which entails the recombination of a V_H gene segment to the DJ_H joint. Pre-B cells are characteristic of the μ heavy chain (black) together with the surrogate light chain (green) on their surface, encoded by the *Vpreβ* and *λ5* genes, in a complex known as the pre-B cell receptor (Sakaguchi et al., 1986; Pillai et al., 1987; Karasuyama et al., 1990; Rolink et al., 1996). After successful rearrangement of the immunoglobulin light chain loci, the conventional B cell receptor (BCR) is expressed on the cell surface as selected cells transform into immature and mature cells. B cells with high affinity BCR may receive signals from T cells ensuring their survival and selection to differentiate into memory B cells and plasma cells, which possess the ability to secrete antibodies (Manz et al., 1997; Blink et al., 2005).

Phenotypically, B cell development can be defined by the status of the Ig gene and the expression of cell surface markers that distinguish stages of cell development. HSC and CLP cells carry CD34 but do not express surface Ig (sIg) or CD19. Pro-B cells committed to the B cell lineage are CD34 and CD19 positive but still do not carry sIg. In the course of development *IGH* and *IGL* loci are successfully rearranged and immature and mature B cells are CD19 positive and express sIg (Poe et al., 2001).

The commitment of HSC to the differentiation in the B lymphoid lineage and the development of B cells are dependent on the activities of several transcription factors. For example, IKAROS and PU.1 are important for the differentiation of HSC to common lymphoid progenitor cells. Mice experiments show that an IKAROS-deficiency prevents the development of lymphoid cells (Georgopoulos et al., 1994) and experiments with PU.1 deficient cells show that PU.1 decides upon B or myeloid lineage differentiation (DeKoter et al., 2000). Differentiation from the CLP to pro-B cells, pre-B cells and further depends on the transcription factors E2A, encoding the helix-loop-helix protein, the early B cell factor (EBF) and PAX5; which encodes the B cell-specific activator protein (BSAP). The deficiency of any of these transcription factors leads to an early differentiation arrest of B cell development and lack of V_H region gene rearrangements (Bain et al., 1994; Zhuang et al., 1994; Nutt et al., 1999). B lymphocyte-induced maturation protein-1 (Blimp-1), which is a repressor of transcription factors that regulate other gene programs, is necessary and sufficient for the differentiation into plasma cells. Blimp-1 represses XBP1, PAX5, Bcl-6, which are important for B cell identity and germinal center function and thus induce plasma cell differentiation and Ig secretion (Turner et al., 1994; Reimold et al., 1996; Lin et al. 1997; Lin et al. 2002).

1.1.2.1 V(D)J recombination in B cells and the role of the Recombination Activating Genes (RAG1/2)

The BCR can recognize and respond to a theoretically unlimited number of pathogens, a feature that is the hallmark of the adaptive immune system. V(D)J recombination is of major importance to the generation of these diverse antigen receptor repertoires by assembling antigen receptor genes from arrays of gene segments (Tonegawa, 1983; Hesslein et al., 2001). The Ig heavy chain contains clusters of V (variable), D (diversity), J (joining) gene segments that make up its variable region and a cluster of C (constant) genes encoding its constant region. In the case of the Ig light chain, the variable region is made up of clusters of V and J gene segments and its constant region (Matsuda, 1997; Matsuda et al., 1998).

V(D)J recombination is a highly regulated process, controlled in several ways (Schlissel et al., 1997). For the *IGH* locus, the somatic recombination is a two step process, starting with the rearrangement of a D_H to a J_H gene segment (DJ_H-joint) at the transition to the pro-B cell stage of development and followed by the rearrangement of a V_H segment to the DJ_H-joint (V_HDJ_H-joint) at the transition of the pre-B cell stage of development. The V_HDJ_H-joint is then joined to the constant region by RNA splicing after transcription as shown on Figure 3 (Tonegawa, 1983; Yancopoulos et al., 1986).

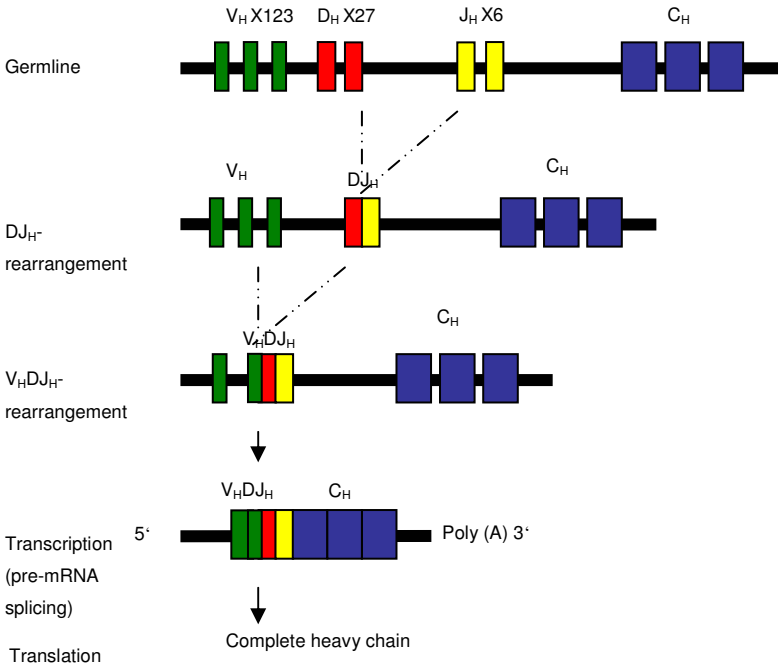


Figure 3: Somatic recombination of the Ig heavy chain locus

The rearrangement of the D_H to the J_H gene segment (DJ_H-joint) occurs at the transition to the pro-B cell stage of development. This is preceeds the rearrangement of the V_H segment to the DJ_H-joint (V_HDJ_H-joint) at the transition of the pre-B cell stage of development. Subsequently, at the level of transcription, V_HDJ_H-joint is linked to the constant region by pre-mRNA splicing.

The Ig gene segments are flanked by conserved recombination signal sequences (RSS), which consist of three distinct elements: a conserved palindromic heptamer sequence and an AT-rich nonamer sequence separated by a nonconserved 12 or 23 bp spacer element (Tonegawa, 1983). V(D)J recombination can occur only between two genes flanked by an RSS with a 12 and a 23 bp spacer. This rule, known as the 12/23 restriction, ensures proper assembly of the Ig and prevents for example wasteful V-to-V or J-to-J rearrangements (Eastman et al., 1996; van Gent et al., 1996). V(D)J recombination is initiated by the lymphoid-specific recombination activating genes 1 and 2 (*RAG1* and *2*). Mouse experiments have shown that in the absence of RAG1 or RAG2 there is no V(D)J recombination and the mice lack mature B and T cells (Mombaerts et al., 1992; Shinkai et al, 1992). RAG introduces single strand breaks between two coding sequences and their flanking RSS. Thereafter, RAG catalyses a *trans*-esterification reaction in which the 3' OH of a coding strand forms a closed hairpin coding end with the opposite DNA strand and a blunt 5' phosphorylated recombination signal end (van Gent et al., 1996). The blunt ends are rapidly rejoined to form a signal joint (SJ) but before coding ends are fused, hairpins must be opened (Ramsden et al., 1995; McBlane et al., 1995). The enzyme DNA protein kinase (DNA-PK) is required for the effective joining of DNA ends (Gottlieb et al., 1993) alongside other proteins of the nonhomologous end-joining (NHEJ) repair pathway like Ku70, Ku80, Artemis and XRCC4, which are necessary for repairing double strand breaks (DSBs) and joining coding and signal ends. During the hairpin opening, an enzyme called terminal deoxynucleotidyl transferase (TdT) can add nontemplated nucleotides to free ends and this phenomenon of N-nucleotide addition contributes to junctional diversity of the coding joint (CJ); the end of the rearranging segments, which code the receptor. If the hairpin is opened on the side, a short overhang is left on one strand. When this overhang is filled, palindromic sequences (P-nucleotides) are generated and this further contributes to the diversity of CJs (Lewis, 1994).

Structurally, both *RAG* genes share no sequence similarity and are both adjacent and convergently transcribed. It is suggested that *RAG* genes might have evolved from a primitive transposon, due to their compact structure and the activity of RAG transposase (Oettinger et al., 1990; Thompson et al., 1995; Agrawal et al., 1998; Hiom et al., 1998). RAG1 has a highly conserved region called the core region, which is sufficient (alongside RAG2) to perform all enzymatic functions and catalyze recombination (Oettinger et al., 1990). The C-terminal domain displays DNA binding activity (Arbuckle et al., 2001) and the N-terminal domain of the protein, which contains a conserved sequence residue carrying a RING motif (a zinc binding motif, which defines a subset of ubiquitin ligases), has a functional significance as an ubiquitin ligase (Lorick et al., 1999; Aravind et al., 2000; Jackson et al, 2000; Yurschenko et al., 2003). Mutation analysis of RAG1 has identified three essential conserved amino acids; D600 and D708 play a role in metal binding and including E962 they are all required for V(D)J recombination (Fugmann et al., 2000; Kim et al., 1999; Landree et al., 1999). RAG2 also possesses a core region sufficient for recombination but contrary to RAG1 does not have the same

sequence conservation with enzymatic function (Sadofsky, et al., 1993). The C-terminal domain has an acidic region, a Cys-His rich PHD motif (found in regulatory proteins and thought to affect chromatin structure) and a binding site for CDK2 (phosphorylation leads to translocation of RAG2 from the nucleus to the cytoplasm) (Lee et al., 1999).

1.1.2.2 Structure of the B cell receptor

The B cell receptor (BCR), which controls the development, activation and maintenance of B lymphocytes, comprises of a membrane-bound immunoglobulin (mIg) molecule and Ig-α and Ig-β (*CD79A/B* genes) heterodimers (Hombach et al., 1990; Matsuo et al., 1991). The mIg differs from secreted Ig by the plasma cells structurally in the carboxyl-terminus, where the secreted form lacks a hydrophobic sequence, which acts as an anchor in the cell membrane (Kitamura et al., 1991).

The Ig molecule comprises of four polypeptide chains; two identical heavy chains and two identical light chains (Figure 3). The heavy chains are joined to one another through disulfide bonds as well as each heavy chain is joined to a light chain through disulfide bonds. There is a mutual association between the V_H and the V_L domains, the C_H1 and the C_L domains, both C_H3 domains but not between the C_H2 domains due to carbohydrate side chains which are joined to these domains (Zouali, 1992; Zouali, 1994).

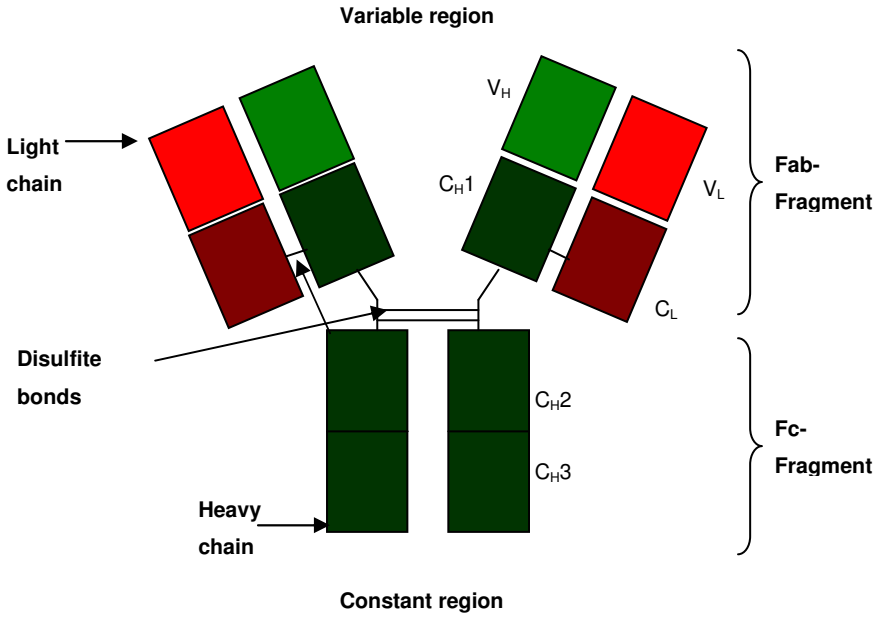


Figure 4: Schematic structure of the immunoglobulin

Showing the heavy chain (green) and the light chain (red), with their respective constant (CH, CL) and variable regions (VH, VL). The "fragment antigen binding" or Fab-fragment contains the C_H1, V_H , C_L and V_L regions and the "fragment crystallizable" or Fc-fragment entails the C_H2 to C_H3/C_H4 domains.

The antibody molecule can be divided into three fragments, two of which are identical and are responsible for antigen binding, thus they are known as the "fragment antigen binding" Fab-fragment, which contains the C_H1 and the V_H domains together with the C_L and V_L regions. The third fragment, the "fragment crystallizable" Fc-fragment entails effector-molecules and is made up of the C_H2 to C_H3/C_H4 domains (Knapp et al., 1982).

The variable region of the Ig heavy chain is made up of V- (variable), D (diversity) and J (joining) gene segments, whereas the light chain, which can either be a lambda (λ) or a kappa (κ) light chain, contains only V- and J- gene segments. The variable region can further be divided into three hypervariable regions (HV), which are separated by framework regions (FR). These hypervariable regions are a binding point for antigens, as they have a structure complementary to the structure of the antigens, thus are also known as the complementarity determining regions, CDR (Figure 5, Wu and Kabat, 1970; Chothia et al., 1989; Morea et al., 1997; Morea et al., 1998).

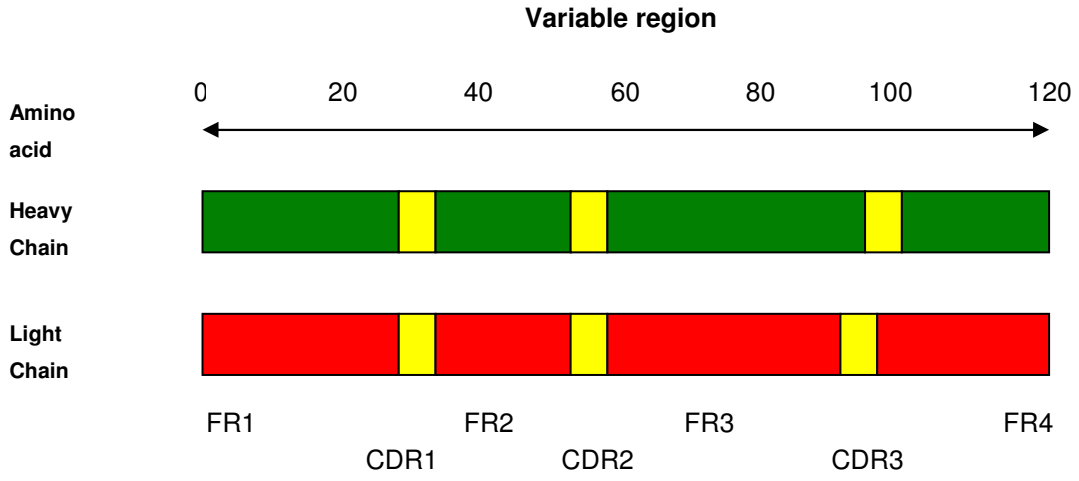


Figure 5: Approximate structure of the variable regions of heavy and light chains

Figure 5 shows the hypervariable or complementarity determining regions (CDR) as well as the framework regions (FR) and their approximate amino acid ranges for the Ig heavy and light chains.

1.1.2.3 Somatic hypermutation and the role of activation-induced cytidine deaminase (*AID*)

Early in B lymphocyte development, a successful V(D)J recombination creates a diverse repertoire of functional variable regions, prior to the contact with antigens (Tonegawa, 1983, Levy et al., 1989). Subsequent antigen recognition and stimulation by helper T cells leads the B cell into the germinal centre (GC) of peripheral lymph organs, where a second phase of antibody diversity occurs through somatic hypermutation (SHM) in human and gene conversion in chicken (Maclennan, 1994; Weill et al., 1996; Reynaud et al., 1996). Another molecular event that alters the Ig loci of B cells, producing a diverse and adaptable antibody repertoire is the class switch recombination (CSR), which joins a rearranged variable region to a new downstream constant region, giving rise to different antibody isotypes with new effector functions (Manis et al., 2002)

Hypermutation targets the variable region of rearranged and expressed Ig genes, with the promoter being the upstream boundary of mutation and about 1.5 kb downstream of the J segment, ending before the intronic enhancer and excluding the C region (Lebecque et al., 1990). Analyses of somatic hypermutation show that there are mutation hotspots, i.e. mutations preferentially target the sequence RGYW and its complement WRCY (R= purine, A or G; Y= pyrimidin, C or T; W= A/T) (Rogozin et al., 1996).

The mechanism and molecular basis of SHM has not yet been completely defined but so far SHM is known to occur in centroblasts and a cytidine deaminase, the activated induced deaminase (AID), which is required for SHM and CSR has been discovered (Muramatsu et al., 1999; Muramatsu et al., 2000; Revy et al., 2000). Analysis of the AID amino acid sequence shows that 34% of its amino acids are identical to the cytidine deaminase APOBEC-1, an enzyme that edits a specific mRNA, converting a C to a U and introducing a premature termination in the *APO-B* mRNA. It is suggested that *AID* and *APOBEC-1* arose by duplication of a common progenitor, as they are both located on chromosome 12p13 (Muto et al., 2000).

In the first phase of SHM, AID deaminates cytidine to uracil C→U (Rada et al., 2002), which results in a G:U mismatch at this position. Subsequent replication will lead to C to T and G to A transitions, meaning one daughter cell will carry the wildtype G:C pair and the other daughter cell will carry a U:A pair. Alternatively, AID induces the releasing of uracil by the highly conserved enzyme, uracil DNA for the apurine/apyrimidine endonuclease (APE), which can lead to a single strand break in the DNA, then a repair polymerase primes synthesis on the free 3' end and finally a DNA ligase seals the nick (Neuberger et al., 2005). Mice experiments have shown that during *UNG* (a gene that encodes the uracil-DNA glycosylase) deficiency, the spectrum of hypermutation was altered, supporting the DNA deamination model of SHM (Rada et al., 2002).

In the second phase of hypermutation, it is thought that the mismatch repair system (MMR) (made up of heterodimer MutS homolog complexes) recognizes single base mispairs and mismatches created by single base deletions or insertions as well as it recognizes mismatches of two to four bps. Subsequently, the MMR system recruits other homologs to excise the mismatch and repair it by DNA synthesis. This phase produces mutation at sites other than C/G pairs (Kolodner et al., 1996; Rada et al., 1998).

1.1.2.4 Kappa deleting element (*KDE*)

Immunoglobulin genes follow a developmentally regulated hierarchy for rearrangements (Hieter et al., 1981; Korsmeyer et al., 1981). The principle of allelic exclusion postulates that when the pre B cell precursor successfully rearranges the V_H, D_H and J_H gene segments on one allele, the recombination machinery is halted, preventing further rearrangement of the second allele (Alt et al., 1984). After the rearrangement of the Ig heavy chain locus, the rearrangement of the Ig light chain gene at the Ig κ L chain (*IGK*) and the Ig λ L chain (*IGL*) loci is initiated. This defines the transition of pre-B cell to immature B cell in the process of B cell development (Klein et al., 2005). Generally, the recombination of the *IGK* initiates prior to the *IGL* gene rearrangement, which means κ -producing B cells usually retain germline λ-genes, while λ-producing B cells have rearranged or deleted κ-genes (Siminovitch et al., 1987). The progression from κ- to λ-gene rearrangement is often associated with a specific deletional recombination, whereby the constant κ (Cκ) and a portion of the joining κ (Jκ)-Cκ, including the kappa enhancer sequence, are uniformly eliminated (Durdik et al., 1984; Siminovitch et al., 1987). This κ gene deletion is mediated by a site-specific rearrangement of a DNA element to an RSS located either in the Jκ-Cκ intron or within the Vκ gene region, which leads to the inactivation of a pre-existing Vκ-Jκ joint in either case (Figure 6). This recombinatorial element is known as the kappa deleting element and is rearranged in all kappa-deleted lambda-producing B cells (Siminovitch et al., 1987).

The rearrangement of the Vκ to Jκ segments follows the same pattern as in the $V_HD_HJ_H$ rearrangements, where DNA double-strand breaks are initiated at the heptamer RSS sequence flanking the Jκ segment, resulting in a hairpin formation at the Jκ-break end and a blunt end at the RSS break, which can be ligated to a blunt-ended linker molecule (Klein et al., 2005).

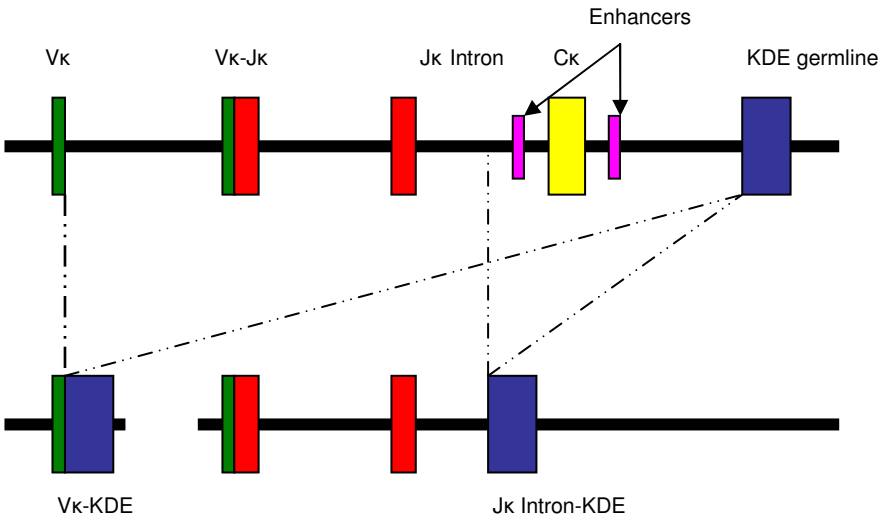


Figure 6: Rearrangement of the *KDE*

Rearrangement of *KDE* shows deletions of a pre-existing Vκ-Jκ joint, Vκ gene segment, Jκ gene segments and Cκ gene segment, when a Vκ RSS is used. Alternatively, when the Jκ intronic RSS is used, *KDE* rearrangement results in deletions of the Cκ gene segment and the κ enhancers (adapted from Klein et al., 2005).

1.1.3 Development of T-lymphocytes

Like B lymphocytes, T lymphocytes originate from hematopoietic stem cells in the bone marrow. It has been shown in mice and humans that the commitment of T cell lineage precursors (TLP) can already occur within the bone marrow, where T cell differentiation begins and they emigrate thereafter to enter the thymus (Dejbakhsh-Jones et al., 2001; Klein et al., 2003). T lymphocyte development is characterized by the expression of cell surface markers and the level of development of the T cell receptor and coreceptors CD4 and CD8. Based on double negative (DN) or double positive (DP) CD4 and CD8 thymocytes, as well as the expression of CD25 and CD44, different levels of T cell development have been defined (Pearse et al., 1989; Godfrey et al., 1993). CD4 is a differentiation antigen expressed on thymocytes and on a subpopulation of mature T cells (Dialynas et al., 1983), CD8 is an antigen coreceptor on the surface of T cells of the thymus (Bierer et al., 1989; Torres-Nagel et al., 1992), CD25 is the interleukin 2 receptor α chain (IL-2Rα) expressed on activated lymphocytes and the glycoprotein CD44 is expressed on HSC but in low levels on leukocytes (Naor et al., 1997).

The most immature thymocytes are $CD4^-$, $CD8^-$, $CD25^-$ and $CD44^+$, which characterizes the DN1 cell-subset, upon entry into the thymus. This cell population has multi-lineage potential and thus the ability to develop into B-cells, T-cells, myeloid-cells, NK- and dendritic cells (Shortman et al., 1996; Wu et

al., 1996). In the DN2 cell-subset ($CD4^-$, $CD8^-$, $CD25^+$, $CD44^+$), which proceeds in the T cell development, the cells still have the ability to develop into multi-lineages as mentioned above, except for the lack of B cell potential (Wu et al., 1996; Schmitt et al., 2004). Final commitment to the T-cell lineage occurs at the DN3 cell stage ($CD4^-$, $CD8^-$, $CD25^+$ $CD44^-$), when extensive rearrangement of the T cell receptor (*TCRB*, *TCRG*, *TCRD*) alleles occurs (Ismaili et al., 1996; Capone et al., 1998). The DN3 cells give rise to cells with in-frame rearranged *TCRB* gene and double positive for $CD4^+$ and $CD8^+$ (DP cells). These cells begin assembling TCRβ and pre-Tα chains to form the cell surface pre TCR complex (Saint-Ruf et al., 1994; Berger et al., 1997). Previous studies have shown a differentiating T cell population in human bone marrow, which expresses the pre-Tα protein on the cell surface (Dejbakhsh-Jones et al., 1999; Klein et al., 2003).

The cortical DP thymocyte population is made up of an unselected repertoire of T cells, which is the main target of positive and negative T lymphocyte selection (Kisielow et al., 1988; Jameson et al., 1995). These thymocytes, which are very motile, interact through their TCR with peptide-(major histocompatibility complex) MHC complexes expressed by stromal and dendritic cells in the cortex (Bousso et al., 2002). After TCR recognition of peptide-MHC ligands during low-avidity interactions, DP thymocytes receive signals for survival and further differentiate into single-positive (SP) thymocytes. In this positive selection process, T cells that are not reactive to self-antigen presented by self-MHC molecules but potentially reactive to foreign antigens are enriched. On the other hand, high-avidity interactions lead to negative selection of T cells by apoptosis in a process, whereby self-reactive T cells are deleted, thus avoiding autoimmunity. Also, DP thymocytes that fail to receive TCR signals are destined to die at this stage. Only 3-5% of developing T cells survives this checkpoint in their development (Egerton et al., 1990). In the T cell development, thymocytes have the ability to determine whether they express either a γδ or αβ TCR complex. A productive rearrangement of the *TCRG/D* genes lead to the expression of a TCR γδ or a productive rearrangement of the *TCRA/B* genes lead to the expression of a TCR αβ (Kearse et al., 1994; Haks et al., 2003).

1.1.3.1 V(D)J recombination in T cells

TCR genes are assembled somatically in differentiating T cells by VDJ recombination in a similar way as B cells (Tonegawa, 1983). Also, RAG initiates the VDJ recombination in the same way, as it introduces DNA double strand breaks (DSBs) between variable region gene segments and the RSS flanking them (Oettinger et al., 1990; Lewis, 1994). Vβs are flanked with 23-RSS and Jβs with 12-RSS, while Dβs have 5' 12-RSS and 3' 23-RSS. Yet, despite the 12/23 compatibility, Vβs do not commonly rearrange directly to Jβs because the 5' Dβ1 12-RSS specifically targets rearrangement of a

Vβ in a precise and position independent manner. This phenomenon is known as the beyond 12/23 restriction (Bassing et al., 2000; Sleckman et al.; 2000).

