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Detection of T-cell clonality in patients with B-cell chronic lymphocytic leukemia

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Abbreviations

ALL	Acute lymphoblastic leukemia
C	Constant
CD	Cluster of differentiation
CDR	Complementarity determining region
CE	Capillary electrophoresis
CLL	Chronic lymphocytic leukemia
D	Diversity
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleoside triphosphatase
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FITC	Fluoroisothiocyanate
IG	Immunoglobulin
IGH	Immunoglobulin heavy chain
IGHV	Immunoglobulin heavy-chain variable
IL	Interleukin
IMGT	Immunogenetics
J	Joining
L	Ladder
Nt	Nucleotide
PAGE	Polyacrylamide gels electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
R-PE	R-phycoerythrin
RFUs	Relative fluorescence units
RSS	Recombination signal sequences
TCR	T-cell receptor
TCRβ	T-cell receptor beta
TCRγ	T-cell receptor gamma
V	Variable
WHO	World health organization

Summary

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease characterized by monoclonal B-cells. In addition to B-cell clonality, minor mono- and oligoclonal T-cell populations have also been observed in CLL patients. Although the precise role of these T-cell expansions remains unknown, these observations indicate that T-cells might be involved in the pathogenesis of CLL. Furthermore, biased expression of certain immunoglobulin heavy-chain variable (IGHV) genes have been reported in CLL, for instance, the IGHV3-21 and IGHV4-34 genes, which may imply that antigen selection could play a role in CLL development.

In this study, potential T-cell clonality was investigated in a total of 10 CLL patients overexpressing the IGHV3-21 and IGHV4-34 genes. Analysis was performed by characterizing T-cell receptor (TCR) β and γ gene rearrangements using multiplex PCR and two sensitive detection techniques, heteroduplex and fragment analysis. Overall, mono- and oligoclonality was indicated using TCRγ in 5 patients overexpressing the IGHV3-21 gene and 2 patients overexpressing the IGHV4-34 gene. Mono- and oligoclonal TCRγ rearrangements, detected in 4 patients overexpressing the IGHV3-21 gene, were then verified by subcloning and sequencing a significant number of clones. Notably, out of 15 sequenced TCRγ rearrangements only one rearrangement displayed functionality. When analyzing for TCRβ rearrangements in the patient samples showing TCRγ mono- and oligoclonality, mono- and oligoclonal patterns were indicated as well. It is known from previous studies that non-functional TCRγ rearrangements can be present in functional TCRβ gene rearrangements, since the TCRγ locus rearranges before the TCRβ locus.

The finding of TCRγ mono- and oligoclonality in this study thus provides support for the observed TCRβ clonality results. However, further investigation by subcloning and sequencing must be performed on cases displaying TCRβ mono- and oligoclonality in order to verify these findings. Whether this clonality results from antigen recognition or a response to the leukemic B-cell clone remains unknown but these results nevertheless strengthen the hypothesis of T-cell involvement in CLL.

1. Introduction

1.1 Leukemia

Cancer of white blood cells is called leukemia and originates in the bone marrow, the soft tissue of larger bones[9]. Precursors of white blood cells, red blood cells and platelets are produced in the bone marrow, which is also the site of maturation for most of these cells, prior to their release to the blood stream[2].

Leukemia begins when one abnormal white blood cell starts to proliferate in an unrestrained manner, eventually resulting in a population of cells that are not able to fight infection or perform other normal functions of a white blood cell[2]. Instead, these cells have acquired the capacity to grow faster and live longer than they should with the unfortunate outcome of preventing the production of other normal blood cells in the bone marrow. Consequently, affected individuals suffer from infections, bleeding and anemia. Furthermore, these accumulating cells spread through the bloodstream, lymph nodes or other organs where they continue to divide and cause swelling and pain[9].

Since different types of white blood cells exist, there are also different types of leukemia[9]. There are five different types of white blood cells; neutrophils, basophils, eosinophils, monocytes and lymphocytes. The majority of lymphocytes are B- and T-lymphocytes (also called B- and T-cells) and they are found in the blood stream and the lymphatic system. These cells recognize foreign substances called antigens and build up an immune response. If lymphocytes are affected, the leukemia is called lymphatic. On the other hand, if cells from the myeloid lineage are affected, they will give rise to myelogenous leukemia[2]. In addition, the pattern of progression of leukemias leads to further categorization. If the onset is abrupt and conditions get worse very fast the leukemia is called acute whereas chronic leukemia develops more slowly[9]. On this basis there are four main types of leukemia; acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML) and chronic lymphocytic leukemia (CLL)[2].

1.2 Chronic lymphocytic leukemia

B-cell chronic lymphocytic leukemia (CLL) is the type of leukemia most often affecting the adult population of Western countries[4]. In Sweden 400-500 patients are diagnosed each year[14]. The incidence of CLL increases with age and the median age of diagnosis is 65-70 years. This leukemia affects males at higher rates than females[14]. Today there is no treatment of CLL and in many patients the disease has an indolent course, whereas it has more rapid progression with fatal outcome in others[4].

CLL is characterized by accumulating neoplastic B-cells expressing membrane molecules; cluster of differentiation (CD) 5, CD19, CD20, CD23 and low levels of immunoglobulins (IGs) on their surface. The cause of CLL is currently unknown. However, previous studies characterizing the IG molecule imply antigen involvement. The exact nature of these antigens to a large extent remains unknown[29].

1.3 Immunoglobulin genes in normal B-cells

Immunoglobulins (IGs) are Y-shaped molecules expressed by all mature B-cells and their task is to recognize antigens. An IG molecule consists of four chains, two large identical heavy (H) chains, and two small identical light (L) chains. Each of these four chains is composed of a variable (V) region and a constant (C) region[29] (Figure 1). The diversity among different IGs resides in the V regions, more precisely, areas called complementarity-determining regions (CDRs). These CDRs make up the antigen binding sites on both light and heavy chains. Thus,

both of these chains participate in antigen interaction[9].

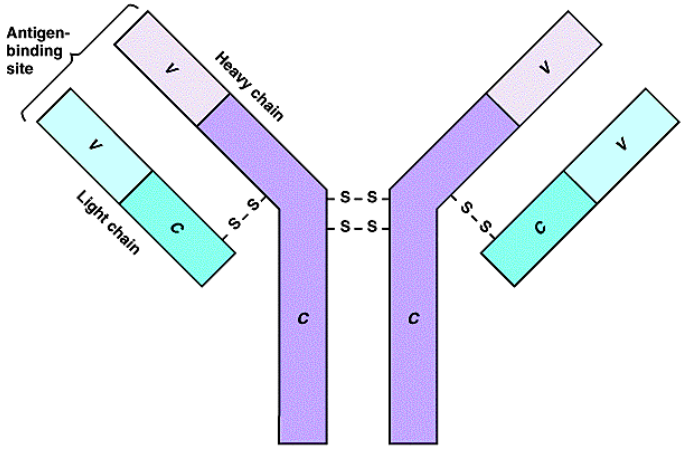


Figure 1. **Schematic structure of the IG molecule.** The IG molecule consists of two identical heavy chains (purple) and two identical light chains (blue). Each chain has a variable region that is highly diverse among IGs and makes up the antigen binding site.

The V region in the heavy and light chains is encoded by multiple gene segments within the IG heavy (IGH) and light chain loci, respectively. The IGH locus contains multiple V (variable), D (diversity) and J (joining) gene segments. There are two types of immunoglobulin light chains called kappa and lambda encoded by multiple V and J gene segments in the IGK and IGL loci, respectively. These gene segments in the heavy and light chain loci create diversity among IGs and this takes place during normal B-cell development. This process, called V(D)J recombination, occurs in all three loci and is necessary in order to enable recognition and response to numerous antigens. In the process of somatic recombination of the IGH locus, one IGHD gene segment joins one IGHJ gene segment, creating an IGHD-J segment. Afterwards, one IGHV gene segment joins the earlier created DJ gene segment resulting in an IGHV-D-J rearrangement (Figure 2). This rearrangement makes up the V region in the heavy chain of the IG molecule. The rearrangement of IGL/IGK genes proceeds in a similar manner and results in joining of one V to one J gene segment creating a V-J rearrangement. Each V gene segment is flanked by recombination signal sequences (RSS), which are recognized by recombination activation genes (RAG 1 and 2). This recognition allows the gene segments to be cut out by RAG1 and 2 and ligated by various protein factors to create the final V(D)J rearrangement[9].