In the α-chain, the Vα gene segment combines to the Jα gene segment to form a functional exon. Due to transcription and splicing of the VJα-exon to the Cα, the mRNA is formed and then translated to the α-chain protein of the TCR. In the β-chain, the Vβ, Dβ and Jβ gene segments combine to form a functional exon, which is transcribed and spliced to the Cβ and the mRNA is then translated to the β-chain of the TCR (Kronenberg et al., 1986). For more details on the influence of RAG protein on VDJ recombination, refer to section 1.1.2.2.

1.1.3.2 Structure of the T-cell receptor

The TCR is composed of a glycolysated heterodimer, either γδ or αβ, which is associated with a membrane-bound complex of proteins collectively known as CD3 (Kappler et al., 1983; Meuer et al., 1983; Ashwell et al., 1990). Each polypeptide chain of the heterodimer is located on the extracellular side of the membrane, having a hydrophobic region toward the C terminus with a short cytoplasmic tail of 2-7 amino acids (Campbell et al., 1994). Both polypeptide chains are linked by a cystein bridge in the extracellular region close to the membrane of the T cell (Bentley et al., 1996). Structurally, the TCR is similar to the Fab-fragment of the immunoglobulin.

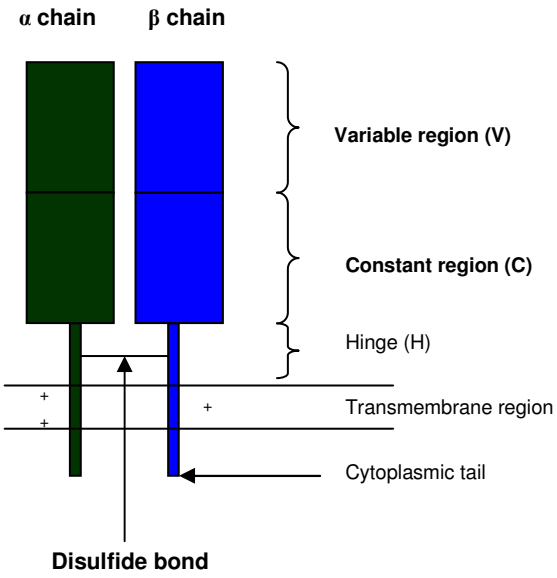


Figure 7: Structure of TCRαβ heterodimer

Figure 7 shows the extracellular variable and constant regions, the transmembrane region and the cytoplasmic tail of the TCRαβ. The transmembrane helices of both chains have positively charged basic rests.

The variable regions are made up of V-, D-, and J- segments in the β chain (like the Ig heavy chain) and V- and J- segments in the α chain (like the Ig light chain). As in B cells, the segments rearrange through V(D)J recombination to form a complete functional V region of the TCR. The gene segments of the TCR are also flanked by nonamer and heptamer RSS, which are also recognized by the same enzymes like in B cells during recombination. Another similarity is the presence of P- and N-nucleotides during the recombination of the TCR gene segments of β, as well as α chain (Schlissel et al., 1993). The constant region of the TCR is much simpler than the Ig constant region: there is only one Cα-gene segment and two Cβ gene segments as Figure 7 illustrates.

The γ and δ polypeptides are found on the T cell surface and associated to the membrane bound CD3-complex (Clevers et al., 1988; Malissen et al., 1993). The *TCRG* and *TCRD* genes also have V-, D-, J-, and C-gene segments. The gene that encodes the TCR δ chain is nested in the *TCRA* gene between the Vα and the Jα gene segments, so that a rearrangement of the TCRα chain inactivates the *TCRD* gene. There are three Dδ, three Jδ, one Cδ gene segments and the Vδ gene segments lie between the Vα gene segments. The γ chain comprises of 12 Vγ gene segments, two Cγ gene segments with three and two Jγs respectively (Chien et al., 1987; Satyanarayana et al., 1988; Hockett et al., 1988; Zhong et al., 1997).

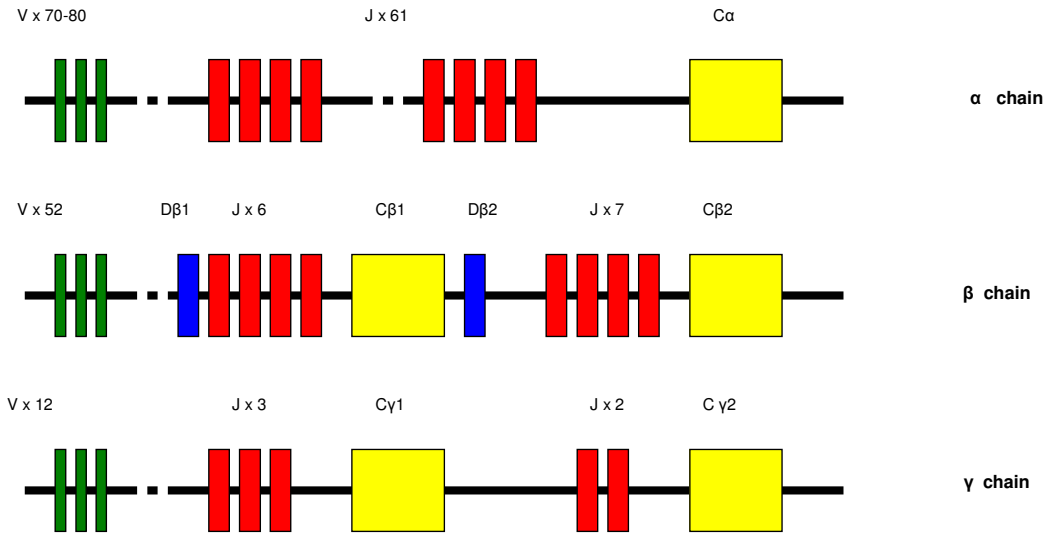


Figure 8: Organization of the TCR α, β and γ chains, showing the V,D,J and C gene segments

1.2 Biology of chronic myeloid leukemia

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder, which is genetically characterized by the presence of the reciprocal translocation t(9;22)(q34;q11), resulting in a *BCR-ABL1* fusion gene on the derivative chromosome 22 called the Philadelphia chromosome (Rowley, 1973). This leukemia develops in three clinical stages, namely: 1) the chronic phase (CP), which is characterized by the accumulation of myeloid progenitors and mature cells in bone marrow, peripheral blood and extramedullary sites (Spiers et al., 1977; Savage et al., 1997). This preliminary stage could last for several years. 2) The accelerated phase (AP), which is characterized by an increase in the frequency of progenitors over a period of about four to six months. 3) Blast crisis (BC) is characterized by a rapid increase in differentiation-arrested blast cells of myeloid or lymphoid origin and lasts only a few months (Kantarjian et al., 1987). In most cases the t(9;22) or its variant is the sole chromosomal anomaly in the CP of the disease but in the course of the disease to the BC phase, 60-80% additional genetic changes are observed (Johansson et al., 2002). The *BCR-ABL1* fusion gene encodes the p210 oncoprotein found in almost all CML patients (Shtivelman et al., 1986; Ben-Neriah et al., 1986). The *BCR-ABL1* fusion gene promotes secondary molecular and chromosomal changes that lead to reduced apoptosis susceptibility, increased proliferation potential and differentiation arrest, as can be observed in CML-BC cells (Shet et al., 2002).

1.2.1 Effect of *BCR-ABL1* translocation

The effects of *BCR-ABL1* on the proliferation and survival of chronic myeloid leukemia cells are characterized by the activation of signal transduction pathways like the RAS, PI-3K/Akt and STAT5 pathways in cells. These activated pathways are responsible for growth factor independence and reduced predisposition to apoptosis of *BCR-ABL1* positive cells. This has been observed by the ectopic expression of p210 oncoprotein in hematopoietic cell lines (Daley et al., 1988; Laneuville et al., 1992; Cortez et al., 1995). The p210 oncoprotein has constitutive tyrosine kinase activity, which is important for cell transformation. P210 is localized in the cytoplasm, thus allowing the assembly of phosphorylated substrates in multiprotein complexes. This results in the transmission of mitogenic and antiapoptotic signals, as described below (Konopka et al., 1985; McWhirter et al., 1991; Lugo et al., 1990; Cortez et al., 1995).

BCR-ABL1 constitutively activates the RAS pathway upon interaction, leading to the enhanced activity of the guanosine di-/tri-phosphate (GDP/GTP) exchange factor SOS, and thus promoting the accumulation of the active GTP-bound form of RAS. It has been observed that down-regulation of the

RAS pathway suppresses proliferation and sensitizes cells to apoptotic stimuli (Sawyers et al., 1995; Skorski et al., 1994). The RAS pathway can be activated, for example when BCR-ABL1 interacts with the growth factor receptor-binding protein (GRB-2)/Gab2 complex and the SHC adaptor is phosphorylated (Goga et al., 1995; Sattler et al., 2002).

Another pathway activated by the ectopic expression of *BCR-ABL1,* as well as in primary CML cells (Skorski et al., 1994) is the phosphatidylinositol-3 kinase (PI-3K)/Akt pathway. Activation of PI-3K pathway provokes an Akt-dependent cascade that regulates localization or activity of targets like *BAD,* MDM2 or IκB-kinase-α. Phosphorylation of BAD suppresses its proapoptotic activity, which is the case in BCR-ABL1- expressing cells (Salomoni et al., 2000). IκB-kinase-α phosphorylation enhances NF-κB activity, which is associated with BCR-ABL1-dependent transformation of primary mouse marrow cells (Reuther et al., 1998). Activation of Akt by BCR-ABL1 may also enhance the proliferation of cells by disruption of the cyclin-dependent kinase (CDK) inhibitor p27 (Gesbert et al., 2000; Parada et al., 2001; Jiang et al., 2000).

The signal transducer and activator of transcription 5 (STAT5)-pathway is an antiapoptotic pathway also activated by BCR-ABL1. When activated, this kinase phosphorylates tyrosine 694 (Y694) of STAT5, translocating it to the nucleus where it functions as a transcription factor. Other target genes of STAT5 which are transcriptionally activated are the antiapoptotic *BCLXL* gene, *A1* and *PIM* which might play a role in *BCR-ABL1* leukemogenesis. STAT5 has been found to be constitutively active in chronic CML and CML-BC primary samples (Chai et al., 1997; Huang et al., 2002).

The p210 activity in stem cells or primitive progenitors promotes both symmetrical (to maintain the pool of stem cells) and asymmetrical (to generate committed late progenitors) cell divisions (Clarkson et al., 2003). A molecular point of view of cell cycle regulation by BCR-ABL1 suggests that BCR-ABL1 induces an exit from quiescence in primitive cells due to its effect on cyclin and CDK inhibitors. Such is the case with cyclin D2 which has increased levels and stimulates proliferation in BCR-ABL1-lymphoblasts or CDK inhibitor, p27, which is down-regulated or cytoplasmic relocated in BCR-ABL1- expressing lymphoid and myeloid cells (Jena et al., 2002; Jonuleit et al., 2000).

BCR-ABL1 expression also has an effect on the activity or levels of proteins involved in DNA repair, especially the repair of DNA double-strand breaks (DSBs). The repair of DSBs by the DNA-PK-dependent pathway (nonhomologous end-joining recombination, NHEJ) is the preferred pathway used by normal human cells (Wang et al., 2001). In BCR-ABL1- expressing cells the DNA protein kinase catalytic subunit (DNA-PK_{CS}) has been observed to be down-regulated by proteasome inhibitors, leading to proteasome-dependent protein degradation (Deutsch et al., 2003). BCR-ABL1 in CML cells has also been associated with down-regulation of BRCA-1, a protein involved in genome integrity

surveillance or the enhanced expression of RAD51, a protein involved in homologous recombination repair (Slupianek et al., 2001). These effects of BCR-ABL1 on DNA-PK_{CS}, BRCA-1 and RAD51 might promote genomic instability, thus causing defective repair of DSBs (Deutsch et al., 2003; Starita et al., 2003; Jasin et al., 2002).

1.2.2 Disease progression in CML

The evolution of CML to CML-BC is accompanied by many cytogenetic, molecular and phenotypic changes in most CML patients. This evolution consists of growth factor-independent proliferation and differentiation arrest, which differentiates blast crisis from chronic phase CML (Sirard et al., 1996; Trotta et al., 2003). The cytoplasm-localized BCR-ABL1 plays a role in the disease initiation and other mutations of genes encoding nucleus localized proteins are involved in most secondary changes of CML-BC. These supplementary mutations add to the effects of BCR-ABL1, when:
1) transcription is directly activated (NOP98/HOXA9) or repressed (AML-1/EVI-1);
2) a nonfunctional transcription factor (e.g. a p53 mutant) is generated;
3) the activity of transcription factors involved in DNA synthesis or in cell cycle check points is indirectly modulated (e.g. the homozygous deletions of the *p16INK4A/ARF* locus leads to the inactivation of the Rb pathway/ enhanced activity of the *E2F* family genes or the MDM2-dependent inactivation of *p53*).
These changes might lead to the inactivation of tumor suppressor genes like *p53*, differentiation arrests by down-regulating differentiation-regulatory genes, as well as proliferation by the activation of proliferation pathways (Sirard et al., 1996; Trotta et al., 2003). The inactivation of p53 when comparing chronic phase and blast crisis CML could be the most common abnormality, as p53 is mutated in 25 to 30% of myeloid CML-BC; deletions of the *p16INK4A/ARF* locus affecting p53 function is found in 50% of lymphoid CML-BC and the expression of MDM2 which down-regulates p53 is detected in mononuclear CML-BC cells (Trotta et al., 2003).

1.3 Flow cytometry

The Fluorescent Activated Cell Sorter (FACS) dates back to the 1960s, when it was invented by Bonner, Sweet, Hulett and Herzenberg for the purpose of flow cytometry and cell sorting of viable cells (Herzenberg et al., 2002). Flow cytometry is a technology used to analyze the physical characteristics of cells/particles as they flow in a fluid stream through a beam of light. This is achieved by using an optical-to-electronic coupling system that detects and shows how the cells scatter laser light and emit fluorescence. The flow cytometer consists mainly of three systems: fluidics, optics and electronics (Herzenberg et al., 1976).

The fluidic system transports cells in a stream to the laser beam for interrogation, whereby only one cell should move through the laser beam at a given moment. The stream flows in a flow chamber with the sample core in the middle of the stream and based on the principles of laminar flow and hydrodynamic focusing, the sample core remains separate but coaxial within the sheath fluid. The sample pressure is always greater than the sheath fluid pressure (Nooter et al., 1990; Scampavia et al., 1995).

The optic system is made up of lasers to illuminate the cells in the sample stream and optical filters to direct the light signals to the detectors. The light scatter occurs as a result of deflection of laser light by a cell and this depends on the physical properties (size and internal complexity like granularity) of the cell. There are two kinds of scatters; the forward scatter (FSC), which is proportional to the cell surface area or size of the cell, is a measurement of light defracted by the cell and the side scatter (SSC), which is proportional to the cell granularity, is a measurement of light refracted and reflected by the cell. Fluorochromes on antibodies for detecting surface markers on cells can be excited with the help of the light emitted by the laser, depending on their absorption and emission spectrums (Kamentsky et al., 1965; Merkel et al., 1992). While the flow cytometry technology was dependent on two or three fluorescent dyes in the beginning, the use of eight to eleven colors simultaneously is now possible in cytometric analyses (Roederer et al., 1997; De Rosa et al., 2001).

The electronic system converts the detected light signals into electronic signals by photo detectors then assigned to the computer. In the cell sorter, the electronic system initiates sorting and deflects cells to be sorted, i.e. single cells or purified cell populations can be physically separated by flow cytometry for further analyses. This technology of separation has a higher degree of specificity when compared to other methods like centrifugation or magnetic bead separation (van den Engh et al., 1980).

1.4 Aim of this thesis

Chronic myeloid leukemia (CML), a disease associated to the Philadelphia chromosome, develops as a tri-phasic myeloproliferative disorder, leading to a fatal, frequently lymphoid blast crisis. This study addresses the evolution of CML in blast crisis (CML-BC) by analyzing the *IGH* genes, *TCRB* and *TCRG* genes of CML-BC cells for lymphoid specific rearrangements and mutations, as well as verifying the expression of lymphoid specific proteins like RAG and AID in CML-BC cells.

The immunoglobulin variable region of antigen-activated B cells in the germinal center is subject to somatic hypermutation, thus creating variants with higher affinity to antigens to become memory B cells or plasma cells (Weigert et al., 1970; Bernard et al., 1978; Jacob et al., 1991). SHM is not restricted to *IGHV* but has also been found in other GC B cell genes like *BCL6*, *CD79A* and *B* as well as *CD95* (Peng et al., 1999; Pasqualucci et al., 1998; Gorden et al., 2003; Müschen et al., 2000). Honjo et al. in 2003 also showed that ectopic expression of *AID* in transgenic mice leads to T lymphoma cells with SHM in the variable region of *TCRB* genes and *c-myc*. AID has been shown to be essential for SHM and class switch recombination (Muramatsu et al., 1999). Previous studies have given evidence that a small population of antigen-specific T cells in the GC acquire mutations in the variable region of *TCRA* genes (Zheng et al., 1994), while SHM has been observed in *TCRB* genes of HIV patients (Cheynier et al., 1998).

Based on these findings, this thesis will focus on whether:

1) Lymphoid specific *IGHV*, κ-deleting element (*KDE*), *TCRBV* and *TCRGV* region gene rearrangements occur in CML-BC cells;
2) Somatic hypermutation (SHM), a characteristic of mature B cells, is observed in the *IGHV*, *TCRBV* and *TCRGV* region genes of CML-BC cells;
3) The expressions of RAG and AID proteins, which in normal lymphoid cells are respectively a prerequisite of V(D)J gene rearrangement and SHM, are found in blast crisis of CML.

Chapter 2

Materials and Methods

2.1 Flow Cytometry analyses

2.1.1 Materials

- 1x PBS [237mmol/l NaCl; 2.7 mmol/l KCl; 8.1 mmol/l Na_2HPO_4; 1.5 mmol/l KH_2PO_4; (pH 7.4)]
- 1x PBS/EDTA [PBS + 0.05% EDTA]
- Propidium Iodide solution (PJ) [2µg/ml in PBS/EDTA] (Sigma)

HSC antibody:

- Anti-CD34 antibody (BD Biosciences)

B-lymphoid antibodies:

- Anti-CD5 antibody (BD Biosciences)
- Anti-CD10 antibody (BD Biosciences)
- Anti-CD19 antibody (BD Biosciences)
- Anti-CD20 antibody (BD Biosciences)

T-lymphoid antibodies:

- Anti-CD3 antibody (BD Biosciences)
- Anti-CD4 antibody (BD Biosciences)
- Anti-CD5 antibody (BD Biosciences)
- Anti-CD7 antibody (BD Biosciences)
- Anti-CD8 antibody (BD Biosciences)
- Anti-human TCRαβ antibody(BD Biosciences)
- Anti-human TCRγδ antibody(BD Biosciences)

Myeloid antibodies:

- Anti-CD13 antibody (BD Biosciences)
- Anti-CD14 antibody (BD Biosciences)
- Anti-IL3Rα antibody (BD Biosciences)
- Anti-GM-CSFRα antibody (BD Biosciences)

Erythroid antibody:

- Anti-Gly A antibody (BD Biosciences)

Cell lines from DSMZ, Braunschweig, Germany:

- BV173
- Nalm1
- CML-T1
- K562
- KCL-22
- JURL
- EM2
- KU182
- LAMA84
- JK1
- MEG01

2.1.2 Method

The flow cytometric characterization of the CML-BC cases was achieved by staining the cells with the above mentioned antibodies against various B lymphoid, T lymphoid, myeloid and erythroid cell surface markers. The antibodies carry a fluorescent-dye for example fluorescein isothiocyanat (FITC) and phycoerythrin (PE). About 10^6 cells are required for the FACS analyses. The cells are first washed in PBS/EDTA then centrifuged at 2000rpm for 5 minutes. The cell pellet obtained after centrifugation is then resuspended in 20µl PBS/EDTA before being stained with the required antibody. 3µl of the FITC antibody is then added to the cells, while only 2µl of the PE antibody suffices, due to the higher intensity of the PE-dye. The cells are then incubated at room temperature for 15 minutes on a shaker, thus enabling equal antibody distribution. After the incubation, the cells are then washed in 500µl PBS/EDTA to remove surplus antibodies and avoid over-dyeing. The probes are then centrifuged at 2000rpm for 5 minutes. The pellet obtained after centrifugation is then resuspended in 300µl PI solution, a fluorescent substance that can only enter cells with defects in the cell
membrane and is hence an indicator for dead cells.The cell/PJ suspension are transfred in the FACS tubes and the cell surface characteristics can then be measured using the BD FACScan.

2.2 Preparing Samples

2.2.1 Cell lines and Primary cases

The above mentioned Ph+ CML-BC cell lines (BV173, Nalm1, CML-T1, K562, KCL-22, JURL, EM2, KU182, LAMA84, JK1, MEG01) were obtained from DSMZ, Braunschweig, Germany and cultured as described below. Five Ph+ CML-BC primary cases were provided by Prof. Hoffmann of the Charité Berlin.

2.2.2 Culturing of cell lines

Material:

- RPMI 1640 GlutaMAX
- Fetal calf serum (Gibco)
- Penicillin/streptomycin
- Incubator CB210 (Binder)
- Sterile work-station (Antair)

Method:
Cell lines were cultured using RPMI 1640 GlutaMAX TM medium supplemented with 20 % fetal calf serum (FCS) and 1% penicillin/streptomycin. The cells were incubated at 37°C in an atmosphere of 5% CO_2. Fresh medium was added to the cell suspension every second or third day.

2.2.3 RNA isolation

Material:

- Rneasy ® Kit (Qiagen)
- B- Mercaptoethanol
- dH_2O_{DEPC}
- chilled 70% ethanol
- centrifuge

Method:

The isolation of RNA was carried out using the Rneasy ® Kit (Qiagen). It is important to be aware of the risks at hand when working with RNases. To avoid RNA degradation by RNases the whole isolation process is done with sterile equipment and work is carried out on ice.
The isolation procedure is divided into four distinct steps: cell lyses, application of RNA to the silica gel membrane, washing the membrane, elution of the RNA.

The cells are lysed by adding RLT buffer containing 10 µl β-Mercaptoethanol/ml, thereby inactivating RNases. One volume of 70% ethanol is then added to the sample and mixed carefully by pipetting. The whole mixture is transferred into a spin column placed in a 2 ml collection tube and later centrifuged. After centrifugation for 15 seconds at full speed, the
RNA binds to the silica gel membrane. The flow-through is discarded and the silica gel is washed through following washing steps:
1) 700 µl of buffer RW1; centrifugation, 15 seconds full speed; discard flow-through
and collection tube, column is transfered into a new collection tube.
2) 500 µl of buffer RPE; centrifugation, 15 seconds full speed; discard flow-through.
3) 500 µl of buffer RPE; centrifugation, 2 minutes full speed; discard flow-through.
4) Centrifugation for 1 minute at full speed to eliminate any buffer RPE carryover, which contains ethanol and can thus inhibit enzymatic reactions.
To elute the RNA, the column is transferred into a 1.5 ml collection tube. 30 µl of RNase-free water is pipetted on the membrane and centrifuged for 1 minute at full speed. Subsequently the RNA can be checked on an agarose gel and subjected to cDNA syntheses.

2.2.4 cDNA synthesis

Material:

- SuperScriptTM III Reverse Transcriptase (RT) (Invitrogen)
- Oligo-dT (MWG)
- dNTP Mix of dTP, dCTP, dGTP, dTTP (Peqlab)
- First-strand buffer (Invitrogen)
- DTT (Invitrogen)
- RNaseOUTTM (Invitrogen)

Method:

The mRNA is the holder of genetic information expressed in cells. RNA is very sensitive to degradation and should therefore be treated on ice and directly transcribed into the much more stable cDNA after RNA isolation. This is achieved by using the enzyme SuperScriptTM III Reverse Transcriptase (RT), which is derived from Moloney Murine Leukaemia Virus and is capable of translating RNA into DNA. This RT is highly thermostable and initiates translation starting from a primer bound to the RNA template. An oligo-dT primer that binds to the polyA-tail of mRNA is used, ensuring that only mRNA and no ribosomal RNA etc is translated into cDNA.

The following components: 2 µl of µM oligo-dT50, 2 µl of 10 mM dNTP Mix are added to the isolated RNA. The sample is then heated at 65°C for five minutes to degrade secondary structures of the RNA and allow the primers to bind to the polyA-tails. Afterwards the sample is incubated on ice for at least one minute and a mixture of the following ingredients is added:
8 µl of First-strand buffer, 2 µl of 0,1 M DTT, 2 µl of 40 units/µl RNaseOUTTM, 2 µl of 200 units/µl SuperScriptTM III RT. After carefully mixing the sample by pipetting, it is incubated at 50°C for 1 hour. To inactivate the RT the sample is finally heated at 70°C for 15 minutes. The obtained cDNA can be stored at -20°C.

2.2.5 Isolation of genomic DNA

Material:

- QIAamp® DNA Blood Mini Kit (Qiagen)
- Ethanol (96-100%)
- Centrifuge
- Vortex-machine

Method:

For the analysis of germline configuration of genes, DNA alterations like mutations or SSBs, the genomic DNA of animal cells can be isolated with help of the QIAamp® DNA Blood Mini Kit. The procedure which is similar to the isolation of RNA is based on four distinct steps:
• Cell lyses
• Application of DNA on a silica gel membrane
• Washing the membrane
• Elution of the DNA

200 µl of cells suspended in PBS are added to 20 µl of Proteinase K and mixed with 200 µl of buffer AL by pulse-vortexing for 15 seconds. The samples are heated to 56°C for 10 minutes and afterwards briefly centrifuged to remove drops from the inside of the lid of the reaction tube. 200 µl of ethanol are added and the mixture is homogenized by pulse-vortexing for another 15 seconds. That makes it necessary to centrifuge the tubes again briefly. The whole mixture is applied to a QIAamp Spin Column placed in a 2 ml collection tube and centrifuged at full speed for 1 minute. The flow-through and collection tube are discarded afterwards and the column is transferred into a new collection tube. Three washing steps follow:
1) 500 µl buffer AW1; centrifugation, 1 minute at full speed; discard flow-through and collection tube, column is transfered into a new one.
2) 500 µl buffer AW2; centrifugation, 3 minutes at full speed; discard flow-through.
3) Centrifugation, 1 minute at full speed to eliminate any buffer AW2 carryover.