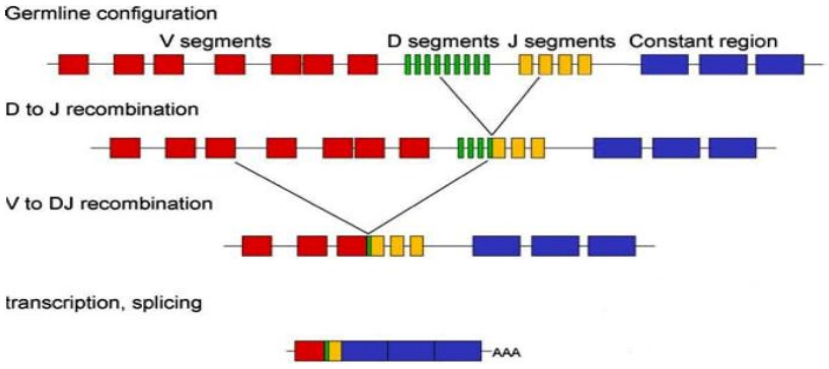


Figure 2. Schematic illustration of the rearrangement process. A unique VDJ rearrangement is created from several V, D and J gene segments in the recombination process. This rearrangement is unique to each B-cell and its progeny.

Following rearrangements of both the heavy and light chain loci, B-cells express IgM and IgD which are IGs expressed by mature B-cells[9,29]. Other mechanisms that also serve to increase diversity of IGs during the rearrangement process are insertions of random nucleotides and base pair deletions in the junction sites of the gene segments[29]. Another mechanism that further gives rise to IG diversification takes place in the germinal centers of lymph nodes and occurs after the B-cell has encountered antigen. This process is called somatic hypermutation (SHM) and involves base substitutions but also insertions and deletions, mainly in the CDRs[9].

1.4 Immunoglobulin genes in chronic lymphocytic leukemia cells

CLL arises from a clonal expansion of a single B-cell which has rearranged IGH and IGL genes that are unique to that cell. These rearrangements can thus be used as a specific marker for the tumour population.

The mutational status of IGHV genes has been demonstrated to give significant prognostic information when dividing CLL into two different prognostic groups, with a more favorable outcome for patients with mutated IGHV genes than for those with unmutated such genes[14]. If the IGHV gene sequence differs from the most similar germline gene by at least or more than 2 %, then the sequence is regarded mutated. IGHV gene sequences that display less than 2 % difference from the germline gene are considered unmutated[4].

Both unmutated and mutated CLL subgroups display usage of certain IGHV genes that differ when compared with the gene usage by normal B-cells. For instance, the IGHV1-69, IGHV3-21, IGHV4-34 and IGHV3-07 genes are overexpressed in CLL patients, which has led to the assumption that antigen(s) may contribute to the disease development[4,14]. Further studies have revealed restricted features of the IG gene rearrangements in subsets of CLL patients, where patients using the same IGHV gene can display similar CDR3 sequences and usage of the same light chain genes[27,28]. Patients displaying highly similar or identical CDR3s have been grouped as "stereotyped" cases whereas then "non-stereotyped" cases have heterogeneous CDR3s. The stereotyped IGHV3-21 subset has been shown to have extraordinary characteristics such as highly restricted and short CDR3s (9 codons), compared to non-stereotyped IGHV3-21[28]. The IGHV3-21 patient group also does not follow the prognostic outcome provided by mutational status, since they have poor outcome regardless of mutational status[28]. Another interesting subgroup identified is the stereotyped IGHV4-34 subgroup, which has a very low median age at diagnosis (43 years) and follows a more indolent course of disease compared to non-stereotyped IGHV4-34 with a median age at diagnosis of 63 years[27]. The observation that CLL patients use a restricted IGHV gene repertoire and show stereotyped B-cell receptors supports the view of antigen involvement. The fact that the region mostly destined to be diverse appears identical among certain CLL patients indicates that the same antigen or groups of structurally similar antigens are recognized by the CDR3. Besides the support of antigen recognition provided by analysis of IGHV, involvement of antigen is supported by the notion that CLL cells express surface markers distinctive for antigen experienced cells, irrespective of mutational status[4]. Interestingly, a recent study has identified potential novel antigens that may account for the proliferation of the CLL clone. Among these are cytoskeletal proteins vimentin, filamin B, and cofilin-1 but also phosphorylcholine-containing antigens (eg *Streptococcus pneumoniae* polysaccharides)[18].

1.5 T-cells in patients with chronic lymphocytic leukemia

The question regarding identification of antigen(s) encountered by the CLL cells is still not fully understood. In addition, another perplexing issue concerning T-cells and their

involvement in CLL has caught attention.

Observations of mono- and oligoclonal T-cell populations in CLL have been reported by several groups, but this area is still under discussion[7,23,25]. These T-cell populations imply a possible involvement of T-cells in the disease process[25]. Speculations have been made that exogenous antigens or antigens released by malignant cells might cause the T-cell expansions[14]. T-cell involvement is further indicated by the notion that the T-cells are capable of producing cytokines, which is evidence that these cells are active. Interestingly, increased cytokine production in patients with progressive disease compared to patients with indolent disease was reported[14]. There are speculations that these cytokines may possibly function as growth factors for CLL cells and have a role in their accumulating process[14].

1.6 T-cells

Before further roles of T-cells in CLL are discussed, a more detailed presentation concerning their function in the normal immune system is necessary.

T-cells are produced in the bone marrow but mature in the thymus[9]. Following maturation, two subpopulations of T-cells can be distinguished by expression of glycoproteins also known as CD on their surface. T-cells expressing CD4 are called helper T-cells (T_H). The other subset of T-cells expresses CD8 and is called cytotoxic T-cells (T_C). Both types express a T-cell receptor (TCR) responsible for recognition of antigen, as well as a complex of five proteins called CD3 that also plays a role in the interaction with antigen and signal cascade.

Nonetheless, there are also differences between these two subsets of T-cells and they carry out very different functions in the immune system[9]. Neither of these subsets recognizes native antigens, meaning that T-cells require that the antigen is digested, degraded and presented on the surface of an antigen presenting cell (APC). The displayed antigen lies in a groove on either the major histocompatibility complex 1 or 2 (MHC).

A single subset of T-cells interacts with only one of the MHC-classes. Once a T-cell has made contact with an antigen and MHC, it requires aid from accessory molecules to become activated, and the roles of the two subsets in the immune system become more obvious[9]. Activation of T_H-cells is initiated by the interaction of the TCR-CD3 complex with the antigen-MHC class 2 molecule on the surface of an APC. MHC 2 is only expressed by specialized APCs like B-cells, phagocytic cells and dendritic cells[9]. This contact initiates a cascade of events that results in proliferation and growth of T_H-cells. Activated T_H-cells become effector cells that secrete cytokines which are required for further development of both B-cells and T_C-cells[9]. B-cells that have encountered antigen need cytokines in order to proliferate and differentiate into antibody secreting B-cells. T_C-cells need cytokines to become fully activated. There are two subsets of T_H-cells that differ in secretion of cytokines upon activation. T_H 1-cells produce interleukin-2 (IL-2) and interferons while T_H 2-cells produce IL-4, IL-5 and IL-6 that provide help for B-cells.

In order to exert their function, which is to destroy other cells, T_C-cells require activation, which is initiated by recognition of antigens in the context of MHC class 1. The fact that almost all cells of the body express MHC class 1 molecules implies that T_C-cells are important in recognizing and destroying self-cells that have been altered and infected, in particular virus-infected and tumor cells[9].

1.7 T-cell receptor

Irrespective of T-cell subpopulations, each T-cell has approximately 10^5 identical T-cell receptors (TCRs) bound to the membrane and expressed on the cell surface. T-cells are able to recognize foreign substances because the TCRs have specificity for a particular antigen[9].