To elute the DNA the column is transferred into a sterile 1,5 ml tube and 200 µl of buffer AE are added. After an incubation of 1 to 5 minutes at room temperature the sample is centrifuged at full speed for 1 minute. The obtained DNA solution can be stored at -20°C.

2.2.6 Isolation of mononuclear cells from peripheral blood by Ficoll density gradient centrifugation

Material:

- Buffy coats from peripheral blood
- Ficoll
- Fetal calf serum (Gibco)
- 1x PBS [237mmol/l NaCl; 2.7 mmol/l KCl; 8.1 mmol/l Na_2HPO_4; 1.5 mmol/l KH_2PO_4; (pH 7.4)]
- 1x PBS/EDTA [PBS + 0.05% EDTA]
- NH4Cl-solution

Method:

B and T lymphocytes from peripheral blood were extracted from the so called Buffy coats which are rich of lymphocytes but still contain a high number of erythrocytes and other cells of the peripheral blood. A density gradient centrifugation is performed to separate the leukocytes from the erythrocytes. Ficoll is a substance with a lower density than erythrocytes but a higher density than lymphocytes, therefore suitable for the separation of these cell types.
First the buffy coat has to be diluted 1:2 (v/v) with PBS supplemented with 5% of FCS. Then the mixture is carefully filled into 50 ml Falcon tubes that already contain 15 ml of ficoll. A mixture of buffy coat and ficoll should be strictly avoided. The tubes are centrifuged at 2000 rpm for 30 minutes without breaks and with the slowest acceleration possible. After centrifugation, the lymphocytes are found in a white ring located just on top of the ficoll and are transferred into sterile 50 ml Falcon tubes. Subsequently the tubes are centrifuged at 2000 rpm for 10 minutes to obtain a concentrated mass. The supernatant is discarded and the pellet is washed once in PBS with 5% FCS. After an additional round of washing by centrifugation the cells are subjected to lyses of the remaining erythrocytes. The majority of erythrocytes has already been depleted by the density gradient centrifugation but in most cases some are still present in the isolated lymphocyte solution. A lysis of the erythrocytes can be performed by resuspending the cell pellet in 15 ml of cold NH_4Cl-solution followed by incubation on ice for 7 minutes. Thereafter, the 50 ml tubes are filled with PBS and centrifuged to discard of the surplus NH_4Cl and the lysed erythrocytes. The obtained lymphocytes can be used for further analyses.

2.2.7 Isolation of $CD19^+$ and $CD3^+$ cells by magnetic cell sorting (MACS)

Material:

- Anti-CD19 MACS Beads (BD Biosciences)
- Anti-CD3 MACS Beads (BD Biosciences)
- LS and LD MACS columns (Miltenyi)
- MACS magnetic stand (Miltenyi)
- 1x PBS/EDTA [PBS + 0.05% EDTA]
- Rotary shaker

Method:

The lymphocyte cell pellet obtained from the ficoll density gradient centrifugation is resuspended as required in PBS. For magnetic cell sorting of CD19 or CD3 positive cells, an anti-CD19 antibody or an anti-CD3 antibody conjugated to magnetic beads is used. 200 µl of the MACS beads are added to 10^8 cells. The sample is incubated at room temperature on a rotary shaker for 15 minutes. During this time an LS or LD MACS column is put into a magnetic stand and equilibrated with PBS. After incubation, unbound MACS beads are removed by adding 15 ml of PBS to the sample and centrifuging at 2000 rpm for 5 minutes. The pellet is resuspended in 3 ml of PBS and applied to the MACS column. To avoid agglutination, PBS containing 0,5% EDTA should be used and the cell suspension should be filtered on application to the column. The $CD19^+$ or $CD3^+$ cells carry magnetic beads on their surface and are therefore held in the MACS column as long as it is placed in the magnetic stand. The flow through can be discarded or subjected to isolation of further cell subsets. To remove unbound cells from the column, the column is washed thrice with 5 ml of PBS. The required cells are eluted by adding 5 ml of PBS onto the column. The column is removed from the magnetic stand and the
PBS together with the cells is pressed out into a 15 ml falcon tube.

2.3 Analyses of prepared samples by polymerase chain reaction (PCR)

The amplification of genes or gene segments important for this thesis was carried out by polymerase chain reaction (PCR). The PCR enables the amplification of enough starting material for a sequence reaction. A solution of DNA or cDNA derived from a sample consisting of a high number of cells serves as template for the amplification process. To analyze the PCR products individually, they are separated by cloning them into a TOPO vector and subsequently transforming them into E.coli. Each emerging E.coli clone harbors one PCR product that can be amplified by Colony-PCR and sequenced thereafter.

Material:

- dNTP mix [10 mmol/l each] (Invitrogen)
- 10x PCR-buffer (Invitrogen)
- $MgCl_2$ (Invitrogen)
- Platinum *Taq* Polymerase (Invitrogen)
- Primers (MWG Biotech)
- T1 thermocycler (Biometra)
- dH_2O

Method:

Polymerase Chain Reaction (PCR) is a method utilized for the amplification of a specific DNA fragment. The PCR starts with the elongation process, whereby an oligonucleotide (primer) is bound to the template-DNA with help of an enzyme called DNA-Polymerase. The DNA-Polymerase links nucleotides complementary to the template strand, during elongation. The resulting double strand DNA (dsDNA) is repeatedly denatured to allow the binding and elongation of new primer molecules. After 30 to 45 rounds of repetition the target DNA fragment is highly multiplied. For the amplification of Ig heavy chain alleles as well as the other genes and gene segments verified in this thesis, Platinum *Taq* DNA-Polymerase was used. This enzyme is highly thermostable and inactive because of an antibody that is bound to the polymerase. An initial heating step of 95°C for two minutes denatures the antibody and thereby activates the enzyme. MgCl2 is important for the PCR reaction as it offers a suitable environment for the *Taq*-polymerase activity. dNTP-mix is made up of the four different desoxy-nucleosid-triphosphates (dNTPs): dATP, dCTP, dGTP and dTTP, which serve as substrate for the enzyme. The annealing temperature is determined by the melting temperature of the primers, while the elongation temperature is dependent on the temperature optimum of the DNA polymerase.

The whole reaction is prepared in a volume of 50 µl and pipetted into a 200 µl reaction tube. The tubes are placed into a thermocycler where the reaction is performed and the success of the reaction can be controlled by an agarose gel electrophoresis.

To analyze the expression pattern of a special gene in different cell types, the RNA of the cells of interest is isolated and the contained mRNA copied into cDNA by a reverse transcriptase (RT). By the use of gene specific primers for PCR on cDNA the expression of this gene can be determined. For the choice of the appropriate primer pairs it is important to keep in mind, that cDNA is derived from mRNA which only contains exon sequences but no introns. It is advisable to choose primers that span exon-exon borders or primer pairs that are separated by large intronic sequences to avoid amplification of genomic DNA. This is of interest because during the RNA extraction small amounts of genomic DNA can also be isolated. PCR products obtained from genomic DNA could lead to false positive results in an RT-PCR.

To ensure the integrity of the cDNA used in a PCR for the amplification of a gene, a housekeeping gene, *GAPDH*, is first analyzed by PCR. This gene is expressed ubiquitously in different cell types and PCR products for *GAPDH* should be obtained independently of the cDNAs used.

For each primer pair used the optimal PCR conditions have to be established. That includes the optimal concentrations of MgCl2, dNTPs and primers, and the appropriate annealing temperature and elongation time.

2.3.1 Amplification of the *GAPDH* housekeeping gene

GAPDH-PCR reation mix:

36 µl	H_2O
5 µl	10 x PCR buffer
2 µl	MgCl2 50mM
1 µl	dNTP mix 10 mM
1 µl	Platinum *Taq* polymerase
2 µl	*GAPDH* forward primer 2,5 µM
2 µl	*GAPDH* reverse primer 2,5 µM
1 µl	template (cDNA)
50 µl	end volume

GAPDH-PCR cycler program: 45 cycles

95°C	2 min.
95°C	50 sec.
57°C	30 sec.
72°C	1 min.
72°C	5 min.
4°C	pause

2.3.2 Amplification of Ig heavy chain genes by polymerase chain reaction

PCR-reaction mix:

36 µl	dH_2O
5 µl	10x PCR buffer
2 µl	$MgCl_2$ 50mM
1 µl	dNTP-Mix 10mM each
1 µl	Platinum Taq Polymerase
2 µl	VH-primer 2,5µM
2 µl	JH-primer-mix 2,5µM each
1 µl	template (can be varied according to the cDNA concentration of the sample)
50 µl	end volume

PCR-program: 45 cycles

95°C	2 min.
95°C	1 min. DNA-denaturation
63°C	1 min.; primer annealing
72°C	1 min. Elongation
72°C	5 min.
4°C	pause

2.3.3 Amplification of Ig germline configuration

PCR-reaction mix:

36 µl	dH_2O
5 µl	10x PCR buffer
1.5 µl	$MgCl_2$ 50mM
1 µl	dNTP-Mix 10mM each
1 µl	Platinum Taq Polymerase
2.5 µl	D_H7-27 -primer 2,5µM
2.5 µl	3' JH-primer-mix 2,5µM each
0.5 µl	template (genomic DNA)
50 µl	end volume

PCR-program:

95°C	2 min.
95°C	50 sec. DNA-denaturation
65°C	30 sec. 45 cycles; primer annealing
72°C	1 min. Elongation
72°C	5 min.
4°C	pause

2.3.4 Amplification of TCR Vβ gene segments by two rounds polymerase chain reaction

First PCR round

PCR-reaction mix:

34 µl	dH_2O
5 µl	10x PCR buffer
2 µl	$MgCl_2$ 50mM
1 µl	dNTP-Mix 10mM each
1 µl	Platinum Taq Polymerase
2.5 µl	Vβ-primer-mix 42µM
2.5 µl	3' Jβ-primer-mix 42µM
2 µl	template (cDNA)
50 µl	end volume

PCR-program: 25 cycles

95°C	2 min.
95°C	50 sec. DNA-denaturation
61°C	30 sec. annealing
72°C	1 min. Elongation
72°C	5 min.
4°C	pause

Second PCR round

PCR-reaction mix:		PCR-program: 45 cycles	
35 µl	dH_2O	95°C	2 min.
5 µl	10x PCR buffer	95°C	50 sec. DNA-denaturation
2 µl	$MgCl_2$ 50mM	61°C	30 sec. annealing
1 µl	dNTP-Mix 10mM each	72°C	1 min. Elongation
1 µl	Platinum Taq Polymerase	72°C	5 min.
2.5 µl	Vβ-primer-mix 42µM	4°C	pause
2.5 µl	5' Jβ-primer-mix 42µM		
1 µl	template (1st round PCR product)		
50 µl	end volume		

2.3.5 Amplification of TCR Cβ1 and Cβ2 germline configurations

PCR-reaction mix:		PCR-program: 45 cycles	
35 µl	dH_2O	95°C	2 min.
5 µl	10x PCR buffer	95°C	1 min. DNA-denaturation
2 µl	$MgCl_2$ 50mM	59°C	30 sec. annealing
1 µl	dNTP-Mix 10mM each	72°C	1 min. Elongation
1 µl	Platinum Taq Polymerase	72°C	5 min.
2.5 µl	Dβ1/ Dβ2-forward primer 2,5µM	4°C	pause
2.5 µl	Cβ1/ Cβ2-reverse primer 2,5µM		
1 µl	template (genomic DNA)		
50 µl	end volume		

2.3.6 Amplification of TCR Vγ gene segments

PCR-reaction mix:	
35 µl	dH_2O
5 µl	10x PCR buffer
2 µl	$MgCl_2$ 50mM
1 µl	dNTP-Mix 10mM each
1 µl	Platinum Taq Polymerase
2.5 µl	Vγ-primer-mix 42µM
2.5 µl	Jγ1.1/ 2.1-primer-mix 42µM
1 µl	template (cDNA)
50 µl	end volume

PCR-program: 45 cycles	
95°C	2 min.
95°C	50 sec. DNA-denaturation
63°C	30 sec. annealing
72°C	1 min. Elongation
72°C	5 min.
4°C	pause

2.3.7 Amplification of kappa deleting element (KDE)

PCR-reaction mix:	
35.2 µl	dH_2O
5 µl	10x PCR buffer
1.8 µl	$MgCl_2$ 50mM
1 µl	dNTP-Mix 10mM each
1 µl	Platinum Taq Polymerase
2.5 µl	Jκ-Cκ intron primer (Forward) 2.5µM
2.5 µl	KDE primer (Reverse) 2.5µM
1 µl	template (genomic DNA)
50 µl	end volume

PCR-program: 45 cycles	
95°C	2 min.
95°C	50 sec. DNA-denaturation
61°C	30 sec. annealing
72°C	1 min. Elongation
72°C	5 min.
10°C	pause

2.3.8 Amplification of kappa deleting element (KDE) germline configuration

PCR-reaction mix:	
35 µl	dH_2O
5 µl	10x PCR buffer
2 µl	$MgCl_2$ 50mM
1 µl	dNTP-Mix 10mM each
1 µl	Platinum Taq Polymerase
2.5 µl	KDE germline primer (Forward) 2.5µM
2.5 µl	KDE primer (Reverse) 2.5µM
1 µl	template (genomic DNA)
50 µl	end volume

PCR-program: 45 cycles	
95°C	2 min.
95°C	50 sec. DNA-denaturation
61°C	30 sec. annealing
72°C	1 min. Elongation
72°C	5 min.
10°C	pause

2.3.9 Amplification of the recombination activating genes (*RAG1* and *RAG2*)

PCR-reaction mix:	
35 µl	dH_2O
5 µl	10x PCR buffer
2 µl	$MgCl_2$ 50mM
2 µl	dNTP-Mix 10mM each
1 µl	Platinum Taq Polymerase
2 µl	*RAG1/ RAG2* primer (Forward) 2.5µM
2 µl	*RAG1/ RAG2* primer (Reverse) 2.5µM
1 µl	template (cDNA)
50 µl	end volume

PCR-program: 45 cycles	
95°C	1 min.
95°C	30 sec. DNA-denaturation
60°C	30 sec. annealing
72°C	30 sec. Elongation
72°C	10 min.
4°C	pause

2.3.10 Amplification of the activation-induced cytidine deaminase (*AID*)

PCR-reaction mix:

35.2 µl	dH_2O
5 µl	10x PCR buffer
1.8 µl	$MgCl_2$ 50mM
1 µl	dNTP-Mix 10mM each
1 µl	Platinum Taq Polymerase
2.5 µl	*AID* primer (Forward) 2.5µM
2.5 µl	*AID* primer (Reverse) 2.5µM
1 µl	template (cDNA)
50 µl	end volume

PCR-program: 45 cycles

95°C	2 min.
95°C	1 min. DNA-denaturation
64°C	1 min. annealing
72°C	1 min. Elongation
72°C	5 min.
4°C	pause

2.4 Agarose gel electrophoresis

Material:

- Agarose UltraPure (Gibco BRL)
- TAE buffer [40 mM Tris, 1 mM EDTA, and 20 mM acetic acid, pH 8.5]
- Ethidium bromide [1 mg/ml]
- 1x loading buffer [0.021% bromphenol blue, 0.021% xylene cyanol, 20 mM EDTA and 5% Glycerol]
- 100 bp DNA ladder (Invitrogen)

Method:

Agarose is a substance isolated from algae, with which gels can be formed. DNA fragments can migrate through an agarose-gel driven by electrical voltage to the anode as DNA is negatively charged. Smaller fragments can move faster through the agarose pores than bigger ones, thus DNA fragments are separated by size in the gel. For the preparation of a 2% agarose gel, 2 g of agarose are diluted in 100 ml of 1x TAE buffer. The dilution is then heated in a microwave. 6 µl of ethidium bromide are added and the liquid agarose is poured into a gel tray, a comb is placed into it and it is left to harden. 5 µl of the PCR product are mixed with 2 µl of 1x loading buffer. When the gel is solid, the comb is removed and the gel tray placed into a gel chamber filled with 1x TAE buffer. The probes and 3 µl of the 100 bp DNA ladder can be loaded into the gel slots and run at 100 - 130 V for 10-15 minutes. The contained ethidium bromide intercalates into the DNA fragments and makes them visible under the UV-light.

2.5 Cloning PCR-products into Topo-vector and transformation of *Escherichia coli* by heat shock

Material:

- TOPO® PCR cloning kit (Invitrogen)
- TOP10 chemocompetent *E. coli* cells (Invitrogen)
- SOC medium [2% Trypton, 0.5% yeast extract, 10 mmol/l NaCl, 2.5 mmol/l KCl, 10 mmol/l $MgCl_2$, 10 mmol/l $MgSO_4$, 20 mmol/l Glucose] (Invitrogen)
- Agar + ampicillin plates [1% bacto-peptone, 0.5% yeast extract, 0.5% NaCl (pH7.5), 1.5% bacto-agar, 100 mg/ml ampicillin]

Method:

The plasmid vector in the TOPO® PCR cloning kit is provided linearized and conjugated with TOPO-isomerase I of Vaccinia virus. PCR products generated with *Taq* polymerases always contain an overhanging adenin at each end. Therefore the vector has to have an overhanging thymidine. The TOPO-isomerase recognizes a PCR product and ligates it into the linearized vector. 4 µl of the PCR product are mixed with 1 µl salt solution provided in the kit and 1 µl of the TOPO vector, in order to perform the cloning reaction. The mixture is then incubated at room temperature for 5 to 30 minutes before being transformed into *E.coli* cells.

The transformation of chemical competent cells is achieved by heat-shock. To this aim, 2 µl of the TOPO cloning reaction are added to a vial of chemical competent *E.coli* cells. The samples are incubated on ice for 30 minutes and then heat-shocked at 42°C for 90 seconds. The tubes are immediately transferred on ice to accomplish the temperature shock. Subsequently 200 µl of S.O.C. medium are added into the tubes to attain a suitable growth environment for the cells. Incubation at 37°C, shaking horizontally at 200rpm for one hour follows. 50 µl of this cell suspension are spread on agar+ampicillin plates and incubated at 37°C over night. The TOPO vector encloses an ampicillin resistance gene, thus only cells that have taken up a TOPO vector are able to grow on the agar plates.

2.6 Analysing the *E. coli* clones

2.6.1 Amplification of the PCR product in the TOPO Vector/ *E. coli* clones by colony PCR

Material:

- DMSO (Sigma)
- BV buffer [166 mM ammonium sulfate, 670 mM Tris-HCl (pH 8.8), 67 mM MgCl2, and 100 mM β-mercaptoethanol] (Invitrogen)
- dNTP-mix 10 mM each (Peqlab)
- M13 forward primer and M13 reverse primer (Invitrogen)
- Platinum *Taq* polymerase (Invitrogen)

Method:

25 µl of the master-mix below are pipetted into a 96 well PCR plate and one *E. coli* clone is transferred into each well. M13 primers bind to vector sequences adjacent to the insert. The colony PCR products are controlled gel electrophoresis and subsequently purified for sequencing.

PCR-reaction mix:

1845 µl	dH_2O
250 µl	BV buffer
125 µl	DMSO
250 µl	dNTP-Mix 10mM each
20 µl	Platinum *Taq* Polymerase
5 µl	M13 forward primer 100 µM
5 µl	M13 reverse primer 100 µM
2500 µl	end volume

PCR-program: 35 cycles

95°C	2 min.
95°C	50 sec.
53°C	1 min.
72°C	1 min.
72°C	5 min.
4°C	pause

2.6.2 Purification of Colony PCR products

Material:

- Filter plates (Corning Inc.)
- Sephacryl™ S-400 (Amersham)
- LiChrosolph® dH_2O (MERCK)

Method:

PCR products are purified in 96 well filter plates filled with Sephacryl. The filter plate is placed on a normal 96 well plate and filled with 300 µl of resuspended Sephacryl in each well. The plates are centrifuged for 2 minutes at 2390 rpm and washed five times with 250 µl LiChrosolph® dH_2O pro well. After the final washing step the filter plate is transferred onto a clean 96 well plate and loaded with PCR products. The plates are once more centrifuged and the PCR products obtained are ready for sequencing.

2.6.3 Sequence reaction

Material:

- BigDye® terminator v1.1 Kit (Applied Biosystems)
- 5X sequencing buffer (ABI Prism)
- BigDye® terminator reaction mix (ABI Prism)
- M13 forward primer or M13 reverse primer (Invitrogen)
- dH_2O

Method:

The principle behind a sequence reaction is similar to a PCR reaction. The difference arises in the addition of dideoxy-nucleotides (ddNTP) which disrupts elongation upon incorporation of a ddNTP. The four different ddNTPs are labelled with different fluorescent dyes. The resulting products are of different length and end with color marked nucleotides. A capillary sequencer finally analyzes these probes to translate them into a DNA sequence.

The sequence reaction is performed with the master-mix shown below:

Sequence reaction mix:

1 µl	BigDye terminator reaction mix
1 µl	BigDye terminator reaction buffer
2 µl	M13 forward primer 10 µM
6 µl	colony PCR product
10 µl	end volume

Sequencing program: 50 cycles

96°C	2 min.
96°C	30 sec.
50°C	15 sec. 50 repeats
60°C	4 min.
10°C	pause

2.6.4 Purification of the sequence reaction

Material:

- MultiScreen®-HV-plate (Millipore)
- SephadexTM G-50 (Amersham)
- MultiScreen column loader plate (Millipore)
- Thermo-Fast® 96 well PCR plate (ABGene)
- LiChrosolph® dH_2O (MERCK)

Method:

The fluorescence-labelled sequencing products are analyzed with a capillary sequencer. Before the analyses, surplus fluorescent ddNTP, which could disturb this process has to be removed by purifying the sequence reaction products. Each well of a 96 well plate MultiScreen®-HV-plate is filled with SephadexTM G-50 with the help of a MultiScreen column loader plate. 300 µl dH_2O are added into each well and the plate is incubated at room temperature for three hours. Thereafter the MultiScreen plate is transferred onto a Thermo-Fast® 96 well PCR detection plate and centrifuged at 2640 rpm for 5 minutes. The Sephadex columns are loaded with the sequence reactions that are later diluted with 10 µl of dH_2O pro well. The sequence products are centrifuged into a clean Thermo-Fast® 96 well PCR detection plate at 2640 rpm for 5 minutes and subsequently analyzed with a capillary sequencer.

2.7 Computer and Internet

2.7.1 Internet programs and websites

- Primer software:

http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

- IMGT:

http://imgt.cines.fr/textes/vquest/

- Reverse complement:

http://www.bioinformatics.vg/bioinformatics_tools/reversecomplement.shtml

- NCBI BLAST:

http://www.ncbi.nlm.nih.gov/blast/Blast.cgi

- Multalign:

http://prodes.toulouse.inra.fr/multalin/multalin.html

- Ensembl human gene:

http://www.ensembl.org/Homo_sapiens/geneview?gene=

- PubMed:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB=pubmed

2.7.2 Software

Microsoft Word Pro, Adope Photoshop 7.0, Microsoft Power Point Pro, Microsoft Excel Pro

Chapter 3
Results

3.1 Phenotypic characterization of chronic myeloid leukemia -blast crisis (CML-BC) in myeloid, B- and T-lymphoid lineages

Using flow cytometry, 11 chronic myeloid leukemia cell lines in blast crisis (CML-BC) and five primary CML-BC cases were analyzed for various HSC (CD34), B-lymphoid (CD5, CD10, CD19, CD20), T-lymphoid (CD3, CD4, CD5, CD7, CD8, TCRαβ, TCRγδ), myeloid (CD13, CD14, IL3Rα, GM-CSFRα) and erythroid (Gly A) surface characteristics. The cell lines, BV173, Nalm1, CML-T1, K562, KCL-22, JURL, EM2, KU182, LAMA84, JK1 and MEG01 were obtained from DSMZ and are characterized as summarized in Table 1 (Drexler et al., 2005). The primary CML-BC bone marrow samples were kindly provided by Prof. Hoffmann of the Charité Berlin.

Table 1: Characterization of the CML-BC cell lines as described in the DSMZ cell lines catalogue, 2005

Cell line	DSMZ description*
BV173	CML in lymphoid blast crisis
NALM1	CML in lymphoid blast crisis
CML-T1	T-lymphoid CML in acute phase
K562	Myeloid CML in blast crisis, with erythroid and megakaryocytic differentiation potential
KCL-22	Myelocytic CML in blast crisis
JURL-MK1	predominantly megakaryocytic CML in blast crisis
EM2	Myelocytic CML in blast crisis
KU812	Basophilic CML in blast crisis, with spontaneous expression of myelocytic, erythrocytic, macrophage, and megakaryocytic features
LAMA 84	Erythroid-megakaryocytic CML in blast crisis
JK1	CML in erythroid blast crisis
MEG-01	Megakaryocytic CML in blast crisis

* Drexler et al., 2005

The phenotypic characteristics of the cell lines on table 1 were verified using the following cell surface markers defined below:

CD34 is an approximately 75-120kD glycoprotein on the surface of hematopoietic stem cells (McSweeney et al., 1998) and endothelial cells (Bhattacharya et al., 2000). CD5 is found on thymocytes, T-lymphocytes and a subset of B-lymphocytes (Kantor et al., 1993). CD10, also known as common acute lymphoblastic leukemia antigen (CALLA), is a transmembrane glycoprotein expressed on a subset of B and T cell progenitors and germinal center B cells (Horejsi et al., 1988; Knapp et al., 1989). During all stages of B-cell differentiation and maturation, the CD19 glycoprotein is highly expressed on B-cells but low on plasma cells (Knapp et al., 1989; Bradbury et al., 1993). CD20 is expressed on pre-B cells, resting and activated B cells but not on plasma cells (Loken et al., 1987; Knapp et al.; 1989). The CD3/TCR complex is found on 70-80% of normal human T lymphocytes and 60-85% of thymocytes (Beverley, 1981; Lanier et al., 1986). CD4 is an antigen coreceptor on the T-cell surface which interacts with MHC class II molecules on antigen-presenting cells (Dialynas, 1983; Bierer et al., 1989; Janeway et al., 1992). CD7 is a transmembrane glycoprotein expressed on thymocytes, mature T cells and NK cells (Knapp et al., 1989). The CD8αβ heterodimer is found on the surface of most thymocytes and a subpopulation of mature T cells (Torres-Nagel et al., 1992). T cells carrying a functional TCR are either γδ or αβ (Kubo et al., 1989). The transmembrane glycoprotein, CD13, binds to granulocytic-monocytic cells and granulocyte-macrophage progenitors but not on lymphocytes, platelets or erythrocytes (Knapp et al., 1989). Human CD14, which is known as a high affinity cell-surface receptor of lipopolysaccharide (LPS) complex, is expressed on circulating monocytes and neutrophils (Wright et al., 1990; Ziegler-Heitbrock et al., 1996). CD123 or α chain IL-3 receptor (IL-3Rα) is expressed on myeloid precursors, myeloid-derived dendritic cells, macrophages and megakaryocytes (Macardle et al., 1996). The human granulocyte macrophage-colony stimulating factor receptor alpha complex (GM-CSF), also known as CD116, is expressed on a variety of myeloid and hematopoietic tumors as well as on normal monocytes, macrophages, neutrophils but not on lymphocytes (Jubinsky et al., 1994; Stacchini et al., 1996). Glycophorin A is a sialoglycoprotein present on human red blood cells (RBC) and erythroid precursor cells (Nakahata et al., 1994).

These antibodies were applied in the flow cytometry analyzes of the CML-BC cases and the results are elaborated on Figure 9.

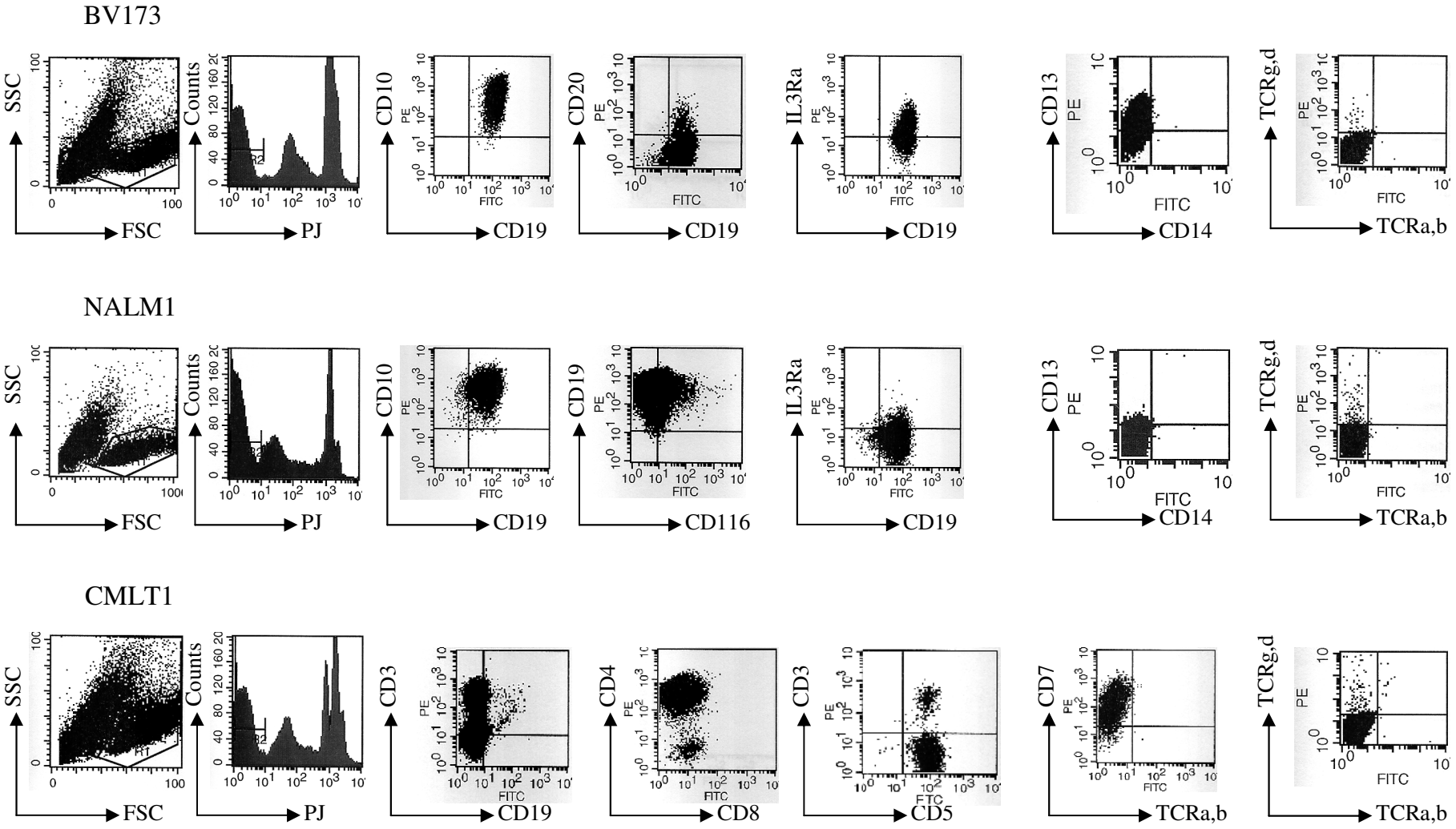


Figure 9a: Phenotypic analysis of the CML-BC cell lines BV173, Nalm1 and CMLT1

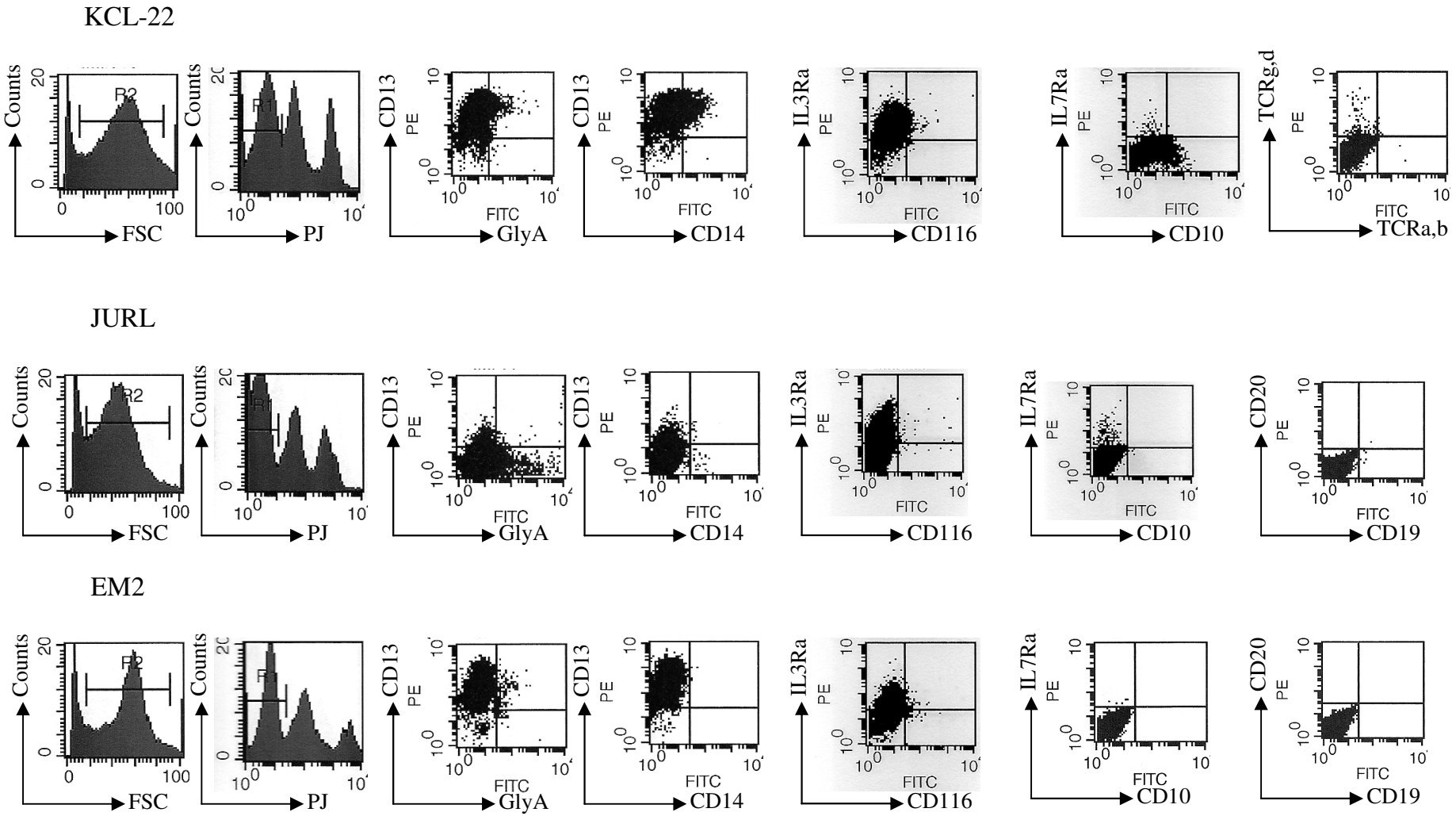


Figure 9b: Phenotypic analysis of the CML-BC cell lines KCL-22, JURL-MK1 and EM2

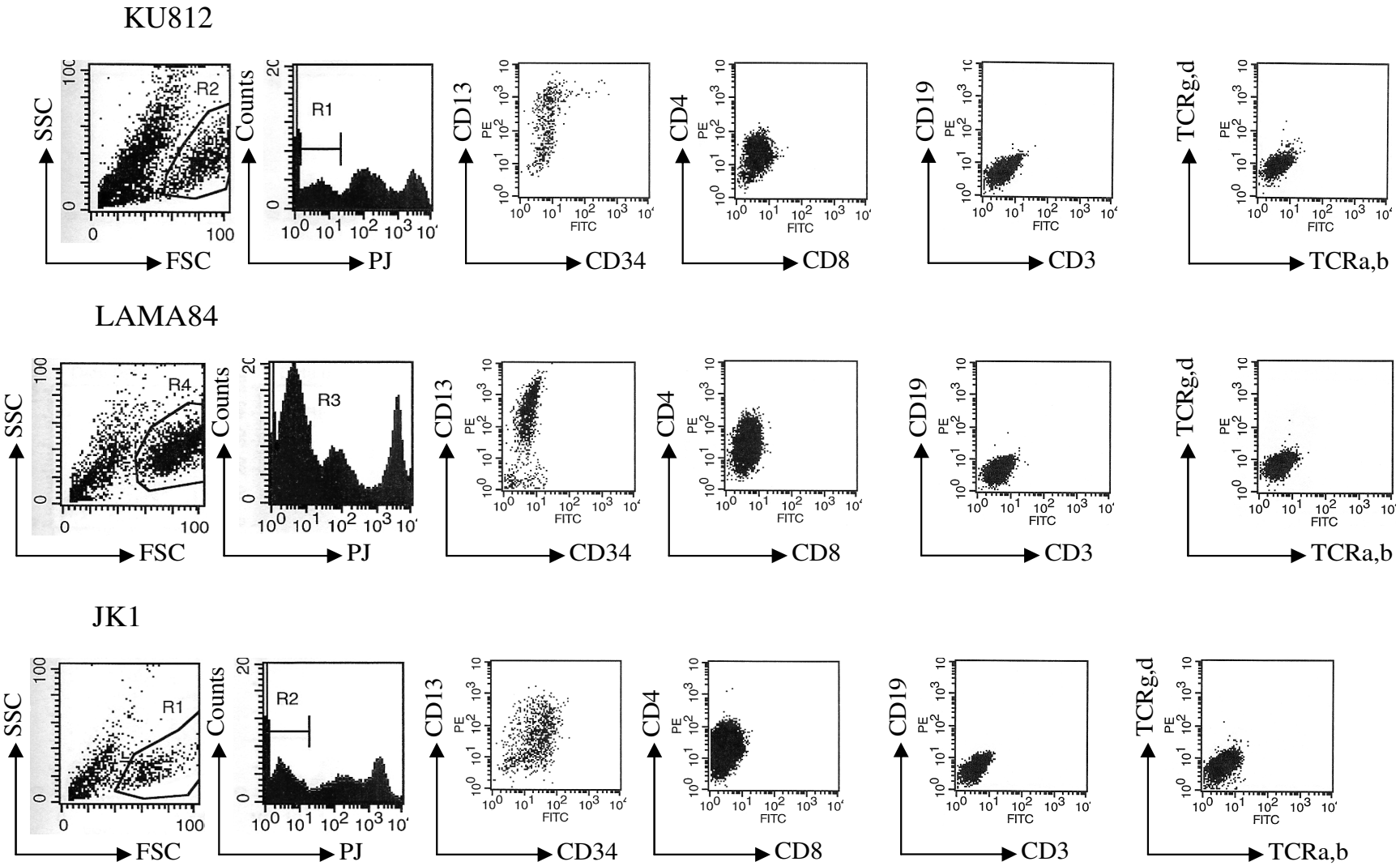


Figure 9c: Phenotypic analysis of the CML-BC cell lines KU812, LAMA84 and JK1.

The CML-BC cell lines are verified for their lineage specificity by FACS using different cell surface markers specific for B-lymphoid, T-lymphoid, myeloid or erythroid lineages. Some CML-BC cell lines are multi-lineage, composing of B lymphoid, T lymphoid, myeloid or erythroid lineages.

Figure 9 shows the phenotypic characteristics of the CML-BC cell lines. The figure shows the scatter plot (FSC/SSC), which reflects the size and granularity of all cell lines as well as the viability histogram of all cell lines, marked by propidium iodide (PI), which facilitates the identification of living and dead cells. The dot plots with the different cell surface markers are gated for lymphocytes and living cells and show that the cell line BV173 is a typical B lymphoid cell line as it expresses CD19, CD10 and partly CD20 on its cell surface. Although being a B lymphoid CML-BC cell line, BV173 also seems to be partly positive for myeloid surface markers like CD13 and IL3Rα as well as having a subpopulation which carries the TCRγδ on its surface. Nalm1 is also a B lymphoid CML-BC as it expresses CD19 and CD10 but this cell line also contains a subpopulation with a myeloid potential as it expresses the granulocyte macrophage-colony stimulating factor (GM-CSF) receptor complex (CD116) and slightly the IL3Rα. Just like BV173, Nalm1 also has a subpopulation of cells carrying the TCRγδ on their surface. CML-T1 has typical T lymphoid characteristics, being CD3, highly CD4, CD5, CD7 and partly TCRγδ positive. Of interest is the finding that CML-T1 has a subpopulation of T cells which also carry the typical B lymphoid CD19 marker. The KCL-22 cell line expresses mainly the myeloid CD13 and IL3Rα cell surface markers but is weakly positive for other myeloid markers like CD14 and CD116. KCL-22 contains an erythroid subpopulation, positive for GlyA and a lymphoid subpopulation, which expresses IL7Rα. The JURL cell line has on the one hand some cells positive for typical myeloid cell surface markers like CD13, CD14, CD116 or IL3Rα but on the other hand it has subpopulations of GlyA positive erythroid cells and IL7Rα positive B lymphoid cells. The EM2 cell line expresses CD13 as well as the IL3Rα and GlyA cell surface markers. The cell line KU182 is myeloid as it expresses CD13 and contains a subpopulation of hematopoietic stem cell progenitors, as this subpopulation is CD34 positive. LAMA84 and JK1 are both CD13 myeloid and partly CD4 T lymphoid. The cell lines K562 and MEG-01 were very difficult to characterize by flow cytometry as they were always negative for most cell surface makers and very difficult to handle. Summarizing these FACS data, these CML-BC cell lines can be defined as either being multi-lineage, composing of B lymphoid, T lymphoid, myeloid or erythroid lineages or singular B lymphoid, T lymphoid, myeloid or erythroid lineages, which might have the potential of differentiating spontaneously into different lineages as some were described by Drexler et al., 2005 DSMZ catalogue of cell lines.

As the FACS data show that some CML-BC cell lines harbor lymphoid subpopulations, it was thus important to verify if these cell lines also express typical lymphoid proteins like RAG1, RAG2 and AID, as well as if they undergo lymphoid specific *IGHV* and *TCRBV* and *TCRGV* gene rearrangements.

3.2 Expression of Recombination Activating Genes (*RAG1*/2) in CML-BC cases

Recombination activating genes (*RAG1* and *RAG2*) are tissue-specific genes required for V(D)J recombination in B and T lymphocytes (Schatz et al., 1989; Oettinger et al., 1990). These proteins are typically expressed in developing lymphocytes during the formation of their antigen receptors. RAG proteins are responsible for the first step of the VDJ reaction by site-specific cleavage of DNA (McBlane et al., 1995). The rearrangement begins with RAG1 and RAG2 binding to high mobility group (HMG) proteins forming a complex which recognizes the RSS flanking the coding sequences to be joined. The coding sequences are then oriented close to one another and RAG is activated to nick a strand of the double stranded DNA. This strand reacts with the complementary strand, breaks it and forms a hairpin structure at the end of the heptamer sequence. Other proteins like Ku70:Ku80 join the complex and RAG breaks the hairpin creating a single stranded DNA end. This end is then modified by TdT by adding or subtracting nucleotides. DNA-ligase IV joins the ends of the gene segments forming a coding joint and the RSS-ends forming a signal joint (van Gent et al., 1996; Oettinger et al., 1990; McBlane et al., 1995; Agrawal et al., 1997).

As the name infers, CML-BC are mainly myeloid cells but the FACS analyses in chapter 3.1 show that some of these CML-BC cell lines do have lymphoid subpopulations. Thus, all eleven cell lines were verified for the expression of these typical lymphoid genes, *RAG1* and *RAG2*, by RT-PCR using *RAG1* and *RAG2* specific primers. RNA was isolated from the cell lines and transcribed into cDNA. The integrity of the cDNAs used for the RT-PCR was verified by amplifying the house-keeping gene *GAPDH* (Figure 10). In six (BV173, Nalm1, K562, CML-T1, JK1 and LAMA) out of the eleven cell lines, the *RAG1* cDNA fragments could be amplified and *RAG2* was amplified in all cell lines except KU812 (Figure 10). The identity of the PCR products was verified by cloning and sequencing. The sequence products were analyzed using NCBI Blast (http://www.ncbi.nlm.nih.gov/BLAST/) and Multalin (http://prodes.toulouse.inra.fr/multalin/multalin.html).

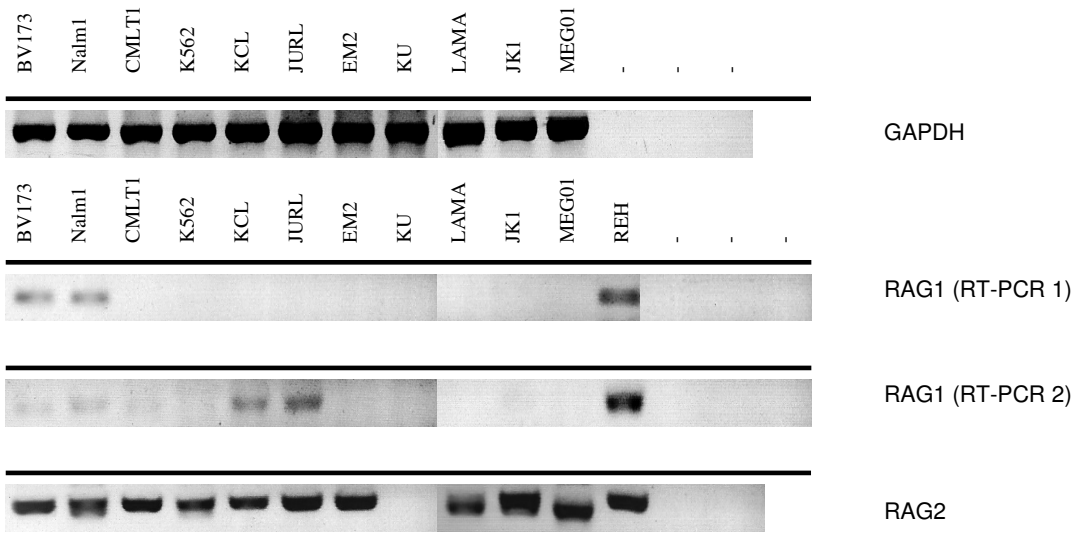


Figure 10: RAG1 and RAG2 expression in CML-BC cell lines

RAG1 and *RAG2* cDNA fragments amplified by RT-PCR using *RAG1* and *RAG2* specific primers. The human B cell precursor leukemia, REH, was used as positive control for the verification of *RAG1*/2 genes. cDNA fragments of the house-keeping gene, *GAPDH*, were amplified to show the integrity of the cell line cDNAs before the *RAG1*/2 RT-PCR. The negative controls of the various PCRs are represented by (-). Because the *RAG1* RT-PCR was not always consistent, different gels are shown.

Figure 10 shows the results of the *RAG1* and *RAG2* RT-PCR analysis. After three trials, weak bands for *RAG1* could be amplified for BV173, Nalm1, CMLT1, K562, KCL, JURL and JK1 after 45 PCR cycles. The analyzes of the *RAG1* PCR products after sequence analysis confirmed and corresponded to the human *RAG1* cDNA fragment in all the above mentioned cell lines. In the contrary, the PCR products/sequences for *RAG2* which seemed at first sight to be positive for all cell lines, except KU812, did not match the human *RAG2* cDNA fragment when blasted. This could be because the primers might not be specific for the *RAG2* gene and might amplify other genes in human or other PCR problems might be responsible for this failure.

Table 2: *RAG1* sequence alignment

RAG1 sequences for Nalm1, BV173, K562, LAMA, JK1, and CMLT1 are aligned to the section of *RAG1* mRNA sequence covered by the *RAG1* specific primers used for the RT-PCR. Primer sequences have been cut-off. The red spaces indicate deletions, the letter in green indicates insertion and blue indicates replacement. Of note: the sequencing was done from one DNA-strand and the mutations still have to be confirmed by sequencing from both DNA-strands.

RAG1-mRNA sequence 5'-	GCAGCCTGCTGAGCAAGGTACCTCAGCCAGCATGGCAGCCTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGATGAAATTCA GCACCCACATATTAAATTTTCAGAATGGAAATTTAA-3
Nalm1-A03	GCAGCCTGCTGAGCAAGGTACCTCAGCCAGCATGGCAGCCTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGATGAAATTCA GCACCCACATATTAAATTTTCAGAA-GGAAATTTAA
Nalm1-B03	GCAGCCTGCTGAGCAAGGTACCTCAGCCAGCATGGCAGCCTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGATGAAATTCA GCACCCACATATTAAATTTTC-GAA-GGAAATTTAA
Nalm1-C03	GCAGCCTGCTGAGCAAGGTACCTCAGCCAGCATGGCAGCCTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGATGAAATTCA GCACCCACATATTAAATT-TC-GAA-GGAAATT-AA
BV173-A06	GCAGCCTGCTGAGCAAGGTACCTCAGCCAGCATGGCAGCCTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGATGAAATTCA GCACCCACATATTAAATTTTC-GAA-GGAAATTTAA
BV173-C06	GCAGCCTGCTGAGCAAGGTACCTCAGCCAGCATGGCAGCCTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGATGAAATTCA GCACCCACATATTAAATT-TC-GAA-GGAAATTTAA
BV173-D06	GCAGCCTGCTGAGCAAGGTACCTCAGCCAGCATGGCAGCCTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGA-GAAATTCA GCACCCACATATTAAATTTTCAGAATGGAAATTTAA
K562-A03	GCAGCCTGCTGAGCAAGGTACCTCAGCCAGCATGGCAGCCTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGATGAAATTCA GCACCCACATATTAAATT-TC-GAA-GGAAATT-AA
LAMA-E03	GCAGCCTGCTGAGCAAGGTACCTCAGCCAGCATGGCAGCCTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGATGAAATTCA GCACCCACATATTAAATT-TC-GAA-GGAAATT-AA
LAMA-F03	GCAGC-TGCTGAGCAAGGTACCTCAGCCAGCATGGCAG-CTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGA-GAAATTC-GGCACCCACATATTAAATTTTCAGAATGGAAATTTAA
LAMA-H03	GCAGC-TGCTGAGCAAGGTACCTCAGCCAGCATGGCAG-CTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGA-GAAATTCA GCACCCACATATTAAATTTTCAGAATGGAAATTTAA
JK1-E02	GCAGCCTGCTGAGCAAGGTACCTCAGCCAGCATGGCAGCCTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGATGAAATTCA GCACCCACATATTAAATTT--C-GAA-GGAAATT-AA
JK1-H02	GCAGC-TGCTGAGCAAGGTACCTCAGCCAGC-TGGCAG-CTC-TTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGA-GAAATTCA GCACCCACATATTAAATTTT-CAGAATGGAAATTTAA
CMLT1-A04	GCAGCCTGCTGAGCAAGGTACCTCAGCCAGCATGGCAGCCTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGATGAAATTCA GCACCCACATATTAAATTT--C-GAA-GGAAATT-AA
CMLT1-B04	GCAGC-TGCTGAGCAAGGTACCTCAGCCAGCATGGCAG-CTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGA-GAAATTCA GCACCCACATATTAAATTTT-CAGAATGGAAATTTAA
CMLT1-C04	GCAGCCTGCTGAGCAAGGTACCTCAGCCAGCATGGCAGCCTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGATGAAATTCA GCACCCACATATTAAATTT--CAGAA-GGAAATT-AA
CMLT1-D04	GCAGCCTGCTGAGCAAGGTACCTCAGCCGGCATGGCAGCCTCTTTCCCACCCACCTTGGGACTCAGTTCTGCCCCAGATGAAATTCA GCACCCACATATTAAATTT--C-GAA-GGAAATT-AA

Table 2 shows a comparison of the *RAG1* sequences for the CML-BC cell lines to the section of *RAG1* mRNA sequence covered by the *RAG1* specific primers, which are not shown in the table. Five clones of each cell line were analyzed and only those clones with differences in the sequences can be seen in the table 2. Three Nalm1 cell line sequences all had mutations in the form of deletions, ranging from one to four deletions, at the 3' end of the sequence close to the *RAG1* reverse primer. The BV173 clones had different mutations, with one to three deletions, while all K562 clones had similar mutations, having the same four deletions. The LAMA84 cell line had three to four deletions distributed over the sequence from the 5' end close to the *RAG1* forward primer, some in the middle and also at the 3' end. Some LAMA84 clones not only had deletions but also a base insertion (guanine; green), not found in any other cell line. JK1 had five to six mutational deletions also spread throughout the sequence. CML-T1 has four to five deletions and also a mutational replacement of adenine by guanine, which was also the sole case and was not found in the other cell lines.

These sequences confirm the genuine expression of lymphoid specific protein *RAG1* in some CML-BC cell lines. This supports the FACS data that suggests that some CML-BC cell lines do harbor lymphoid subpopulations. If this is the case, and taking into consideration the fact that RAG is a prerequisite for *IGH*, *TCRB* and *TCRG* gene recombination, one might expect an abnormal existence of rearranged B and T cell receptor genes in these CML-BC cell lines.