A TCR is a heterodimer composed of two polypeptide chains, either α and β or γ and δ[9]. The two chains are covalently linked to each other by disulfide bonds creating either αβ TCR or γδ TCR[1] (Figure 3). The total T-cell population in humans is dominated by T-cells expressing αβ TCR (90-99%)[9]. A significantly smaller fraction of T-cells express γδ TCRs, and most of these are negative for both CD4 and CD8[13]. Much less is known about the functions of T-cells expressing γδ TCR. However, the two TCR types are thought to recognize different types of antigens. The majority of γδ TCRs react with antigen that is not processed or presented in the context of an MHC molecule. Thus, there are notable differences between the two receptor types but their fundamental role, antigen recognition, is the same. The pivotal role played by the TCR is enhanced through the chains that build up a TCR.

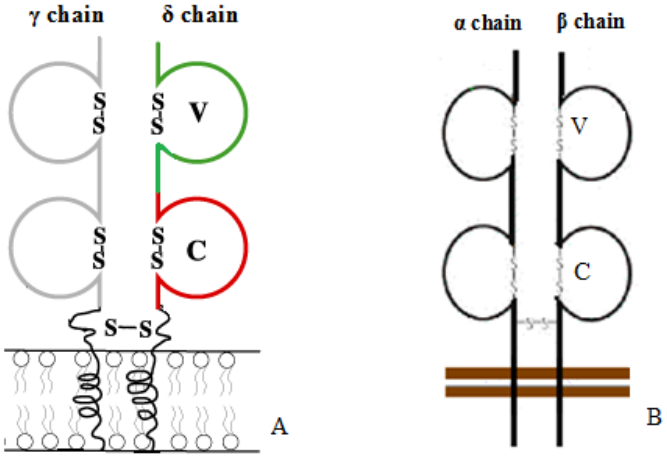


Figure 3. **Schematic illustration of TCRs.** In figure A, γδ TCR and in figure B, αβ TCR. Each α, β, γ and δ chain has a V region where the antigen binding site reside.

Each chain (α, β, γ and δ) contains a V domain and a C domain[9]. The C domains have conserved regions that reside within the sequences responsible for linking the two chains together in a TCR. The V domains, as the name implies, demonstrate sequence variation in which the diversity among different TCRs resides. V domains contain short amino acid segments that make up three hypervariable regions also called CDRs. These regions directly contact the antigen and contribute to the unique binding site displayed by each TCR and hence the specificity for its particular antigen. In the TCR, CDRs from each chain are juxtaposed to form the antigen-binding site of the TCR[9.] Among the three CDRs, the one with greatest variability and with the major role in the recognition of antigen is CDR3. How diversity in the variable domain is achieved has its origin in the rearrangement process that occurs in each chain, in parallel with the IG genes. TCRα and TCRγ genes are encoded by several V, J and C gene segments, whereas β and δ chain are encoded by V, D, J and C gene segments. Each V, D and J gene segment is flanked by RSS. Pre-T-cells express RAG1-and RAG2-coding enzymes that recognize RSS and cut out the gene segment. One V gene segment is joined by various protein factors with one J gene segment to create a V-J joining in α- and γ chains. Similarly, β- and δ chains are formed after the V(D)J recombination has occurred. Following ligation of the segments, a gene sequence is formed that is transcribed, the RNA is processed and translated[9]. This rearrangement process takes place during T-cell development and follows a hierarchical order[14]. TCRδ genes rearrange first, followed by TCRγ genes; if this rearrangement is functional TCRγδ is expressed. However, if the initial rearrangement of TCRγδ is non-functional, the rearrangement process proceeds to TCRβ and TCRα[5].

As several gene segments exist (V, D and J) this process enables a large number of combinations and produce diversity among TCRs in the immune system. It has been estimated that each individual possesses a repertoire of 10^8 TCRs corresponding to a billion different populations of T-cells with a unique TCR for each cell and its progeny[14]. TCR gene rearrangements are present in almost all immature and mature T-cells as they occur early in lymphoid differentiation. Each T-cell has rearranged TCR genes unique for that cell[5].

1.8 T-cell clonality

TCR gene rearrangements occur early in T-cell differentiation and are present in almost all immature and mature T-cells. T-cell malignancies are derived from a single malignant T-cell and this malignantly transformed cell gives rise to a progenitor cell, which gives rise to the tumor cell population. All tumor cells have identical TCR rearrangements as they originate from a common cell. Thus, these TCR rearrangements can be used for analysis of clonal T-cell populations, as one or several clonal rearrangements are present in the leukemic population[16,5].

Importantly, clonality is not only associated with malignantly transformed cells. In contrast to polyclonal T-cells found in healthy individuals, mono or oligoclonal T-cell populations can exist, originating from a few progenitor cells that undergo clonal expansion due to, for instance, antigen exposure.

1.9 Methods to analyze T-cells

As previously mentioned, T-cells may possibly be involved in the disease development of CLL. T-cell analysis in CLL patients requires sensitive separation of cells since the majority of cells in tumor samples are CLL cells. Following separation of T-cells from the dominating CLL cells, a method that can confirm presence of T-cells is required. These objectives can be addressed by performing cell isolation and fluorescence activated cell sorting (FACS).

1.9.1 Cell isolation

Various methods for isolation of cells can be employed depending on for what type of application the cells of interest will be used subsequently. Magnetic dynalbeads coated with monoclonal antibodies can be used for positive or negative selection of cells. In positive selection, the monoclonal antibodies bind to the cells that are to be sorted out from the cell suspension. The process of negative selection operates through the use of monoclonal antibodies that target specific cells. The cells to be isolated are left unbound and can be separated from the targeted cells.

1.9.2 Fluorescence activated cell sorting

Fluorescence activated cell sorting (FACS) is a sensitive method that separates and characterizes phenotypically different cells, one by one[19]. As this technique is based on fluorescence, the cells of interest are labeled with a fluorescent dye. The dye is coupled to a monoclonal antibody that only binds those cells that express its antigen[15]. Thereafter, the cells are injected into a flow component of liquid that delivers the cells individually to a laser beam, which illuminates the cells[19].

In order to analyze different parameters of cells, which include physical and chemical traits, a measurement method specific for detection of that parameter is employed. As cells are illuminated by the laser beam they emit and also scatter some of the light. When scattered light is along the same axis as the laser light, it is detected in the forward scatter channel which is proportional to the size of the cell. Light scattered to the side or perpendicular to the

axis travelled by the laser light, is detected in the side scatter channel and is proportional to the cell shape. Forward scatter and side scatter allow for differentiation of cell types in a heterogeneous cell population. The information obtained can be used to perform gating which is a process that selects cells of interest whereas unimportant cell populations are discriminated in the analysis. Thus, subsets of cells can then be analyzed and one can look at parameters specific only to that subset. In addition to scattered light, fluorescence signals from the labeled antibodies are created that can be measured as single or multiple parameters. The collected data is converted to digital values and sent to a computer for analysis[15].

The acquired data is plotted by the software program and displayed in diagrams. Dot plot diagrams are most commonly used when the analysis involves two fluorescent parameters. In such a diagram two parameters are plotted on the x- and y-axis and each cell is a spot on this graph according to its parameter intensities.

1.10 Targets to study clonal T-cell populations

Analysis of T-cell populations in CLL is possible and relies on the rearrangement process of the TCRs. As each T-cell has unique TCR rearrangements, occurrence of a certain rearrangement in large proportions of cells will indicate clonality. A target to study T-cell clonality is the TCRγ locus, which encodes the γ chain in the γδTCR. The gene segments in the TCRγ locus are rearranged at an early stage of T-cell development[5]. The human TCRγ locus is located on chromosome 7 and contains multiple Vγ and Jγ segments. In total there are 14 Vγ segments, of which 10 have been shown to undergo rearrangement and 6 are expressed. The TCRγ locus also contains 5 Jγ segments[5]. All of these different V and Jγ segments have been subdivided into different families (Figure 4).