3.3 Rare occurrence of immunoglobulin gene rearrangements in CML-BC

The immunoglobulin heavy chain is made up of clusters of variable (V), diversity (D) and joining (J) gene segments encoding its variable region and a cluster of constant (C) genes encoding its constant region (Ravetch et al., 1981; Matsuda et al., 1998). Early B cell differentiation is characterized by somatic rearrangements within the immunoglobulin heavy chain locus which are necessary for the expression and functionality of the B cell receptor and prerequisite for the selection for further differentiation. This somatic recombination process also known as V(D)J recombination is guided by recombination signal sequences flanking the gene segments and V(D)J recombinase enzymes, including the recombination activating genes (*RAG1* and *RAG2*) (Tonegawa et al., 1983; Schatz et al., 1989). To find out if immunoglobulin gene rearrangement is a characteristic of CML-BC cells, *IGH* region genes were amplified by RT-PCR using 6 V_H-specific 5'-primers and a J_H-specific 3'-primer mix. All 11 CML-BC cell lines were analyzed for *IGH* V(D)J recombination as well as the five primary cases, using memory B cells as a positive control for the PCR. The PCR products were then cloned and sequenced as previously described and the sequences were analyzed using the IMGT program (www.imgt.cines.fr). Also, Ig D_H7-JH gene rearrangements were amplified in analogy to the PCR strategy for the Ig V_H(D)J gene rearrangement. In this PCR, a D_H7-27 specific primer, which amplifies rearrangements of this gene segment (D_H7-27) to any J_H gene segment and also germline configuration of the *IGH* locus (Kanzler et al., 1996), was used together with a J_H-specific 3'-primer mix to verify the germline configuration of CML-BC genomic DNAs. This is based on the fact that in almost all IgH DJ and all IgH VDJ rearrangements the primer binding site upstream of D_H7-27 is deleted (Figure 11).

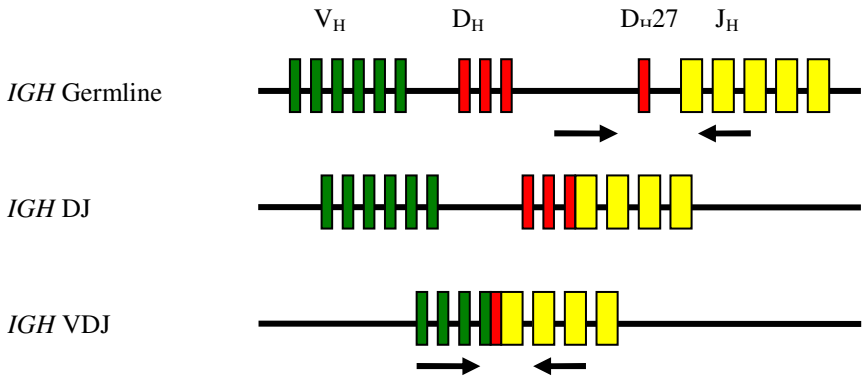


Figure 11: An illustration of the human *IGH* locus

showing the germline configuration, *IGH* DJ and *IGH* VDJ with the DH7-27 gene segment deleted in both cases of recombination. Arrows indicate the approximate location of the primers used in the RT-PCR.

While most human B cells carry rearrangements on both *IGH* alleles (Walter et al., 1991); either two *IGHV* rearrangements (where one is functional and the other nonfunctional) or a *IGHV* rearrangement together with a D_HJ_H joint, non-B cells have *IGH* loci in germline configuration (Kanzler et al., 1996). In the case of CML-BC cells, all cell lines except the lymphoid CML-BC Nalm1 and BV173 as well as all primary cases have at least one unrearranged *IGH* locus as detected by PCR and illustrated on Figure 12. $CD3^+$ T cells which are expected to have *IGH* loci in germline configuration were used as positive control for the PCR.

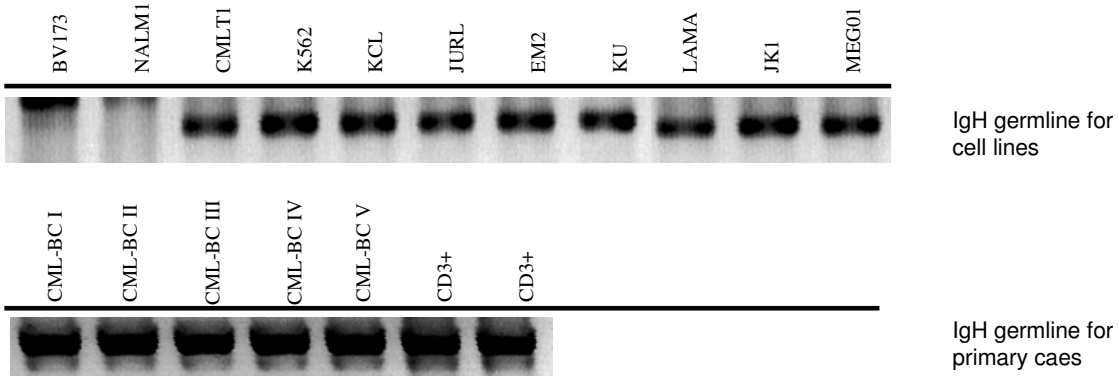


Figure 12: Germline configuration of the IGH locus of CML-BC cell lines and primary cases using a D_H7-27 primer and a J_H primer mix. The BV173 and Nalm1 cell lines show bands on the gel that are higher than expected, as compared to the bands amplified in $CD3^+$ T cells, which serve as positive control for this PCR.

Table 3 shows various characteristics of the CML-BC cases and especially the occurrence of $V_HD_HJ_H$ rearrangements in cell lines, as well as primary cases of CML-BC. Apart from the cell lines KU812 and MEG01, which probably have both alleles of the *IGH* locus in germline configuration, all other cases have at least one rearranged *IGH* allele. The rearrangements involve different V_H, D_H and J_H segments and the rearrangement types range from one as in BV173, KCL-22, JURL or CML-BC V to eight different rearrangements as in Nalm1. The sequences of the V_H region also reveal an accumulation of somatic mutations with evidence of ongoing mutations as demonstrated on evolutionary trees. These will be elaborated in chapters 3.9.1 and 3.9.2.

Table 3a: V_H Rearrangements, mutations and other characteristics of chronic myeloid leukaemia in blast crisis

Case	V_H	D_H	J_H	Number of mutations [x 10^{-3} bp]	Cytogenetics	Genes involved	*AID*	*RAG1*	CDKN2A/B**	Phenotype
BV173	V3-21	D2-15	J3	6 (22); intraclonal diversity	t(9;22)(q34;q11)	*BCR-ABL1*	+	+	deletion	B lymphoid (CD19), Myeloid (CD13, IL3Rα, GM-CSFRα)
NALM1	V1-8 V2-5 V2-70 V3-9 V3-43 V4-31 V4-59 V4-51	none D3-16 D3-16 D2-21 D3-9 D3-16 D3-16 D3-16	J2 J4 J4 J3 J5 J4 J4 J4	10 (37); intraclonal diversity 3 (11) 7 (26); intraclonal diversity 6 (22); intraclonal diversity 7 (26); intraclonal diversity 6 (22); intraclonal diversity	t(9;22)(q34;q11)	*BCR-ABL1*	+	+	n.k.	B lymphoid (CD19, VpreB, μ chain), Myeloid (IL3Rα, GM-CSFRα)
CML-T1	V4-34 V3-74 germline	D2-8 none	J2 J5	6 (22) 37 (137); intraclonal diversity	t(9;22)(q34;q11)	*BCR-ABL1*	+	+	n.k.	T lymphoid (CD3, CD4, CD7, CD8, TCRγ), Myeloid (CD13, GM-CSFRα, IL3Rα), B lymphoid (CD19)
K562	V3-43 V3-21 V6-1 germline	none D4-23 D3-9	J4 J6 J6	3 (11) 6 (22)	t(9;22)(q34;q11)	*BCR-ABL1*	+	+	deletion	Erythroid (CD36, CD71, Glycophorin A), Myeloid (CD13, CD14, IL3Rα,GM-CSFRα)
KCL-22	V4-39 germline	D3-3	J6	1 (3)	t(9;22)(q34;q11)	*BCR-ABL1*	-	-	n.k.	Myeloid (CD13, CD14, IL3Rα, GMCSFrα)
JURL	V1-46 germline	D3-10	J6	3 (6)	t(9;22)(q34;q11)	*BCR-ABL1*	+	-	n.k.	Myeloid (CD13, CD14, IL3Rα, GMCSFrα)
EM2	V6-1 V1-2 germline	D3-9 D7-27	J4 J4	1 (3) 1 (3)	t(9;22)(q34;q11)	*BCR-ABL1*	+	-	n.k.	Myeloid (CD13, CD14, IL3Rα, GMCSFrα)
KU 812	germline			no mutation	t(9;22)(q34;q11)	*BCR-ABL1*	+	-	n.k.	T lymphoid (CD4, TCRγ), Myeloid (CD13)
LAMA 84	V6-1 V6-1 germline	D5-5 D5-18	J3 J4	6 (19) 3 (10)	t(9;22)(q34;q11)	*BCR-ABL1*	+	+	deletion	T lymphoid (CD4, TCRγ), Myeloid (CD13)

Note: Intraclonal diversity denotes the presence of multiple sequences for one V_H gene rearrangement, which share common mutations but differ in diversifying mutations.
** Drexler HG et al., DSMZ Catalogue of cell lines
n.k.: not known

Table 3b: Continuation of V_H Rearrangements, mutations and other characteristics of chronic myeloid leukaemia in blast crisis

Case	V_H	D_H	J_H	Number of mutations [x 10^{-3} bp]	Cytogenetics	Genes involved	*AID*	*RAG1*	CDKN2A/B**	Phenotype
JK1	V6-1 V1-2 germline	D5-5 D7-27	J4 J4	6 (19) 4 (13)	t(9;22)(q34;q11)	*BCR-ABL1*	-	+	n.k.	T lymphoid (CD4, TCRγ), Myeloid (CD13)
MEG01	germline			no mutation	t(9;22)(q34;q11)	*BCR-ABL1*	+	-	deletion	Myeloid (CD13)
CML-BC I	V1-18 V3-21 Germline	D2-2 D6-25	J6 J6	7 (26); intraclonal diversity 4 (15)	t(9;22)(q34;q11)	*BCR-ABL1*	(CD13+) – (CD19+) +	n.k.	n.k.	Myeloid (CD13), B lymphoid (CD19)
CML-BC II	V1-3 V3-7 V3-30 germline	D5-18 D2-2 D5-5	J6 J6 J4	0 4 (15); intraclonal diversity 11 (41)	t(9;22)(q34;q11)	*BCR-ABL1*	(CD13+) – (CD19+) +	n.k.	n.k.	Myeloid (CD13), B lymphoid (CD19)
CML-BC III	V1-8 V1-46 Germline	D5-18 D2-21	J6 J5	0 36 (133); intraclonal diversity	t(9;22)(q34;q11)	*BCR-ABL1*	(CD13+) – (CD19+) +	n.k.	n.k.	Myeloid (CD13), B lymphoid (CD19)
CML-BC IV	V3-33 V3-30 V3-23 V3-30 germline	D2-2 D6-6 D2-2 D2-15	J4 J4 J4 J6	24 (89); intraclonal diversity 21 (78); intraclonal diversity 14 (52) 17 (63); intraclonal diversity	t(9;22)(q34;q11)	*BCR-ABL1*	(CD13+) – (CD19+) +	n.k.	n.k.	Myeloid (CD13), B lymphoid (CD19)
CML-BC V	V2-5 **g**ermline	none	J5	2 (7)	t(9;22)(q34;q11)	*BCR-ABL1*	(CD13+) – (CD19+) **+**	n.k.	n.k.	Myeloid (CD13), B lymphoid (CD19)
Mean:				**8.4 (30,74)**						

Note:
Intraclonal diversity denotes the presence of multiple sequences for one V_H gene rearrangement, which share common mutations but differ in diversifying mutations.
* (CD13+) or (CD19+) depicts sorted myeloid and lymphoid populations respectively.
** Drexler HG et al., DSMZ Catalogue of cell lines
n.k.: not known

Characteristics of the CML-BC cases; summerizing the cytogenetics, some genes and proteins involved, phenotype and $V_HD_HJ_H$ rearrangements. 14 of the 16 cases have at least one rearranged *IGH* allele. Somatic hypermutation events were observed in the V_H gene segments of most CML-BC cases.

3.4 Rearrangement of the κ-deleting element (KDE)

As soon as B cell precursors successfully rearrange V_H, D_H and J_H gene segments on one allele, the recombination machinery is suggested to be halted, preventing further rearrangement of the second allele and thus enabling allelic exclusion of the *IGH* locus (Alt et al., 1984). At the end of the recombination process of the *IGH* locus, a recombination process of the *IGL* gene is initiated, which involves the rearrangement of the Ig κ light chain and subsequently the Ig λ light chain loci. This process marks the transition of pre-B to immature B cells (Hardy et al., 1991). The progression from κ- to λ-gene rearrangement is often associated with a deletion of the constant κ (Cκ) and a portion of the joining κ (Jκ)-Cκ and the kappa enhancer sequence (Durdik et al., 1984; Siminovitch et al., 1987). This recombination is mediated by a site-specific rearrangement of a DNA element to an RSS located either in the Jκ-Cκ intron or within the Vκ gene region, which leads to the inactivation of a pre-existing Vκ-Jκ joint. This recombinatorial element is known as the kappa deleting element (*KDE*) and is rearranged in all kappa-deleted lambda-producing B cells (Siminovitch et al., 1987). To verify if cells in CML-BC go further than the rearrangement of the *IGH* locus, it was investigated whether *KDE* is rearranged CML-BC by performing a one round PCR of 45 cycles using a JκCκ intron primer and a *KDE* specific primer. Figure 13 shows the *KDE* recombination event and where the primers used for the PCR are situated.

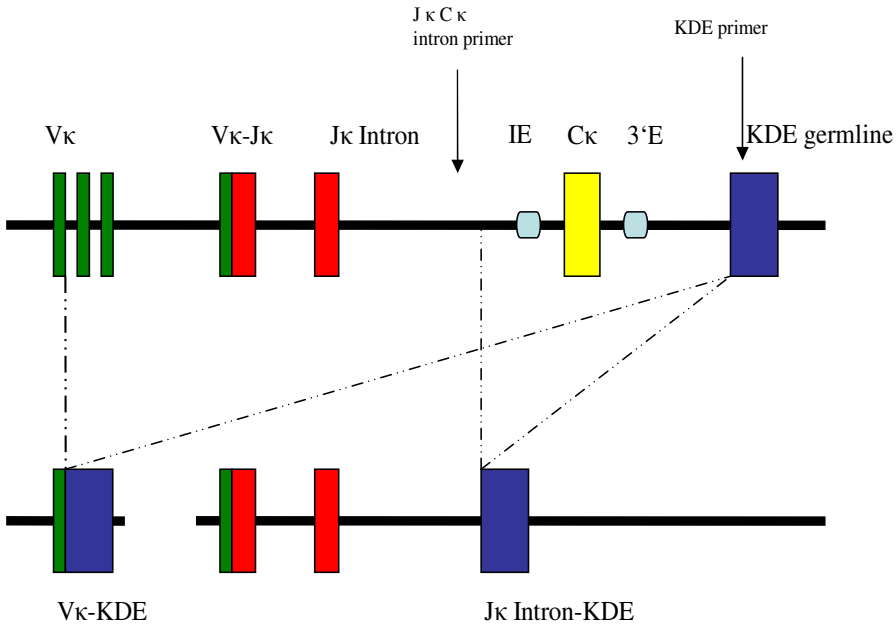


Figure 13: Two types of *KDE* rearrangements

By using an upstream Vκ-RSS of an unrearranged Vκ gene segment, downstream unrearranged Vκ gene segments, a pre-existing VκJκ joint, unrearranged Jκ gene segments and Cκ genes are deleted. Otherwise an intronic JκCκ RSS can be used during the *KDE* rearrangement resulting in the deletion of the Cκ gene and both κ enhancers. Primers were situated to localize the latter rearrangement in the PCR.

Table 4: Sequence alignment of rearranged *KDE* to intronic JκCκ RSS

as seen in two CML-BC cell lines compared to germline (GL) sequence and rearranged *KDE* in a B cell line (697). The upper alignment shows the Jκ intron domain covered by the primer while the lower alignment shows the junction of the Jκ intron to the *KDE*, highlighting the heptamers of the recombination signal sequences (brown) involved in the recombination. Depicted on the table are mutations in the form of replacements (red), insertions (green), deletions (blue) and recurring mutations that define germline polymorphism (underlined).

	Jκ-intron
GL	CTTTGGTGGCCATGCCACCGCGCTCTTGGGGCAGCCGCCTTGCCGCTA TG CCGTGGCCACC
JURL	CTTTGGTGGCCATGCCACCGCGCTCT_GGGGCAGCCGCCTTGCCGCTAGTGGCCGTGGCCACC
NALM1	CTTTGGTGGCCATGCCACCGCGCTCT_GGGGCAGCCGCCTTGCCGCTAGTGGCCGTGGCCACC
697	CTTTGGTGGCC_TGCCACCGCGCTCTTGGGGCAGCCGCCTTGCCGCTAGTGGCCGTGGCCACC

	Jκ-intron RSS		**KDE-RSS**
GL	CTGTGTCTGCCCGATTGATGCTGCCGTAGCCAGCTTTCCTGATG**CACAGTG**>RSS...		...RSS<**CACTGTG**GGAGCCCTAGTGGCAGCCCAGGGCGACTCCTCA
JURL	CTGTGTCTGCCCGAT_AATGCTGCCGTAGCCAGCTTTCCT	C	GGAGCCCTAGTGGCAGCCCAGGGCGACTCCTCA
NALM1	CTGTGTCTGCCCGAT_AATGCTGCCGTAGCCAGC	CCTTCGGG	AGTGGCAGCCCAGGGCGACTCCTCA
697	CTGTGTCTGCCCGAT__GTGCTGCCGTAGCCAGCTTTCCT	CCT	GCCCTAGTGGCAGCCCAGGGCGACTCCTCA

Of all eleven CML-BC cell lines, two showed rearrangements of the *KDE* to an intronic Jκ-Cκ RSS. This event typical to B lymphocytes was observed in the Nalm1 and JURL cell lines and the sequences obtained from the PCR products showed various forms of mutations when compared to germline Jκ-Cκ intron and *KDE* sequences. An insertion of guanine was observed in all cases as well as a deletion of thymine. Comparing the sequences to other *KDE* rearrangement sequences in literature (not shown in table 4) (>gi|12750934|emb|AJ291993.1|HSA291993, >gi|2612957|gb|AF026483.1|AF026483, gi|33285|emb|X03959.1|HSIGKREC for Hodgkin lymphoma, MCL12-Mantle cell lymphoma, GM-cell line GM2132 respectively) recurring mutations were observed which depicted germline polymorphism. These results further justify the fact that CML-BCs have B lymphoid characteristics and behave like B cell lymphoma.

3.5 Sporadic occurrence of *TCRBV* region gene rearrangements in a fraction of CML-BC cases

T lymphocytes mediate cellular immunity through their receptors, which consist of two distinct heterodimers (TCRαβ or TCRγδ). Like in B lymphocytes *TCRB* genes are assembled somatically in differentiating T cells by V(D)J recombination, initiated by the RAG endonuclease (Tonegawa et al., 1983; Kronenberg et al., 1986). RAG protein is expressed by six CML-BC cases and 14 out of 16 CML-BC cases have rearranged *IGH* loci. Considering the fact that the principles of *IGH* gene rearrangements apply to the *TCRB* locus (as far as rearrangements and germline configuration for one of the two Cβ clusters are concerned), it was worthwhile verifying if the CML-BC cases do also have rearranged *TCRBV* region genes. This was achieved by a two round PCR of 70 cycles in total, using cDNA amplified from the CML-BC cases, a collection of 24 Vβ-family-specific primers and two Jβ-specific primer mixes. Using genomic DNA amplified from the CML-BC cases, the germline configuration of the *TCRB* locus was detected with primers binding intronic sequences 5' and 3' of the Dβ1 and Dβ2 gene ssegments (Figure 14).

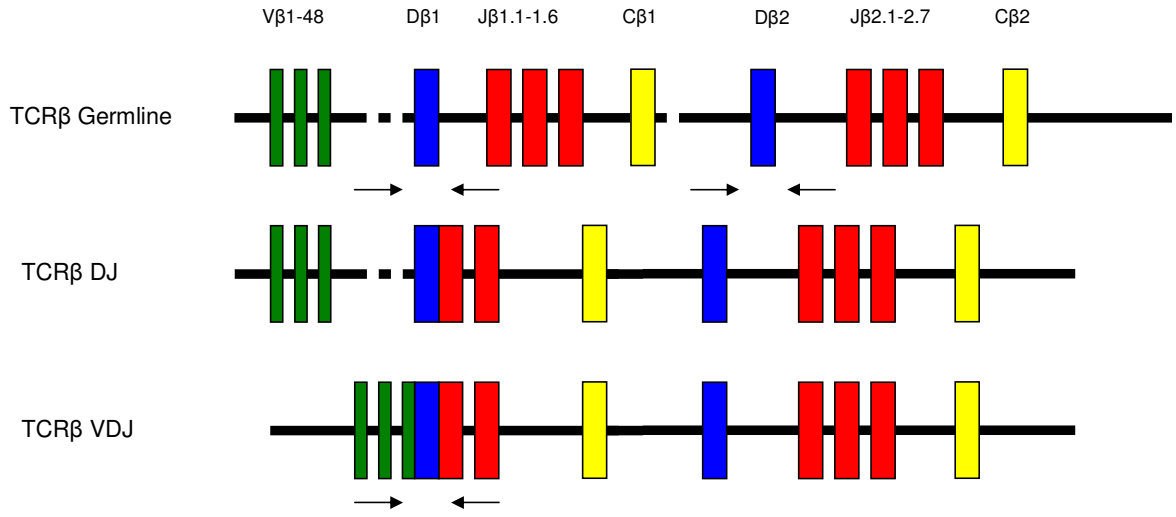


Figure 14: An illustration of the human *TCRB* locus

showing the germline configuration, *TCRB* DJ and *TCRB* VDJ. Arrows indicate the approximate location of primers.

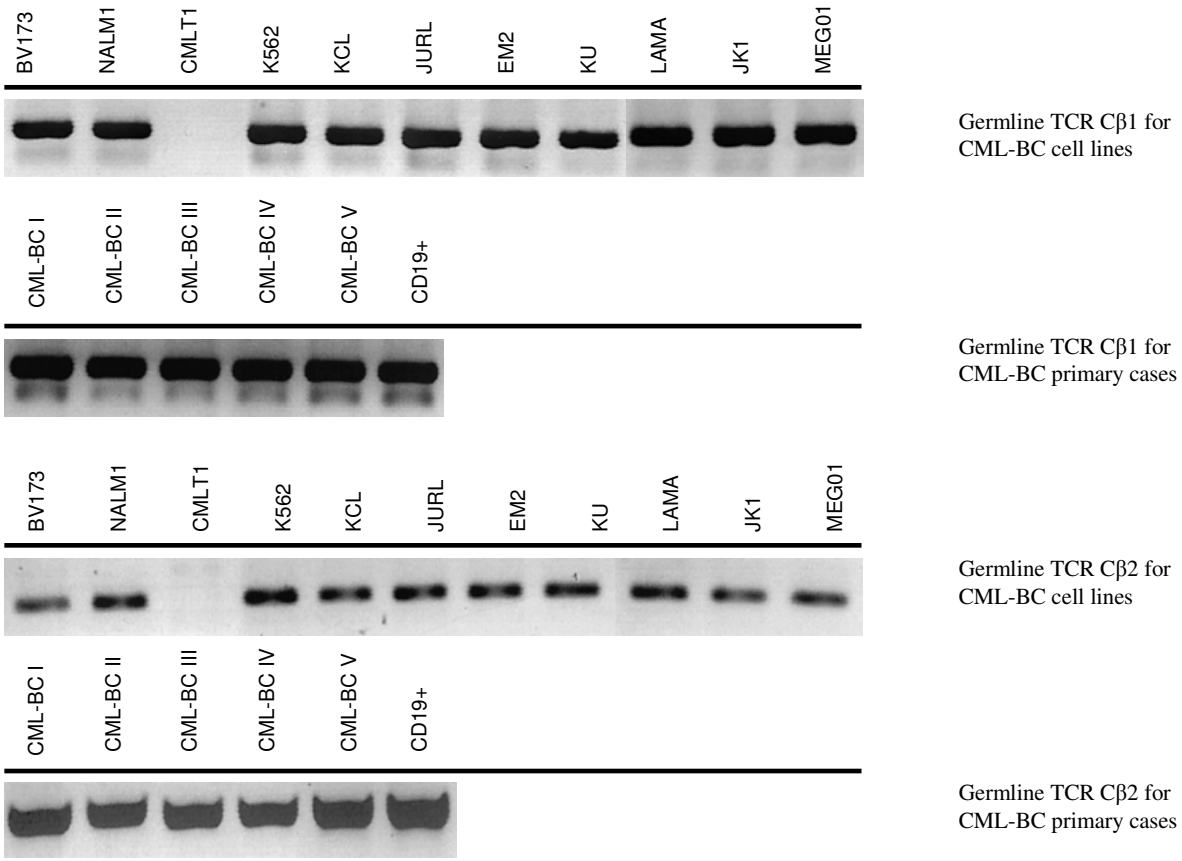


Figure 15: Germline configuration of the *TCRB* locus of CML-BC cell lines and primary cases using primers binding intronic sequences 5' and 3' of the Dβ1 and Dβ2 gene segments. $CD19^{+}$ B cells serve as positive control for this PCR as they are expected to have unrearranged *TCRB* loci.

Illustrated in Figure 15 are the germline configurations of both *TCRB1* and *TCRB2* of the 16 CML-BC cases. CML-T1 is the only case where both Cβ clusters are rearranged and as it will be shown later both *TCRB* loci are rearranged. The other 15 cases have at least an unrearranged cluster in one of the *TCRB* loci or in both loci. Table 5 shows the characteristics of the CML-BC cases but especially the results of the *TCRB* VDJ RT-PCR. The sequences were analyzed using the IMGT tool for human Vβ region gene and it was observed that seven cases, including CML-T1, K562, JURL, EM2, CML-BC III, IV and V had undergone somatic recombination in their Vβ region genes. The other cases; BV173, Nalm1, KU182, LAMA84, JK1, MEG01, CML-BC I and II therefore have both *TCRB* loci in germline configuration. Still, these sporadic *TCRB* gene rearrangements detailed on table 5 below add-up to the other unusual events observed in CML-BC.