Another important target to analyze in studies of T-cell clonality is the TCRβ locus, which encodes the β chain in TCR. The TCRβ locus is also located on chromosome 7 but in contrast to TCRγ, its gene composition is of much greater complexity[5]. In addition to multiple Vβ and Jβ genes, it encodes Dβ genes. Of these, there are approximately 44 functional Vβ genes, 2Dβ genes and 13 Jβ genes, subdivided into different families. The combinatorial diversity of TCRβ rearrangements is thus much larger than that of TCRγ[5].

Although rearrangements occurring in TCRs should only be present in T-cells, unexpected TCR rearrangements have been observed in B-cells[30]. Thus special consideration must be taken when analyzing T-cell clonality in CLL due to the possibility that the CLL cells also carry TCR rearrangements. If CLL cells are analyzed simultaneously with T-cells, a rearrangement can be assigned to being a clonal TCR rearrangement if it is absent in CLL cells.

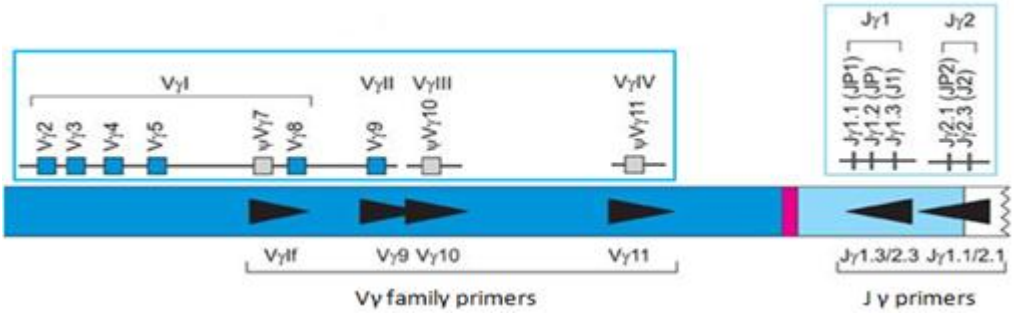


Figure 4. Schematic diagram of the TCRγ locus. The TCRγ locus contains several V- and J gene segments, grouped into families, with most members in the Vγ1 family. The black arrows in both directions represent the position of forward- and reverse primers used to amplify TCRγ rearrangements. (Figure adapted with permission from Leukemia 2003; 17:2257-2317, van Dongen *et al.*)

1.11 Techniques to study T-cell clonality

1.11.1 Multiplex polymerase chain reaction

Clonal rearrangements in TCR genes can be detected using the polymerase chain reaction (PCR). Since multiple gene segments exist, several primers are necessary and hence a multiplex PCR approach is the method of choice. In multiplex PCR, multiple primers are used within a single PCR reaction in order to produce different amplicons. A European collaborative study, Biomed, has developed and standardized PCR assays for detection of clonal rearrangements of TCRγ and TCRβ genes[5]. The design of primers is based on the different families of TCRγ and TCRβ genes and detects almost all possible rearrangements in these two loci. To detect clonal TCRβ rearrangements 23Vβ, 6Jβ1, 7Jβ2, 13Jβ and 2Dβ primers are utilized. For TCRγ only 4Vγ and 2Jγ primers are necessary to identify TCRγ clonal rearrangements.

1.11.2 Heteroduplex analysis

In order to avoid interpreting PCR products on agarose gels as false positive results, separation techniques discriminating between PCR products from mono- oligoclonal and polyclonal TCR gene rearrangements have to be applied. PCR products can be subjected to heteroduplex analysis which is a simple but sensitive method and important in this context. PCR products are denatured and shortly thereafter renatured resulting in the production of heteroduplex with diverse junctional regions if fragments originate from polyclonal T-cell populations. This is visualized as a smear when analysed on polyacrylamide gel. However, if fragments are from monoclonal T-cell populations, homoduplexes with identical junctional regions will be formed and a distinct band can be observed[5] (Figure 5).