Table 5a: Vβ Rearrangements, mutations and other characteristics of chronic myeloid leukaemia in blast crisis cell lines

Case	Vβ	Dβ	Jβ	Number of mutations [x 10^{-3} bp]	Cytogenetics	*RAG1*	Phenotype
BV173	germline Cβ1, Cβ2			no mutation	t(9;22)(q34;q11)	+	B lymphoid (CD19), Myeloid (CD13, IL3Rα, GM-CSFRα)
NALM1	germline Cβ1, Cβ2			no mutation	t(9;22)(q34;q11)	+	B lymphoid (CD19, VpreB, μ chain), Myeloid (IL3Rα, GM- CSFRα)
CML-T1	V4-2 V4–3 V18-01 V19-01 V19-01	none none none D2-1 D2-1	J2-3 J2-3 J2-7 J2-5 J2-6	3 (7) 0 5 (13) 0 0	t(9;22)(q34;q11)	+	T lymphoid (CD3, CD4, CD7, CD8, TCRγ), Myeloid (CD13, GM-CSFRα, IL3Rα), B lymphoid (CD19)
K562	V4-3 germline Cβ1, Cβ2	D1-01	J1-2	3,8 (11) intraclonal diversity	t(9;22)(q34;q11)	+	Erythroid (CD36, CD71, Glycophorin A), Myeloid (CD13, CD14, IL3Rα,GM-CSFRα)
KCL-22	germline Cβ1, Cβ2			no mutation	t(9;22)(q34;q11)	-	Myeloid (CD13, CD14, IL3Rα, GMCSFrα)
JURL	V4-1 V20-1 V7-3 germline Cβ1, Cβ2	none none D2-2	J1-2 J1-2 J2-6	4 (6) intraclonal diversity 0 6 (18)	t(9;22)(q34;q11)	-	Myeloid (CD13, CD14, IL3Rα, GMCSFrα)
EM2	V30-02 V18-01 germline Cβ1, Cβ2	D1-01 D2.01	J1-2 J1-1	2 (5) 6 (18)	t(9;22)(q34;q11)	-	Myeloid (CD13, CD14, IL3Rα, GMCSFrα)
KU 812	germline Cβ1, Cβ2			no mutation	t(9;22)(q34;q11)	-	T lymphoid (CD4, TCRγ), Myeloid (CD13)
LAMA 84	germline Cβ1, Cβ2			no mutation	t(9;22)(q34;q11)	+	T lymphoid (CD4, TCRγ), Myeloid (CD13)
JK1	germline Cβ1, Cβ2			no mutation	t(9;22)(q34;q11)	+	T lymphoid (CD4, TCRγ), Myeloid (CD13)
MEG01	germline Cβ1, Cβ2			no mutation	t(9;22)(q34;q11)	-	Myeloid (CD13)

Note:

Intraclonal diversity denotes the presence of multiple sequences for one Vβ gene rearrangement, which share common mutations but differ in diversifying mutations.

Table 5b: Vβ Rearrangements, mutations and other characteristics of chronic myeloid leukaemia in blast crisis primary cases

Case	Vβ	Dβ	Jβ	Number of mutations [x 10^{-3} bp]	Cytogenetics	*RAG1*	Phenotype
CML-BC I	germline Cβ1, Cβ2			no mutation	t(9;22)(q34;q11)	n.k	Myeloid (CD13), B lymphoid (CD19)
CML-BC II	germline Cβ1, Cβ2			no mutation	t(9;22)(q34;q11)	n.k	Myeloid (CD13), B lymphoid (CD19)
CML-BC III	(**CD19+**)*				t(9;22)(q34;q11)	n.k	Myeloid (CD13), B lymphoid (CD19)
	V6-5	D1-01	J1-1	1 (3)			
	V20-1	D1-01	J1-2	0			
	V28-01	D2-01	J2-5	8 (23)			
	(**CD13+**)*						
	V3-1	D1-01	J1-1	0			
	germline Cβ1, Cβ2						
CML-BC IV	(**CD19+**)*				t(9;22)(q34;q11)	n.k	Myeloid (CD13), B lymphoid (CD19)
	V28-01	D2-02	J2-7	0			
	V12-5	D2-02	J2-3	3 (5)			
	germline Cβ1, Cβ2						
CML-BC V	(**CD19+**)*				t(9;22)(q34;q11)	n.k	Myeloid (CD13), B lymphoid (CD19)
	V20-1	D2-02	J2-7	1 (3)			
	germline Cβ1, Cβ2						
Mean:				**3.91 (11.73)**			

Note:
* (CD13+) or (CD19+) depicts sorted myeloid and lymphoid populations respectively.
n.k.: not known

Table 5c: Vβ Rearrangements and mutations of control $CD3^+$ T cells

Case	Vβ	Dβ	Jβ	Number of mutations [x 10^{-3} bp]	Phenotype
$CD3^+$	V6-1	D1-01	J2-2	0	T lymphoid (CD3)
cells	V6-1	D2-01	J1-5	0	
	V7-2	D1-01	J2-7	0	
	V28-01	none	J2-2	0	
	V28-01	D2-01	J2-2	0	
	V28-01	D1-01	J1-4	0	
	V28-01	D2-01	J1-1	0	
	V28-01	D1-01	J1-5	0	
	V12-4	D1-01	J2-2	0	
	V12-4	D2-02	J2-2	0	
	V12-3	D2-01	J2-2	0	
	V2-01	D1-01	J1-5	1 (3)	
	V3-1	D1-01	J1-2	1 (3)	
	V7-7	D2-02	J2-7	1 (3)	
	V20-1	D2-01	J2-1	1 (3)	
	V28-01	D1-01	J2-2	1 (3)	
	V28-01	D2-01	J1-1	1 (3)	
	V28-01	D1-01	J1-2	1 (3)	
	V28-01	D2-01	J2-2	1 (3)	
	V6-5	D1-01	J1-5	2 (6)	
Mean:				**0,5 (1,47)**	

Tables 5a, 5b and 5c show *TCRB* gene rearrangements observed in CML-BC and $CD3^+$ control T cells, also showing the number of aberrant mutation events.

CML-T1 had five different *TCRBV* rearrangements while the other cases ranged between one and three rearrangements. The primary CML-BC cases were previously sorted in $CD13^+$ myeloid and $CD19^+$ B lymphoid populations, from which cDNA was later amplified and the occurrence of *TCRBV* gene rearrangements was investigated using these populations. In one case, CML-BC III, both B lymphoid and myeloid populations had rearranged *TCRBV* region genes and in two other cases, CML-BC IV and V, the B lymphoid population had rearranged *TCRBV* region genes. This argues against lineage specificity in CML-BC, as some cells carrying the CD19 B cell surface marker or the CD13 myeloid surface marker also seem to have rearranged *TCRB* instead of germline *TCRB*. One might argue that these observations (Sporadic occurrence of *TCRBV* gene rearrangements in CML-BC cases) were as a result of the 70 cycles of amplification carried out in the 2-round PCR. To disprove these assumptions, 22 B cell leukemia and lymphoma cell lines (KASUMI, GRANTA, SEM, BEL1, U266, JNN, JEKO1, MEC, NALM6, SP49, LP1, MC116, RS4-11, MN60, KMH2, MHH-CALL3, MHH-PreB, JVM, DB1, HPBnull, SD1, SUP-B15) were investigated for the expression of rearranged *TCRBV* genes in a 2-round PCR of 70 cycles (Figure 16). Only one cell line, SP-49, had a rearranged *TCRBV* gene. This shows that rearranged *TCRB* genes are not necessarily amplified in non T cells after a 70 cycle PCR. Table 5 also shows that cases of aberrant somatic hypermutation were observed in the *TCRBV* gene with the possibility of intraclonal diversity in two cases. This will be discussed in chapters 3.9.3 and 3.9.4.

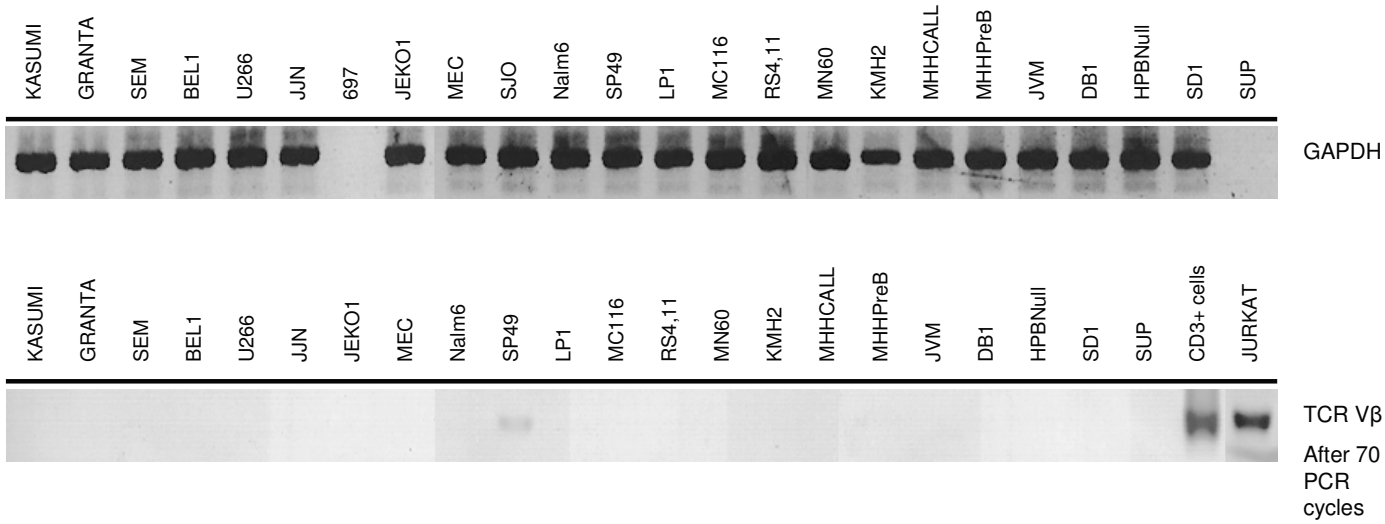


Figure 16: B lymphoid leukemia and lymphoma cell lines analyzed for *TCRB* gene rearrangements

21 B lymphoid leukemia and lymphoma cell lines were verified for the expression of *TCRBV* after 70 PCR cycles. Only SP49 was positive. $CD3^+$ T cells and the T cell line, Jurkat were used as positive controls for this PCR.

3.6 Rearrangements in the *TCRGV* region gene of CML-BC cases

The *TCRG* locus has a similar structure as the *TCRB* locus, having 2 constant region genes with each having its own Jγ gene segments but without Dγ gene segments. There are relatively fewer Vγ gene segments as compared to the *TCRB* locus (Triebel et al., 1988; Huck et al., 1988). Like in *TCRB*, *TCRG* genes are assembled somatically in differentiating T cells by VJ recombination. CML-BC cases were verified for the expression of rearranged Vγ region genes by RT-PCR using a TCR Vγ specific primer combination and two TCR Jγ specific primers which recognize both clusters. The PCR products were cloned, sequenced and the sequences were analyzed using IMGT as previously described.

Table 6 shows that three cell lines (BV173, Nalm1 and CML-T1) and all primary cases had rearranged *TCRG* loci. No rearranged Vγ was amplified in the other cell lines after three control PCRs. The frequency of mutations in the Vγ region genes ranged between zero in most cases and three in one case which might be caused by *Taq* DNA polymerase errors.

Table 6: Vγ Rearrangements, mutations and other characteristics of chronic myeloid leukaemia in blast crisis

Case	Vγ	Jγ	Number of mutations [x 10^{-3} bp]	Cytogenetics	*RAG1*	Phenotype
BV173	V3-01 V8-01	J2-01 Jp1-01	1 (1) 2 (6)	t(9;22)(q34;q11)	+	B lymphoid (CD19), Myeloid (CD13, IL3Rα, GM-CSFRα)
NALM1	V5-01 V5-01	J2-01 Jp1-01	0 0	t(9;22)(q34;q11)	+	B lymphoid (CD19, VpreB, μ chain), Myeloid (IL3Rα, GM-CSFRα)
CML-T1	V4-02 V4-02	J2-01 J1-01	0 1 (5)	t(9;22)(q34;q11)	+	T lymphoid (CD3, CD4, CD7, CD8, TCRγ), Myeloid (CD13, GM- CSFRα, IL3Rα), B lymphoid (CD19)
K562	no product*			t(9;22)(q34;q11)	+	Erythroid (CD36, CD71, Glycophorin A), Myeloid (CD13, CD14, IL3Rα,GM-CSFRα)
KCL-22	no product*			t(9;22)(q34;q11)	-	Myeloid (CD13, CD14, IL3Rα, GMCSFrα)
JURL	no product*			t(9;22)(q34;q11)	-	Myeloid (CD13, CD14, IL3Rα, GMCSFrα)
EM2	no product*			t(9;22)(q34;q11)	-	Myeloid (CD13, CD14, IL3Rα, GMCSFrα)
KU 182	no product*			t(9;22)(q34;q11)	-	T lymphoid (CD4, TCRγ), Myeloid (CD13)
LAMA 84	no product*			t(9;22)(q34;q11)	+	T lymphoid (CD4, TCRγ), Myeloid (CD13)
JK1	no product*			t(9;22)(q34;q11)	+	T lymphoid (CD4, TCRγ), Myeloid (CD13)
MEG01	no product*			t(9;22)(q34;q11)	-	Myeloid (CD13)
CML-BC I	V4-02	J2-01	0	t(9;22)(q34;q11)	n.k.	Myeloid (CD13), B lymphoid (CD19)
CML-BC II	V3-01	J2-01	0	t(9;22)(q34;q11)	n.k.	Myeloid (CD13), B lymphoid (CD19)
	(CD19+)‡					
CML-BC III	V3-01	J2-01	0	t(9;22)(q34;q11)	n.k.	Myeloid (CD13), B lymphoid (CD19)
	V4-02	J2-01	0			
	V8-01	J2-01	1 (5)			
	V9-01	J2-01	0			
	(CD13+)‡					
	V2-01	J2-01	1 (2)			
	V2-02	J2-01	1 (1)			
CML-BC IV	V2-02	J2-01	3 (14)	t(9;22)(q34;q11)	n.k.	Myeloid (CD13), B lymphoid (CD19)
	V4-02	J2-01	1 (1)			
CML-BC V	V3-01	J2-01	0	t(9;22)(q34;q11)	n.k.	Myeloid (CD13), B lymphoid (CD19)
	V4-02	J2-01	0			
	V10-02	J2-01	0			
Mean:			**0.4 (1.84)**			

Note: * No PCR product in 3 control PCRs.
‡ (CD13+) or (CD19+) depicts sorted myeloid and lymphoid populations respectively.
n.k.: **not known**.

3.7 Activation-induced cytidine deaminase (AID) observed in CML-BC cell lines and primary cases

The activation-induced cytidine deaminase (AID) is known to be an essential enzyme in the regulation of class switch recombination (CSR) and somatic hypermutation (SHM) in B cells (Muramatsu et al., 2000; Revy et al., 2000). AID is suspected to function either by editing unknown mRNA to generate specific endonucleases for CSR and SHM or by deaminating cytosine in DNA, then DNA is cleaved by base excision repair enzymes. Independent on how it works, AID has been proven to be essential and sufficient for CSR and SHM as demonstrated by studies on *AID*-deficient animals and patients (Muramatsu et al., 2000; Revy et al., 2000). Honjo et al. have previously shown that the over-expression of AID in non-B cells can induce SHM in a wide variety of genes with little specificity. For example *AID* transgenic mice developed T cell lymphomas with SHM found in the TCR Vβ region gene (Kotani et al., 2005). If this is the case, it will be interesting to find out if *AID* is expressed in CML-BC and if yes, whether it has the effect of ectopic expression of *AID* in non-B cells i.e. the induction of SHM in genes other than the Ig variable region genes, thus explaining the SHM observed in *TCRBV* region genes (Table 5a and b). The verification of *AID* expression in CML-BC was achieved by an *AID* RT-PCR using *AID* specific primers based on the human cDNA sequence, after creating cDNA from the RNA isolated from the cell lines. The integrity of all cDNAs was constantly verified by the amplification of a *GAPDH* cDNA fragment. Figure 17 shows a summary of different *AID* RT-PCRs carried out with CML-BC cell lines. Two cell lines, JK1 and KCL-22 were repeatedly negative for *AID* in three experiments. In the different experiments AID is sometimes activated/ expressed and sometimes not, as observed in some cell lines like Nalm1, EM2 and MEG01. The expression of *AID* in these CML-BCs might either imply that AID is not a B cell specific enzyme or that the expression of AID is as a result of the existing B lymphoid populations in CML-BC, thus explaining the weak expression of *AID* in some cell lines. The expression of *AID* in the primary cases (Figure 18 and 19) is B cell specific as *AID* was found only in the $CD19^+$ sorted population but not in the $CD13^+$ population of all five cases, except in one case (CML-BC I) where there was a weak *AID* band seen after 45 *AID* RT-PCR cycles of the T lymphoid population (Figure 18).

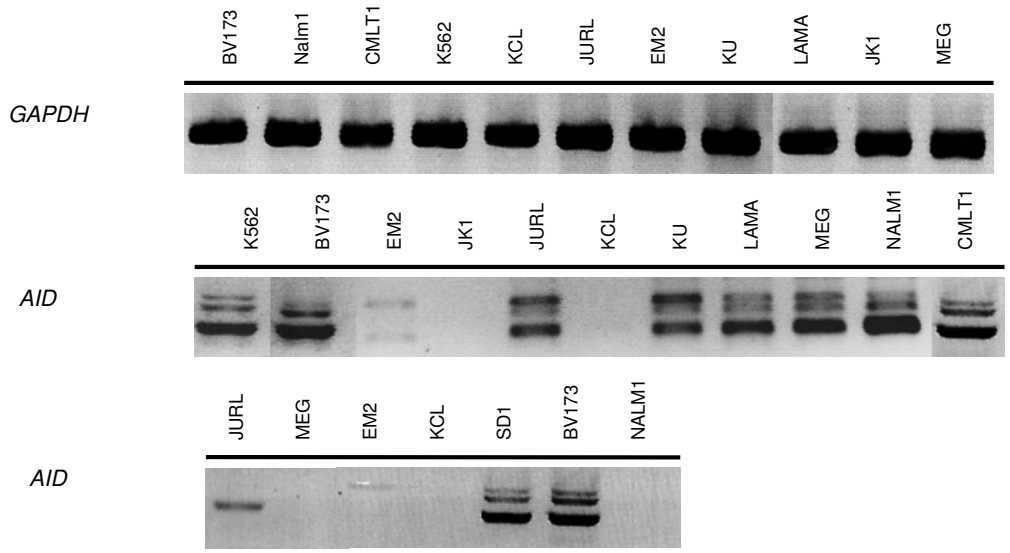


Figure 17: Summary of *GAPDH* and *AID* RT-PCRs for CML-BC cell lines

The middle and bottom pictures show different RT-PCRs, whereby an *AID* cDNA fragment is sometimes amplified in Nalm1, EM2 and MEG01 and sometimes not. In both cases, the expression of *GAPDH* was verified before using the cDNAs as templates for the *AID* RT-PCR. *AID* cDNA fragments could not be amplified in JK1 and KCL in three independent RT-PCRs.

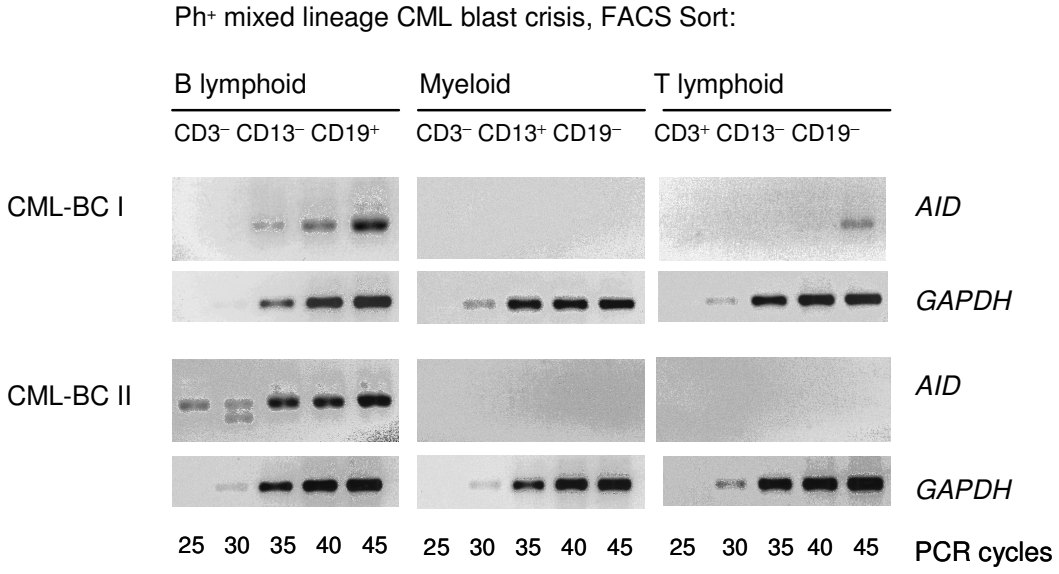


Figure 18: Two CML-BC primary cases were sorted in B lymphoid, T lymphoid and myeloid populations and verified for their expression of *AID* by semi-quantitative RT-PCR

The house-keeping *GAPDH* gene was also amplified and shown in the different cycles. The B lymphoid populations express *AID* and the T lymphoid population of CML-BCI has a weak expression of *AID* after 45 PCR cycles (Adapted from Henke et al., unpublished).

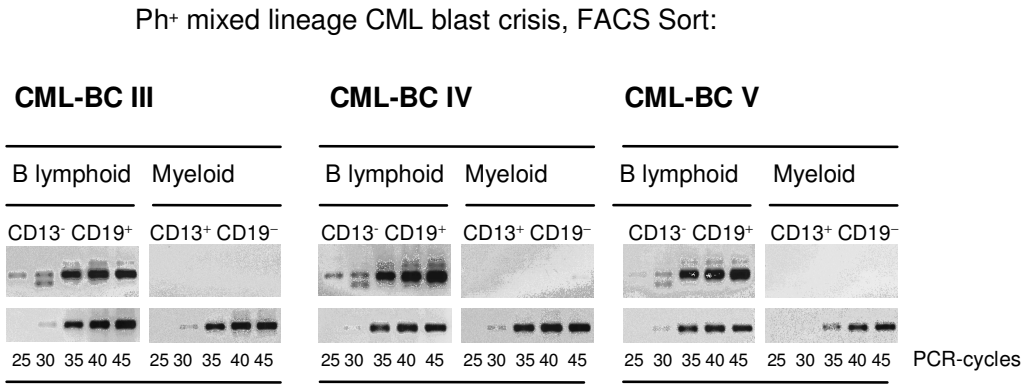


Figure 19: AID semi-quantitative RT-PCR for three other CML-BC primary cases

The three other CML-BC primary cases were sorted in B and T lymphoid populations and also semi-quantitatively verified for the expression of *AID*. *AID* cDNA fragments were amplified in the B lymphoid sub population of all cases (Adapted from Henke et al., unpublished).

3.8 Aberrant somatic hypermutation in CML-BC cells

Somatic hypermutation (SHM) of IgV genes is normal in B cells and is strongly correlated to B cell differentiation (Berek et al., 1991). The hypermutation process is suggested to be concentrated in, or restricted to germinal center environment and shuts off when cells leave the germinal center microenvironment (Berek et al., 1991). Functionally, SHM represents the molecular basis of affinity maturation of naïve B cells after exposure to and interaction with antigens. Molecularly, it represents the hypermutation capability of IgV genes from the 5' of the IgV gene promoter to around 1.5 kb downstream excluding the constant region (Müller-Hermelink et al., 1998). The mutations consist mainly of point mutations, which accumulate mainly within the antigen-binding complementary determining regions (CDR) and rarely duplications or deletions (Goossens et al., 1998). SHM has been found in marginal zone B cell lymphoma and diffuse large B cell lymphoma independent of the germinal centre microenvironment showing that in malignant B cell populations the proliferation-associated hypermutation process is independent of the germinal center microenvironment. SHM has also been found in a wide variety of genes with little specificity when AID is over-expressed in non-B cells, for example *AID* transgenic mice developed T cell lymphomas with SHM found in the TCR Vβ region gene (Kotani et al., 2005). In the course of analyzing the sequences of the V_H and Vβ region genes of CML-BC cases, several cases of aberrant SHM were observed in various cell lines and primary leukemias (elaborated in sections 3.8.1- 3.8.4). These findings fit to the somewhat peculiar and abnormal nature of this leukemia, considering the malignancy of CML-BC, the abnormal expression of RAG and AID proteins in CML-BC cells and the sporadic occurrence of rearrangements of V_H and Vβ region genes in CML-BC.

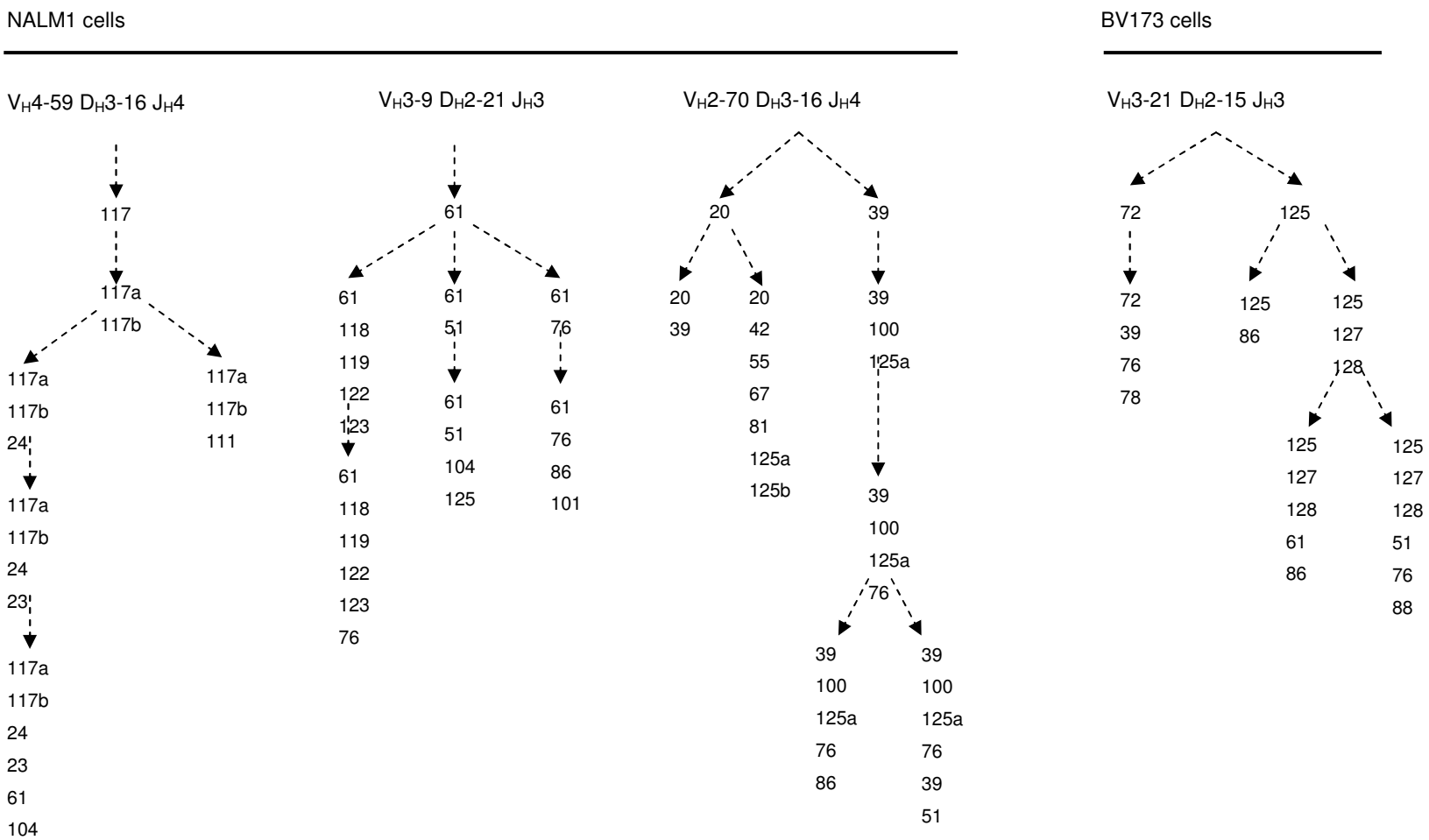
3.8.1 Somatic Hypermutation in *IGHV* region genes of CML-BC cells

In all but two CML-BC cases (CML-BC II: V_H1-3 D_H5-18 J_H6 and CML-BC III: V_H1-8 D_H5-18 J_H6) where rearranged V_H region genes were observed, somatic mutations were found in the IgV region gene. These mutations ranged from $3/10^3$ bp, as in KCL-22 and EM2, to $133/10^3$ bp in CML-BC III (Table 3), whereby mutations higher than $10/10^3$ bp were considered significant allowing for mutation errors attributed to the *Taq* DNA polymerase (whose mutation frequency was calculated at $2.5/10^3$ bp). The CML-BC cell lines of B lymphoid origin, Nalm1 and BV173, have high levels of somatic hypermutation of up to $37/10^3$ bp and $22/10^3$ bp respectively. Both cell lines also showed evidence of ongoing mutations in some rearrangements, as will be shown later. The CML-BC cell line of T lymphoid origin, CMLT1, had $22/10^3$ bp mutations in the V_H4-34 D_H2-8 J_H6 rearrangement which is as much as the mutations in BV173. The other CMLT1 rearrangement had a high level of mutations of up to $137/10^3$ bp. K562, LAMA84 and JK1 also had up to 22 and $19/10^3$ bp mutations, respectively. Two primary cases also had a SHM accumulation of up to $24/10^3$ bp in CML-BC IV and $133/10^3$ bp in CML-BC III as already mentioned. Taking all 16 cases into perspective (Table 3), there is a correlation between the expression of *AID* and the harboring of somatic hypermutation: in 11 cases where *AID* was found to be expressed, there is also a considerable accumulation of SHM, except EM2, KU182 and MEG01 which are either unmutated or have a low mutation frequency. JK1, which lacks *AID* expression, had two rearrangements with 19 and $13/10^3$ bp mutations, which might be because AID might have been active in earlier stages of the disease, thus causing hypermutated V_H region genes, and later became inactive.

3.8.2 Intraclonal diversity in *IGHV* region genes of some CML-BC cases

As mentioned above, evidence of continuous accumulation of mutations by ongoing hypermutation was observed in the V_H gene regions of seven out of the 16 CML-BC cases (Table 3), exemplified by evolutionary trees of receptor mutations of two cell lines (Figure 20). This leads to clonal heterogeneity, which is consistent with an active somatic hypermutation process. This implies that some clones harbor common mutations shared by multiple other subclones, with the difference that these subclones have individual diversifying mutations, reflecting their clonal evolution.

Figure 20: Tracing the clonal evolution of two CML-BC cell lines of lymphoid origin by V_H region gene mutations (Feldhahn et al., unpublished)



Evolutionary trees of Nalm1 and BV173 showing subclones, which share mutations and subsequently acquire individual mutations. Numbers stand for mutated codons and letters represent different mutations in the same codon. For example in the Nalm1 V_H4-59 D_H3-16 J_H4 rearrangement many clones share the

mutation on codon 117. Further subclones were then found with another mutation of this same codon (mutations 117a and b) and the evolution proceeds on the one hand with subclones which share 117a,b obtaining mutations on codon 111. On the other hand, subclones were found sharing 117a, b and later acquired mutations on codons 24, 23, 61 and 104 progressively.

3.8.3 Somatic Hypermutation in *TCRBV* genes of CML cells but not in *TCRBV* genes of normal T cells

In a previous study, single nucleotide replacements in Vβ genes reflecting low levels of SHM or polymorphism have been described (Concannon et al., 1986). Also, germinal centre T cells have previously been isolated and their receptor genes were sequenced, finding out that these genes accumulated mutations (Zheng et al., 1994). These studies raise the intriguing possibility that T cell receptors might have somatic hypermutation potential. Upon ectopic expression of *AID* in non-B cells, SHM has been observed in *TCRBV* genes (Kotani et al., 2005). Taking these data into consideration, *TCRBV* genes of CML-BC cases, normal CD3+ T cells, an *AID* positive T-ALL and the B lymphoma cell line SP49 (which surprisingly carried a rearranged *TCRBV* gene) were investigated for SHM. Five CML-BC cases, CML-T1, K562, JURL, EM2 and CML-BC III, out of seven with rearranged *TCRB* genes have significant numbers of mutations ranging from $7/10^3$ bp to $23/10^3$ bp (Table 5a and b). CML-T1 has *TCRBV* genes with up to $13/10^3$ bp mutations, JURL and EM2 with up to $18/10^3$ bp mutations and the primary case CML-BC III with up to $23/10^3$ bp. Table 5c shows normal CD3+ T cells which were analyzed for rearrangements in their *TCRB* locus and for mutations in their Vβ region genes. Out of the 20 clones sequenced, 11 had no mutations i.e. $0/10^3$ bp in the Vβ region genes. In eight clones, $3/10^3$ bp mutations were observed and in one case, $6/10^3$ bp mutations. Subsequently, an *AID* expressing T-ALL was analyzed for rearrangements in the *TCRB* locus and for mutations in its Vβ region genes. Table 7 below shows the rearrangements obtained and the number of mutations observed. A low mutation frequency is observed ranging from $3/10^3$ bp to $7/10^3$ bp mutations. The B lymphoma cell line SP49, which was the only one out of 22 B cell leukemia and lymphoma cell lines to express the TCRβ is also shown on Table 7. Only two clones of SP49 were successfully sequenced and both sequences were mutated, namely $7/10^3$ bp and $13/10^3$ bp mutations. Summarizing these results, normal $CD3^+$ T cells have an average of $1.5/10^3$ bp mutations, an *AID* positive T-ALL has an average of $5/10^3$ bp mutations and CML-BC cells have an average of $12/10^3$ bp mutations in their Vβ region genes. This implies that CML-BC cells have *TCRBV* genes which undergo aberrant SHM, while *AID* positive T-ALL mutates in a much lower frequency and normal T cells do not undergo SHM in their *TCRBV* genes.

Table 7: Vβ Rearrangements and mutations of a T-ALL primary case positive for *AID* and the B cell lymphoma cell line SP49

Case	Vβ	Dβ	Jβ	Number of mutations [x 10^{-3} bp]	*AID*	Phenotype
Primary T-ALL (1202)	V2-1	none	J1-3	1 (3)	+	T lymphoid
	V3-1	D1-01	J1-3	0		
	V4-1	D1-01	J2-6	3 (7)		
	V4-2	D1-01	J1-3	2 (5)		
	V18-1	D1-01	J1-2	3 (7)		
	V18-1	D2-02	J1-2	2 (5)		
	V20-1	D2-01	J1-3	1 (3)		
Mean				**1.74 (5.22)**		
SP-49	V4-2	D2-02	J1-2	5 (13)	+*	B lymphoid
	V4-1	D2-01	J1-2	3 (7)		
Mean				**4 (9.2)**		

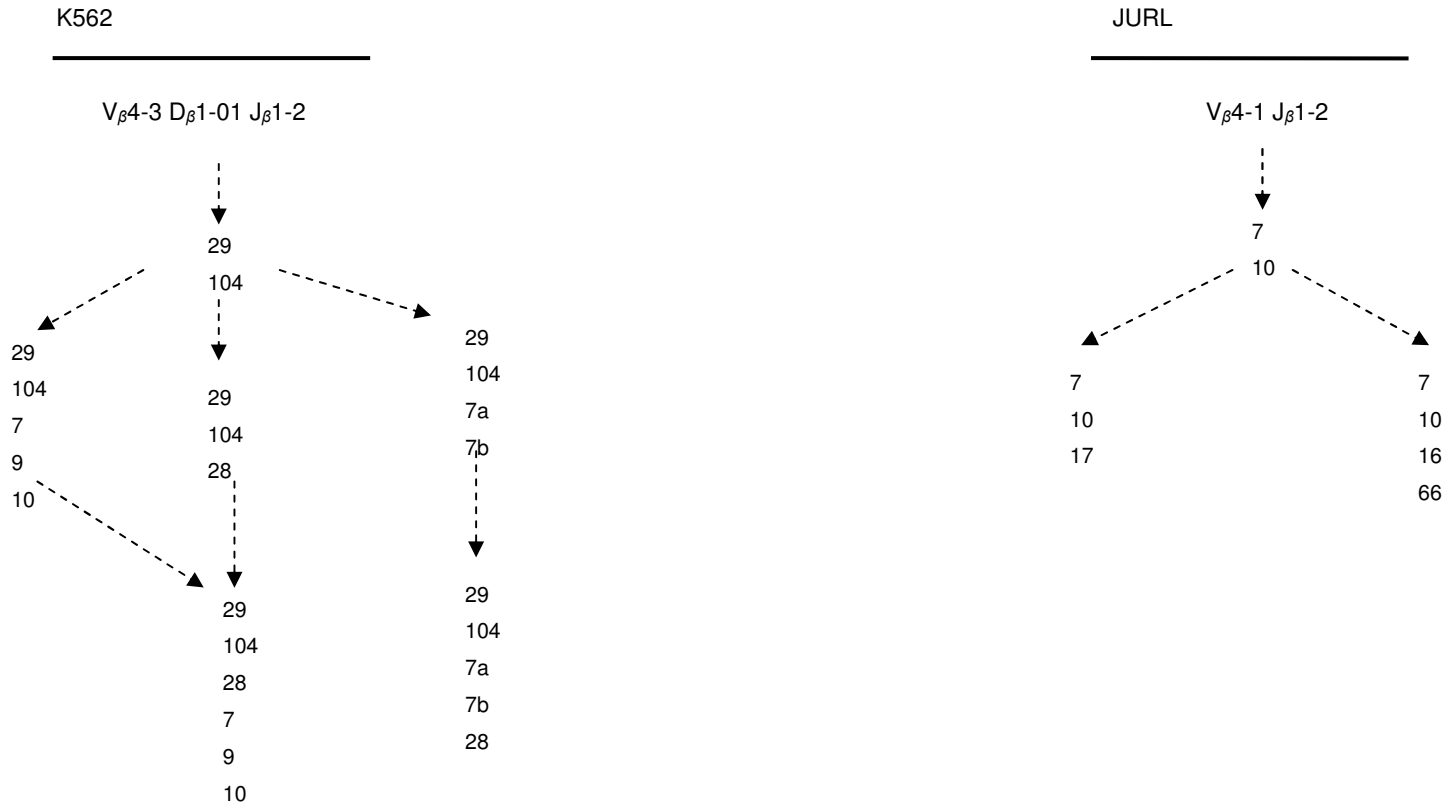
*: Klapper et al., 2006

TCRB gene rearrangements and mutations observed in an *AID* positive T-ALL as well as the B cell lymphoma SP49. A low mutation frequency ranging from $3/10^3$ bp to $7/10^3$ bp mutations is observed in the T-ALL, while the *AID* positive SP49 had $7/10^3$ bp and $13/10^3$ bp mutations in the respective clones.

3.8.4 Intraclonal diversity in *TCRBV* region gene of some CML-BC cases

Evidence of continuous accumulation of mutations by ongoing hypermutation is shown in the Vβ region genes of two CML-BC cell lines, K562 and JURL (Figure 21). This leads to subclonal diversification, which is consistent with an active somatic hypermutation process as seen in B cells. This means that some clones have common mutations shared by multiple other subclones. These subclones differ from each other, as they have individual diversifying mutations, reflecting their clonal evolution. This especially confirms the authenticity of the SHM as the mutations can be traced to a common origin.

Figure 21: Tracing the clonal evolution of two CML-BC cell lines of myeloid/megakaryocytic origin by Vβ region gene mutations



Evolutionary trees of K562 and JURL showing subclones, which share mutations and subsequently acquire individual mutations. Numbers stand for mutated codons and letters represent different mutations in the same codon. For example, the "mother" clone of K562 Vβ4-3 Dβ1-01 Jβ1-2 rearrangement has mutations in codons 29 and 104 and evolves by gaining mutations in three directions as seen in the subclones. In the first direction it gains mutations on codons 7, 9 and 10 and subsequently codon 28 in the third generation. In the second direction it gains a mutation in codon 28 in the second generation and codons 7, 9 and 10 in the third generation. In direction three it gains two mutations on codon 7 and subsequently codon 28. In this way, a family of many clones develops from the same origin

Chapter 4
Discussion

In this thesis, several CML-BC cases have been investigated for the expression of typical lymphoid genes and proteins. Thanks to the possibility of analyzing the human *IGHV* and *TCRBV* region genes and comparing sequences of CML-BC cases to germline sequences, aberrant rearrangements and somatic hypermutation have been observed in the *IGHV* and *TCRBV* region genes of several CML-BC cases. Other genes, like the recombination activating genes (*RAG*) and the activation-induced cytidine deaminase (*AID*), which are normally exclusively expressed in lymphocytes have also been detected in CML-BC cells.

4.1 Lymphocyte phenotype, lymphoid-specific proteins and protein alterations in CML-BC

4.1.1 Some CML-BC cases express B lymphoid, T lymphoid and myeloid surface markers

Flow cytometry analyses show that most CML-BC cases are mixed lineage blast crisis. Most cell lines express antigens of at least two hematopoietic lineages, i.e. some cell lines like BV173 and Nalm1 which are predominantly B lymphoid (CD19, CD10) in their phenotype also express the CD13 myeloid surface marker and also have a T lymphoid subpopulation carrying the TCRγδ (Figure 9). CML-T1 which is T lymphoid of origin has a subpopulation which is positive for the T cell specific CD3 surface marker and simultaneously carries the B cell specific CD19 cell surface marker (Figure 9). The other cell lines are predominantly myeloid in phenotype but some possess cells with the lymphoid characteristic IL7Rα, the erythroid glycophorin A surface marker or the hematopoietic stem cell marker CD34 (Figure 9). These CML-BC cells can be defined as either being a multi-lineage culmination of B lymphoid, T lymphoid, myeloid or erythroid lineages, or singular B lymphoid, T lymphoid, myeloid or erythroid lineages, which might a hematopoietic differentiation potential as some were described by Drexler et al., 2005 in the DSMZ cell line catalogue. These findings are consistent with findings in literature, where CML have been described to have an immature myeloid phenotype in 60-70% of cases and lymphoid phenotype in 25-30% of cases (Jaffe et al., 2001; Motoji, 2001) or studies where CML-BC has been described to be mixed-lineage (Lopez-Karpovitch et al., 1997).

In some cases these B and T lymphoid phenotypes are reflected in the genotype characteristics of the cells, but not vice versa. In all cell lines with a B lymphoid phenotype (BV173, Nalm1, CMLT1), there is also a rearrangement of the *IGHV* region gene. Rearranged *IGHV* region genes are also found in cell

lines, which show no typical lymphoid phenotypes, like K562, LAMA84, KU182, EM2, JK1 or KCL and JURL (Table 3). This means that CML-BC cells which are cytogenetically B lymphoid might not be phenotypically B lymphoid, which implies that cytogenetic analyses might be required to establish CML-BC. The cell lines with rearranged *TCRGV* region genes are positive for surface TCRγ but cell lines with rearranged *TCRBV* region genes had no clear-cut TCRβ phenotype. This shows that the phenotypic characteristic of CML-BC cells does not always correspond to their genotypic characteristic.

4.1.2 The lymphoid specific recombination activating genes (*RAG1* and *RAG2*) are expressed in CML-BC

The recombination activating gene 1 is expressed in six out of 11 CML-BC cell lines (Figure 10). *RAG1* and *RAG2* are tissue-specific genes required for V(D)J recombination in B and T lymphocytes (Schatz et al., 1989). These proteins are typically expressed in developing lymphocytes during the formation of their antigen receptors. RAG proteins carry the first step of the VDJ reaction by site-specific cleavage of DNA (van Gent et al., 1995). The results in figure 10, tables 3, 5 and 6 show the lymphoid origin of the CML-BC cell lines and justify their lymphoid phenotype and genotype, providing an explanation for the observed *IGHV* and *TCRBV* gene rearrangements in CML-BC. The cell lines positive for *RAG1*; BV173, Nalm1, CMLT1, K562, LAMA84 and JK1, all have rearranged *IGHV* region genes and four have rearranged *TCRBV* region genes (Table 3 and 5). Table 3 shows that some cell lines not expressing the RAG protein still possess rearranged *IGHV* region genes. This might imply that RAG was active in the early stages of the clonal evolution, where *IGHV* gene rearrangements occur, and RAG might have become inactive in later stages of the disease.
Difficulties were encountered in the amplification and sequencing of the *RAG2* cDNA fragment. The RT-PCR products for *RAG2*, which were sequenced and analyzed by blast did not correspond to the human *RAG2* gene, maybe because the primers used for the RT-PCR were not specific for *RAG2* and were likely to amplify other genes in human.

The expression and activity of RAG protein also means continuous DNA double strand breaks and accumulation of secondary transforming events, which might have an implication on the clonal evolution of CML cells and the transformation from CML acute phase to blast crisis. RAG activity has also been shown to target non-Ig genes and thus causing other genetic aberrations in lymphoma and leukemia. For example the deletions affecting the *hprt* locus in T cells (Fuscoe et al., 1991) or the transcription factor *TAL1* (Aplan et al., 1990) are suspected to be as a result of V(D)J recombinase activity. In these examples, heptamer-like sequences were found near the break-points of the deletions

and the break-points also show deletion and addition of nucleotides, thus suggesting the enzymatic activity of RAG.

Also the *RAG1* sequences of the cell lines analyzed showed various mutations in the form of insertions, deletions and replacements when aligned to *RAG1* mRNA sequence (Table 2). Mutations in the *RAG* genes have previously been described to lead to V(D)J rearrangement deficiencies, e.g. immunodeficiencies like Omenn Syndrome or B-SCID, which have a reduction or even complete lack of B and T lymphocytes (Mombaerts et al, 1992; Shinkai et al, 1992; Villa et al., 1998). This is not the case with CML-BC cells as most cell lines do not lack B and T lymphocytes but these mutations might lead to yet unknown secondary changes that might be vital for the progression to blast crisis.

CML-BC is characterized by the *BCR-ABL1* fusion gene, which amongst other effects also has an effect on the activity or levels of proteins involved in DNA repair, especially the repair of DNA double-strand breaks. The repair of DSBs by the DNA-PK dependent pathway (NHEJ) is the preferred pathway used by normal human cells (Wang et al., 2001). In BCR-ABL1- expressing cells the catalytic subunit DNA-PK_{CS} has been observed to be down-regulated and reversed by proteasome inhibitors, leading to proteasome-dependent protein degradation (Deutsch et al., 2001). For example, mice deficient in DNA-PK_{CS} can initiate V(D)J recombination but cannot form coding joints (Gao et al., 1998). BCR-ABL1 in CML cells has also been associated with the enhanced expression of RAD51, a protein involved in homologous recombination repair (Slupianek et al., 2001). These examples mentioned above show opposite effects of BCR-ABL1 on DNA-PK_{CS} and RAD51, which may just lead to promoting genomic instability and secondary genetic abnormalities causing defective repair of DSBs (Calabretta et al., 2004), leading to defective recombinase activity.

4.1.3 Activation-induced cytidine deaminase (*AID*) in CML-BC might lead to aberrant mutations in Ig and non-Ig genes of CML-BC cells

Another B lymphoid-specific protein observed in most CML-BC cases is the activation-induced cytidine deaminase (Figures 17, 18 and 19). Using RT-PCR, *AID* could be observed in nine out of 11 CML-BC cell lines, whereby JK1 and KCL-22 were repeatedly negative in three RT-PCRs. The amplification of *AID* by RT-PCR in some CML-BC cell lines was not consistent, as sometimes *AID* was expressed and sometimes not. This was the case with Nalm1, EM2 and MEG01 (Figure 17). The expression of *AID* in the CML-BC primary cases is B cell-specific as *AID* was expressed only in the $CD19^+$ sorted population but not in the $CD13^+$ population of four cases, but in one case (CML-BC I)

there was a weak *AID* band seen after 45 *AID* RT-PCR cycles of the T lymphoid population (Figure 18 and 19).

AID is an essential enzyme in the regulation of class switch recombination (CSR) and somatic hypermutation (SHM) in B cells (Muramatsu et al., 2000; Revy et al., 2000). It is suggested to function either by editing unknown mRNA to generate specific endonucleases for CSR and SHM or by deaminating cytosine in DNA, then DNA is cleaved by base excision repair enzymes. Independent on how it works, AID has been proven to be essential and sufficient for CSR and SHM, as no mutation, switch or gene conversion is observed in mouse models, chicken models and in patients with *AID* deficiency (Muramatsu et al., 2000; Revy et al., 2000; Harris et al., 2002; Arakawa et al., 2002). In support of the importance of AID, transfection of *AID* gene in fibroblasts is sufficient to induce isotype switching and mutation events just as transfection of *RAG* is sufficient to induce rearrangement (Schatz et al., 1988; Yoshikawa et al., 2002). Taking these facts into consideration, it is no surprise that mutations are found on the *IGHV* region genes of CML-BC cells, if *AID* is found in these cell lines. But surprising was the observation that these mutations were not limited to *IGHV* genes, as SHM was found in the *TCRBV* region genes of CML-BC cells. This will imply that *AID* not only affects *IGH* genes (or other B cell genes) but might also have an effect on *TCR* genes. This suggestion is supported by the fact that Honjo et al., 2005 had shown that the over-expression of AID in non-B cells can induce SHM in a wide variety of genes with little specificity. For example, *AID* transgenic mice developed T cell lymphomas with SHM found in the *TCRBV* region gene, *c-myc*, *CD4* and *CD5* genes (Kotani et al., 2005). They proposed that the target selection of SHM is regulated in the same way between T and B cells when AID is expressed. AID is involved in DNA cleavage (Nagaoka et al., 2002; Begum et al., 2004) and may be responsible for accumulation of mutations in *IGHV* and *TCRBV* region genes in cells, thus AID might play a role in the evolution of CML-BC.

4.1.4 Homozygous deletions of the *CDKN2A* and *CDKN2B* genes is found in CML-BC and also characteristic of some B and T cell leukemia and lymphoma

Research was carried out on lymphoid specific proteins found in CML-BC but previous studies also show similarities between CML-BC and B and T cell leukemia and lymphoma. One of such similarities shown in other studies is the alteration of the *CDKN2A* and *CDKN2B* tumor suppressor genes, which are deleted in BV173, K562, LAMA84 and MEG01 cell lines (Drexler et al., 2005).
The cyclin-dependent kinase (CDK) inhibitors *CDKN2A* and *CDKN2B* have long been identified as tumor suppressor genes (Knudson et al., 1971), which are inactivated either by deletion, missense mutation or promoter hypermethylation in many cancers (El-Naggar et al., 1997; Tao et al., 1997;

Danahey et al., 1999). CDK inhibitors can block cell cycle progression by inhibiting the kinase activity of CDKs and therefore have a negative control on cell proliferation (Drexler et al., 1998), but *CDKN2B* has been found to be the most frequently altered gene in cancer (Hirama et al., 1995) including CML-BC, AML, and ALL (Sill et al., 1995).

Of interest to this thesis is the finding that deletions of *CDKN2A* in T-ALL lines may be caused by misdirected activity of RAG recombinase (Cayuela et al., 1997; Kitagawa et al., 2002), as the location of T-ALL *CDKN2A* breakpoints has been found near recognition sequences of these enzymes (Raschke et al., 2005). This might be the case with CML-BC cells which express RAG activity and the deletions of the *CDKN2A* might not be limited to CML-BC with a lymphoid phenotype as described by Sill et al., 1995. Alterations of this nature leading to the lack or inactivation of these tumor suppressor genes in *BCR-ABL1* positive CML cells might play a role in the secondary changes observed in CML to CML-BC transformation.

4.2 Lymphoid specific rearrangements (*IGHV*, *KDE*, *TCRBV* and *TCRGV* region genes) in CML-BC

Rearrangements of the *IGH* and *TCRB/G* alleles have been shown to be useful clonal markers in lymphoproliferative disorders of B lymphoid and T lymphoid lineages (Seeger et al., 2001) but not necessarily expected in myeloproliferative disorders like in CML-BC. Figure 9 shows that some CML-BC cell lines carry B lymphoid, T lymphoid and myeloid lineage phenotypes and Figure 10 shows that some express the recombination activating genes (*RAG1* and *RAG2*), which is a prerequisite for somatic rearrangements within the *IGH* and the *TCRB/G* genes (Tonegawa et al., 1983; McBlane et al., 1995; van Gent et al., 1995; van Gent et al. 1996). In accordance to the above mentioned findings of RAG and lymphoid phenotype in CML-BC, *IGHV*, *TCRBV* and *TCRBG* gene rearrangements could be amplified in CML-BC cases (Table 3 and 5).

Out of the 16 CML-BC cases studied, 14 had rearranged *IGHV* region genes (Table 3). In the cases of BV173 and Nalm1 which are CML-BC of lymphoid origin, rearrangements of the *IGH* gene might be expected but CML-T1 which is T lymphoid of origin also carried rearranged *IGHV* region genes. Such was the case with the other cell lines (K562, KCL, JURL, EM2, LAMA and JK1) which were predominantly myeloid but all had rearranged *IGH* alleles, as well as the five primary cases which also had somatic recombined *IGHV* region genes.

Of all eleven CML-BC cell lines, two showed rearrangements of the *KDE* to an intronic JκCκ RSS (Table 4). This recombination is also mediated by a site-specific rearrangement of a DNA element to an RSS located either in the Jκ-Cκ intron or within the Vκ gene region (Siminovitch et al., 1987).This event typical to B lymphocytes was observed in the Nalm1 and JURL cell lines.

Rearrangements of the *TCRBV* region genes were observed in seven out of the 16 CML-BC cases (Table 5). The cases CMLT1, K562, JURL, EM2, CML-BC III, IV and V had rearrangements in their *IGHV* and *TCRBV* region genes. The cell lines BV173 and Nalm1 of B lymphoid origin had both *TCRB* alleles in germline configuration but on the other hand both cell lines had rearranged *TCRGV* region genes. The rearrangement of *TCRGV* region genes was observed in eight out of the 16 CML-BC cases (Table 6).

These genes all have the RSS and are probably disposable to the common recombinase activity by RAG. The existence of rearranged *IGH* and *TCRB/G* genes in CML-BC has been rarely described. Previously, *IGH* rearrangements have been described in CML-BC patients with CD10 expression i.e. lymphoid blast crisis (Ohyushiki et al., 1991). They also observed frequent *TCR* gene rearrangements in these cases and rightly attributed this phenomenon to the common recombinase activity. These observations are confirmed by the results on Tables 3, 5 and 6, which also assert that aberrant *IGH* and *TCR* gene rearrangements are not only limited to the phenotypically lymphoid CML-BC but are also found in phenotypically non-lymphoid CML-BC, as seen in K562, KCL, JURL, EM2, LAMA and JK1 cell lines.

The simultaneous expression of both myeloid and lymphoid immunophenotype by CML-BC as well as the simultaneous existence of *IGHV* and *TCRB/G* gene rearrangements in some CML-BC cases poses the question of aberrant antigen expression and aberrant gene rearrangements or is it a case of lineage infidelity in CML-BC as observed in acute mixed lineage leukaemia (AMLL) (Schmidt et al., 2001). A model of a “common myeloid/lymphoid progenitor cell” was described which might be responsible for AMLL. This theoretical very early hematopoietic progenitor cell shows temporary expression of myeloid, B and T lymphoid characteristics and might have rearranged its *IGH* and *TCR* genes. This hypothesis proposes that the myeloid or lymphoid markers are subsequently down-regulated and the cell enters either a myeloid, T lymphoid or B lymphoid pathway (Schmidt et al., 2001). This might be the case with CML-BC cells but this must be verified in further studies by single cell PCR to answer the question if a single cell expresses both myeloid and lymphoid characteristics, as well as both rearranged *IGH* and *TCR* genes, thus having a common origin. At this point of research, this thesis postulates that most CML-BC cells have aberrant rearrangements of the *IGH* gene and sporadic but also aberrant rearrangements of the *TCRB* and *TCRG* alleles.

4.3 Lymphoid specific mutation mechanisms in CML-BC

Normally, antigen-activated B cells undergo clonal expansion in germinal centers, where the immunoglobulin variable region genes are subject to SHM. In this process, single nucleotide substitutions are introduced, creating variants with high antigen affinity and thus selected to become memory B cells or plasma cells (Berek, 1991).

4.3.1 Aberrant somatic hypermutation observed in *IGHV* region genes of some CML-BC cases, with some having an intraclonal diversity potential

After having observed the aberrant expression of RAG, AID (an enzyme whose expression is specifically induced in activated B lymphocytes and required for SHM as described by Muramatsu et al., 2000) and the somatic recombination of *IGH* genes in CML-BC, the research focused on the intriguing finding that in some CML-BC cells, *IGH* genes are targeted by SHM. These mutations ranged from $3/10^3$ bp, as in KCL-22 and EM2, to $137/10^3$ bp in CML-T1 (Table 3a), whereby mutations higher than $10/10^3$ bp were considered significant to avoid mutation errors attributed to the *Taq* DNA polymerase (whose mutation frequency was calculated at $2.5/10^3$ bp). Nalm1 and BV173 had mutations of up to $37/10^3$ bp and $22/10^3$ bp respectively. CMLT1 had up to $137/10^3$ bp. K562, LAMA84 and JK1 also had up to 22 and $19/10^3$ bp mutations respectively. Two primary cases had a SHM accumulation of up to $24/10^3$ bp in CML-BC IV and $133/10^3$ bp in CML-BC III (Table 3b). In 11 cases where *AID* was found to be expressed (Figure 17), there is also accumulation of SHM, except EM2, KU182 and MEG01 which are either unmutated or have a low mutation frequency. The JK1 cell line, which lacks *AID* had two rearrangements with $19/10^3$ and $13/10^3$ bp mutations. This might be explained by the possibility that AID had been active in earlier stages of the disease development causing hypermutation of the *IGHV* region genes, but the AID was turned off in later phases of the disease.

The analyses of the *IGHV* region genes hypermutation showed fingerprints of clonal developments in ongoing mutations of some CML-BC cell lines. This characteristic of antigen-activated proliferating germinal center B cells (Jacob et al., 1991) was exemplified by evolutionary trees of IgV mutations of BV173 and Nalm1 in Figure 20. This leads to subclonal diversification, consistent with an active somatic hypermutation process. Implying that some clones harbor common mutations shared by multiple other subclones, which bear individual diversifying mutations, reflecting their clonal evolution.

4.3.2 Aberrant somatic hypermutation observed in Vβ region genes of some CML-BC cases, with some having an intraclonal diversity potential

It has previously been observed that the SHM process is not limited to Ig loci, as other genes like *BCL6* (Peng et al., 1999; Pasqualucci et al., 1998), *CD95* (Müschen et al., 2000) or *CD79A* and *CD79B* (Gordon et al., 2003) have been found mutated in normal germinal centre B cells. Aberrant SHM process has been reported to affect proto-oncogenes like *PIM1* and *c-myc* in DLBCL (Pasqualucci et al., 2001) or in classic and nodular lymphocyte-predominant Hodgkin lymphoma (Liso et al., 2006) i.e. SHM has been found to target activated B cells and B cell lymphoma. The TCR though similar to the Ig genes is believed to be incapable of hypermutation. The possibility that germinal center TCRα but not *TCRBV* region genes undergo SHM has previously been raised (Zheng et al., 1994). Okazaki and Honjo, 2003, showed that extensive mutations accumulate in the *TCRBV* region genes of AID-induced T cell lymphomas similar to SHM in the Ig gene of B cells. These T cell lymphomas obtained from ectopic expression of AID also showed SHM in *PIM1* and *c-myc* oncogenes, comparable to the somatic mutations observed in their counterparts in B cells. This raised the conclusion that SHM is regulated similarly between B and T cells upon AID over-expression.

Five CML-BC cases, CML-T1, K562, JURL, EM2 and CML-BC III, out of seven with rearranged *TCRB* region genes have significant numbers of mutations ranging from $7/10^3$ bp to $23/10^3$ bp (Table 5a and b). CML-T1 cells carry rearranged *TCRBV* region genes with up to $13/10^3$ bp mutations, JURL and EM2 with up to $18/10^3$ bp mutations and the primary case CML-BC III with up to $23/10^3$ bp. To show that this new finding was a distinctive characteristic of T cells in CML-BC and not an artifact of PCR, normal $CD3^+$ T cells isolated from healthy blood were analyzed in the same way as the CML-BC cases, for rearrangements in their *TCRB* locus and for mutations in their *TCRBV* region genes. Out of the 20 clones sequenced, 11 had no mutations i.e. $0/10^3$ bp in the *TCRBV* region genes. In eight clones $3/10^3$ bp mutations were observed and in one case $6/10^3$ bp mutations (Table 5c). Subsequently, *AID* expression was investigated in T-ALL primary cases to find out if *AID*-positive T-ALL cells do have somatic mutated *TCRBV* region genes. A mutation frequency was observed ranging from $3/10^3$ bp to $7/10^3$ bp mutations (Table 7). Summarizing these results, normal $CD3^+$ T cells have an average of $1.5/10^3$ bp mutations, an *AID* positive T-ALL has an average of $5/10^3$ bp mutations and CML-BC cells have an average of $12/10^3$ bp mutations in their Vβ region genes. These results might suggest that CML-BC cells have TCR Vβ region genes which undergo aberrant SHM, while *AID* positive T-ALL mutates in a much lower frequency and normal T cells do not undergo SHM in their Vβ region genes.

There is also evidence that the aberrant hypermutation activity is ongoing, as observed in the *TCRBV* gene regions of two CML-BC cell lines, K562 and JURL (Figure 21). This also leads to clonal heterogeneity, which is consistent with an active somatic hypermutation. This especially confirms the authenticity of the SHM as the mutations can be traced to a common origin.

The aberrant SHM observed in *IGHV* and *TCRBV* region genes of CML-BC cells might be a result of an extensive but unspecific activity of AID in this stage of the disease. The importance of these findings is still unknown but these aberrant mutations in addition to other secondary genetic alterations may eventually play a role in the evolution and transformation of the disease.

Chapter 5
Conclusion

1) Most CML-BC cases are lineage heterogeneous, expressing at least two hematopoietic lineage cell surface markers. They thus have a mixed lineage phenotype of B lymphoid, T lymphoid and myeloid characteristics, with the potential of differentiating into lymphocytic or myelocytic blast crisis or in both. In some CML-BC cases, the phenotype does not correspond to the genotype as some cell lines might not express any B or T lymphoid surface markers but still have rearranged V_H or Vβ region genes respectively.
2) The recombination activating gene (RAG1), which is specifically expressed in lymphocytes and required for V(D)J recombination in B and T cell receptors was amplified from six out of eleven CML-BC cell lines. This finding correlates with the somatic recombination of V_H, Vβ and Vγ region genes observed in most CML-BC cases on the one hand. On the other hand, the expression and activity of RAG might also lead to continuous and maybe unspecific DNA double strand breaks on yet unknown genes (for example the deletions of *CDKN2A* and *CDKN2B* in CML-BC may be caused by misdirected activity of RAG recombinase), thus the accumulation of secondary transforming events, which might have an implication on the clonal evolution of CML cells and the transformation from CML acute phase to blast crisis.
3) In 14 out of 16 CML-BC cases, the activation-induced cytidine deaminase (AID), an essential enzyme in the regulation of class switch recombination (CSR) and somatic hypermutation (SHM) in B cells could be amplified. AID might be responsible for the accumulation of mutations on *IGHV* region genes and *TCRBV* region genes of CML-BC cells and thus might play an important role in the evolution of CML-BC.
4) In accordance to finding RAG protein and lymphoid phenotype in CML-BC, extensive rearrangements of *IGH*, *TCRB* and *TCRG* alleles were also observed in some CML-BC cases. Out of the 16 CML-BC cases studied, 14 had rearranged V_H region genes. Of all eleven CML-BC cell lines, two showed rearrangements of the *KDE* to an intronic JκCκ RSS. Rearrangements of the *TCRBV* region genes were observed in seven out of the 16 CML-BC cases. In seven cases, simultaneous rearrangements in the *IGHV* and *TCRBV* region genes could be observed. The rearrangement of the *TCRGV* region genes was observed in eight out of the 16 CML-BC cases. These region genes are all flanked by the RSS, which are a target for common recombinase activity by RAG. These results assert that aberrant *IGH* and *TCR* gene rearrangements are not limited to the phenotypically lymphoid CML-BC but are also found in phenotypically non-lymphoid CML-BC, a characteristic which might be attributed to the malignancy of the blast crisis.

5) The aberrant expression of AID enzyme, whose expression is normally induced in activated B lymphocytes and required for somatic hypermutation (SHM), in CML-BC correlates to the finding of *IGHV* region genes targeted by SHM in CML-BC. In eight of the 11 cases where *AID* was expressed, there was an accumulation of SHM in the *IGHV* region genes. *TCRBV* region genes were also found to be somatically mutated in some CML-BC cases. Although similar to the *IGH* gene, the *TCR* is considered to be incapable of hypermutation. Five CML-BC cases out of seven with rearranged *TCRBV* genes harbored significant numbers of mutations. The aberrant SHM observed in *IGHV* and *TCRBV* region genes of CML-BC cells might be the result of an extensive but unspecific activity of AID in this stage of the disease. The clonal evolution of some CML-BC cases can be traced by the ongoing mutations observed in the *TCRBV* and *IGHV* region genes of these cases. This subclonal diversification is consistent with an active somatic hypermutation process as seen in B cells. It confirms the authenticity of the SHM as the mutations can be traced to a common origin.

The implications of these findings still have to be elucidated but it is imaginable that the aberrant expression of lymphoid-specific proteins, the existence of lymphoid specific rearrangement- and mutation-mechanisms in CML-BC coupled with other genetic alterations may eventually concur in the evolution and transformation of the disease from the chronic to the blast crisis stage.

Abstract

Lymphoid-Specific Gene- Rearrangement and –Mutation Mechanisms in Chronic Myeloid Leukemia Blast Crisis

Chronic myeloid leukemia (CML), a hematological malignancy defined by the Philadelphia chromosome, develops as a tri-phasic myeloproliferative disorder, leading to fatal, frequently lymphoid, blast crisis. This study addresses the secondary events in the evolution of CML that initiate disease progression and, ultimately, blast crisis (CML-BC).

Using RT-PCR and flow cytometry, 11 CML-BC-derived cell lines and five primary CML-BC cases were analyzed. Phenotypic analysis showed that CML-BC leukemias frequently comprise of subclones of B- lymphoid, T- lymphoid and myeloid lineages. Analyses by RT-PCR showed multiple immunoglobulin variable region (*IGHV*) gene rearrangements in 9 of 11 cell lines and in all five primary cases. Somatic hypermutation (SHM), a characteristic of mature B cells, was observed in the *IGHV* region genes of these CML-BC cells, with intraclonal diversity in eight of the 16 CML cases. Consistent with aberrant SHM in lymphoid CML-BC clones, these cells also exhibit aberrant expression of the mutator enzyme AID, which is required for SHM in normal germinal center B cells. Also rearrangement of the κ-deleting element (*KDE*) observed in one CML-BC cell line, is consistent with a B cell-origin of at least subclones in this case of CML-BC. In addition, rearranged T cell receptor β (*TCRB*) alleles were amplified from seven of 16 CML cases but only from one of 21 B lymphoid leukemia and lymphoma cases used as controls. V(D)J recombination in lymphoid CML-BC clones seems to be constitutively active at the *IGHV* and/or *TCRB* loci, because mRNA expression for the recombination activating gene (RAG) proteins could be detected in most of the CML-BC cases analyzed. Surprisingly, traces of somatic hypermutation were also observed in the *TCRBV* region gene rearrangements amplified from CML cells, whereas *TCRBV* genes amplified from normal T cells were devoid of somatic mutations. Also the clonal evolution of two cell lines could be traced by mutations within *TCRBV* region genes.

Extensive and deregulated activity of lymphoid-specific gene- rearrangement and -mutation mechanisms carry the risk of secondary genetic aberrations, likely contributing to progression of chronic phase CML. These findings collectively indicate, that increased genomic instability owing to deregulated V(D)J recombination and SHM may ultimately give rise to a highly transformed malignant clone leading to blast crisis at the final stage of CML.

Deutsche Zusammenfassung

Lymphoid-spezifische Umlagerungs- und Mutationsmechanismen in chronischer myeloischer Leukämie

Die chronisch myeloische Leukämie (CML) ist eine Krankheit für die das Philadelphiachromosom charakterisiert ist. Diese myeloproliferative Krankheit verläuft in drei Phasen: der akuten Phase, der beschleunigten Phase und der fatal endenden Blastenkrise. In dieser Studie werden die sekundären Ereignisse der Entwicklung der CML in der Blastenkrise (CML-BC) behandelt.

Mit Hilfe von RT- PCR und Durchflußzytometrie wurden 11 CML-BC Zelllinien und fünf primäre CML-BC Fälle untersucht. Die CML-BC Fälle bestanden aus B-Lymphozyten, T-Lymphozyten und myeloiden Subklonen. Anhand der RT-PCR-Analysen konnten Umlagerungen der V_H Gensegmente in 9 von 11 Zelllinien und in allen primären Fällen nachgewiesen werden. Somatische Hypermutation (SHM), eine Eigenschaft von reifen B-Zellen, wurde in den V_H Gensegmenten der CML-BC Zellen gefunden und in acht der 16 Fälle konnte eine intraklonale Diversität festgestellt werden. Weiter wurde eine Umlagerung des „κ-deleting element“ (KDE) in einer Zelllinie festgestellt, welches die B-lymphoide Eigenschaft dieser Zelllinie bestätigt.

Zusätzlich wurden in T Zellen, T-lymphoiden Leukämien und Lymphomen Umlagerungen der TCR Vβ Gensegmente nachgewiesen. In B-lymphoiden Leukämien und Lymphomen konnten dagegen nur in einem von 21 Fällen Umlagerungen beobachtet werden. Im Gegesatz dazu wurden umgelagerte TCRβ Allele in sieben von 16 CML-BC Fällen amplifiziert. Überraschenderweise wurden Spuren von somatischer Hypermutation im TCR Vβ-Locus der CML-BC Zellen festgestellt, wobei TCR Vβ Gene in normalen T-Zellen nicht dem Prozess der SHM unterliegen. Die klonale Evolution von zwei CML-Linien konnte anhand dieser Mutationen verfolgt werden.

Die Anwesenheit von RAG bzw. AID in normalen lymphoiden Zellen ist eine Voraussetzung für die V(D)J Genumlagerung bzw. die SHM. Die Expression von RAG und AID konnte in CML-BC Fällen nachgewiesen werden. Das passt zu den Ergebnisse bezüglich der Gensegmentumlagerungen und SHM von Ig V_H - und TCR Vβ.

Die Versuchsergebnisse deuten an, dass die Aktvitäten der lymphoid-spezifischen Genumlagerungs- und Mutationsmechanismen letzendlich zu einem Ausbruch der Blastenkrise in der Entwicklung der CML führen könnten.

Chapter 6

Appendix

6.1 List of abbrviations

AID	activated induced cytidine deaminase
AP	acute phase
APE	apurine/apyrimidine endonuclease
APOBEC-1	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1
Bcl-6	B cell lymphoma 6
BCR	B cell receptor
Blimp-1	B lymphocyte-induced maturation protein-1
BSAP	B cell-specific activator protein
CDKN	cyclin dependent kinase
cDNA	copy deoxyribonucleic acid
CDR	complementary determining region
CJ	coding joint
CLP	common lymphoid progenitor
CML-BC	chronic myeloid leukemia- blast crisis
CMP	common myeloid progenitor
CP	chronic phase
CSR	class switch recombination
DN	double negative
DNA-PKcs	DNA protein kinase catalytic subunit
dNTPs	deoxynucleotide triphosphates
DP	double positive
DSB	double strand breaks
EBF	early B cell factor
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescent activated cell sorting
FITC	fluorescein isothiocyanat
FR	framework region
FSC	forward scatter
GAPDH	glyseraldehyde-3-phosphate dehydrogenase
GC	germinal center
GC-SF	granulocyte colony-stimulating factor

GDP	guanosine di-phosphate
GL	germline
GRB-2	growth factor receptor-binding protein-2
GTP	guanosine tri-phosphate
HSC	hematopoietic stem cell
HV	hypervariable
Ig	immunoglobuline
KDE	kappa deleting element
MMR	mismatch repair
NHEJ	nonhomologous end-joining
PAX5	paired box gene 5
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI-3K	phosphoinositide-3 kinase
RAG	recombination activating gene
RNA	ribonucleic acid
RSS	recombination signal sequence
RT	reverse transcriptase
SHM	somatic hypermutation
SP	single-positive
SSC	side scatter
STAT5	signal transducer and activator of transcription 5
TAE	Tris-Acetate-EDTA
TCR	T cell receptor
TLP	T lymphoid progenitor
XBP1	X-box binding protein 1

6.2 List of Primers

AID_F	TGCTCTTCCTCCGCTACATC
AID_R	CCTCATACAGGGGCAAAAGG
GAPDH_F	TTAGCACCCCTGGCCAAGG
GAPDH_R	CTTACTCCTTGGAGGCCATG
M13_F	GTTTTCCCAGTCACGAC
M13_R	CAGGAAACAGCTATGAC
OligodT Primer	TTTTTTTTTTTTT
RAG1_Meffre_F	TGCAGACATCTCAACACTTTGGCCAG
RAG1_Meffre_R	TTTCAAAGGATCTCACCCGGAACAGC
RAG2_Meffre_F	AGCAGCCCCTCTGGCCTTCAG
RAG2_Meffre_R	CATGGTTATGCTTTACATCCAGATG
V_H1	CAGTCTGGGGCTGAGGTGAAGA
V_H2	GTCCTRCGCTGGTGAAACCCACACA
V_H3	GGGGTCCCTGAGACTCTCCTGTGCAG
V_H4	GACCCTGTCCCTCACCTGCRCTGTC
V_H5	AAAAAGCCCGGGGAGTCTCTGARGA
V_H6	ACCTGTGCCATCTCCGGGGACAGTG
D_H1	GTGTGCAGGCCTCRGTCTCTGTG
D_H2	GCACTGGGCTCAGAGTCCTCTC
D_H3	CCTCAGGTCAGCCCTGGACATC
D_H4	TGAGATCCCCAGGACGCAGCAC
D_H5	TCCCTGGGAAGCTCCTCCTGAC
D_H6	GACACCAGACAGAGGGGCAGGC
D_H7-27	AGAGTGACTGGCAGGGTTGAGG
$J_H1.4.5$	GACGGTGACCAGGGTKCCCTGGCC
J_H2	GACAGTGACCAGGGTGCCACGGCC
J_H3	GACGGTGACCATTGTCCCTTGGCC
J_H6	GACGGTGACCGTGGTCCCTTKGCC
TCRB_D1_F	CCCCTTCGCCAAACAGCCTTA
TCRB_GL1_Rex	GAGTGAGGCAGAGGCATTCTGAAC
TCRB_GL1_Rint	GCAGAGGCATTCTGAACCAAATTG
TCRB_D2_F	TCAGGGTGATGCATGTTCCAAGGA
TCRB_GL2_Rex	GGGACCCTGCAAGACCACAGCT
TCRB_GL2_Rint	ACTCTTCCCACCTGGTAGCTGCAT
TCR_BV1/5	ACAGCAAGTGAC(AGT)CTGAGATGCTC
TCR_BV2	GAGTGCCGTTCCCTGGACTTTCAG
TCR_BV3	GTAACCCAGAGCTCGAGATATCTA
TCR_BV4	TCCAGTGTCAAGTCGATAGCCAAGTC
TCR_BV6.1	ATGTAACT(CT)TCAGGTGTGATCCAA
TCR_BV6.2	GTGTGATCCAATTTCAGGTCATAC
TCR_BV7	TACGCAGACACCAA(AG)ACACCTGGTCA

TCR_BV8	GGTGACAGAGATGGGACAAGAAGT
TCR_BV9	CCCAGACTCCAAAATACCTGGTCA
TCR_BV10	AAGGTCACCCAGAGACCTAGACTT
TCR_BV11	GATCACTCTGGAATGTTCTCAAACC
TCR_BV12	CCAAGACACAAGGTCACAGAGACA
TCR_BV13	GTGTCACTCAGACCCCAAAATTCC
TCR_BV14	GTGACCCAGAACCCAAGATACCTC
TCR_BV15	GTTACCCAGACCCCAAGGAATAGG
TCR_BV16	ATAGAAGCTGGAGTTACTCAGTTC
TCR_BV17	CACTCAGTCCCCAAAGTACCTGTT
TCR_BV18	TGCAGAACCCAAGACACCTGGTCA
TCR_BV19	ACAAAGATGGATTGTACCCCCGAA
TCR_BV20	GTCAGATCTCAGACTATTCATCAATGG
TCR_BV21	CAGTCTCCCAGATATAAGATTA(CT)AGAG
TCR_BV22	GGTCACACAGATGGGACAGGAAGT
TCR_BV23	CTGATCAAAGAAAAGAGGGAAACAGCC
TCR_BV24	CAAGATACCAGGTTACCCAGTTTG
TCR_BV25	GACAGAAAGCAAAATTATATTGTGCC
TCR_3'BJ1.2	TACAACGGTTAACCTGGTCCCCGA
TCR_5'BJ1.2	TAACCTGGTCCCCGAACCGAAGG
TCR_3'BJ1.3	CACCTACAACAGTGAGCCAACTT
TCR_5'BJ1.3	GCCAACTTCCCTCTCCAAAATATATGG
TCR_3'BJ1.5	CCAACTTACCTAGGATGGAGAGTCGA
TCR_5'BJ1.5	GATGGAGAGTCGAGTCCCATCAC
TCR_3'BJ1.6	CCTGGTCCCATTCCCAAAGTGGA
TCR_5'BJ1.6	CCCATTCCCAAAGTGGAGGGGTG
TCR_3'BJ2.2	CCTTACCCAGTACGGTCAGCCTA
TCR_5'BJ2.2	AGTACGGTCAGCCTAGAGCCTTCT
TCR_3'BJ2.3	TCCCGGGGCGCCCCCTCCCCAGTT
TCR_5'BJ2.3	GAGCCCCCGCTTACCGAGCACTGTCA
TCR_3'BJ2.6	CAGCCGCCGCCTTCCACCTGAAT
TCR_5'BJ2.6	CCGGCCCCGAAAGTCAGGACGTT
TCR_3'BJ2.7	TCCATCGTTCACCTTCTCTCTAAACA
TCR_5'BJ2.7	GCCCGAATCTCACCTGTGACCGTG
TCRg_V11-J1.IN.INT	TCAGGAGTTATAAGCATTCACC
TCRg_V11-J1.IN.EXT	TGTAACACAGGCCCACTGGG
TCRgV1-9_int_E.D.	ACGGCGTCTTCAGTACTATGAC
TCRgV1-9_ext_E.D.	TGCAGCCAGTCAGAAATCTTCC
TCRgJ1.1/2.1 V.D.	TTACCAGGCGAAGTTACTATGAGC
TCRgJ1.3/2.3 V.D.	GTGTTGTTCCACTGCCAAAGAG

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6.4 Publications

Feldhahn, N., Rio, P., Soh, B. N., Liedtke, S., Sprangers, M., Klein, F., Wernet, P., Jumaa, H., Hofmann, W. K., Hanenberg, H., Rowley, J. D., & Muschen, M. 2005, "Deficiency of Bruton's tyrosine kinase in B cell precursor leukemia cells", *Proc.Natl.Acad.Sci.U.S.A*, vol. 102, no. 37, pp. 13266-13271.

Soh, B. N., Klein, F., Feldhahn, N., & Muschen, M. 2005, "B-lymphoid or myeloid lineage identity of cell lines derived from chronic myeloid leukemia blast crisis", *Cancer Genet.Cytogenet.*, vol. 161, no. 2, pp. 187-188.

Eidesstattliche Erklärung

Ich erkläre an Eides Statt, daß die vorliegende Dissertation in allen Teilen von mir selbständig angefertigt wurde und die benutzten Hilfsmittel vollständig angegeben worden sind.
Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht oder veroffentlicht worden.
Weiter erkläre ich, daß ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotionseröffnungsverfahren beantragt habe.

Düsseldorf, den Bonaventure, Soh Bejeng Ndikung

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