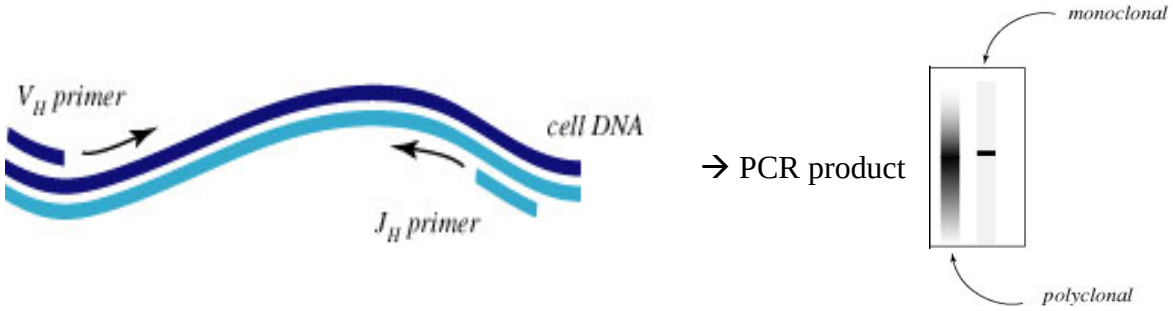


Figure 5. The principle of heteroduplex analysis. The amplified rearrangements are subjected to heteroduplex analysis which results in heteroduplexes visualized as a smear if the rearrangements originate from polyclonal T-cell populations. A rearrangement resulting in one band originates from a monoclonal cell population. (http://www.acor.org/ped-onc/diseases/MRD/pcr.html)

1.11.3 Capillary electrophoresis - Fragment analysis

Electrophoresis performed in capillary tube is called capillary electrophoresis (CE) and fragment analysis is a form of CE. In fragment analysis the DNA-molecules are separated based on their size. This technique has high resolution and allows separation of fragments that only differ by one nucleotide[6]. First, PCR products to be analyzed are amplified with fluorescently labeled primers. The fluorescence signal arising is proportional to the amount of PCR product, measured in relative fluorescence units (RFUs). Before the samples are injected into the capillary, dilution of the PCR product is recommended to avoid the fluorescence intensity exceeding the recommended range. All samples are analyzed together with a size standard, for comparison of the fragment size. The results are interpreted using a software program, and displayed as an electropherogram in which PCR products are visualized as

fluorescent peaks[10]. The fluorescence intensity of the peak is proportional to the amount of the TCR rearrangement and thus clonal rearrangements can be revealed. A single peak or a few peaks of certain intensity are seen if the clonal TCR rearrangement originates from mono- or oligoclonal T-cell populations. On the other hand, if the TCR rearrangements originate from polyclonal T-cell populations many peaks are observed resembling a hedgehog profile.

1.12 Aim

The aim of this study was to investigate T-cell (mono- or oligo-) clonality in CLL patients. This was performed by characterisation of TCRγ and TCRβ gene rearrangements in T-cell populations from CLL patients overexpressing the IGHV3-21 and IGHV4-34 genes. Most of the IGHV3-21+ patients included displayed restricted IG gene features with stereotyped B-cell receptors, whereas the majority of the IGHV4-34 cases showed non-stereotyped receptors. Methods utilized for this analysis were cell sorting, FACS, PCR, heteroduplex analysis, fragment analysis, cloning and sequencing.

2. Results

2.1 Fluorescence activated cell sorting

CLL cells were isolated from tumour samples of 5 IGHV4-34 patients by negative depletion using monoclonal antibodies that targeted non-B-cells. T-cells are present among these non-B-cells, although in very low amount. Following isolation of CLL cells and prior to fluorescence activated cell sorting (FACS) analysis, B-and T-cells were fluorescently labelled. Cell samples were gated using an IgG2 mouse isotype control. Pure CD5/CD19 B-CLL cells were detected in corresponding CLL samples. Even though low levels of T-cells were detected in the patient samples, FACS analysis verified pure T-cell populations.

FACS analysis on sorted CLL cells, CD4 and CD8 T-cells from 5 IGHV3-21 patients was performed and revealed pure cell populations[17].

2.2 DNA concentration and quality

DNA from sorted IGHV3-21 patient´s CD4- and CD8 T-cells and corresponding CLL cells previously isolated[17] , as well as DNA isolated from both CLL cells and T-cells from the group of IGHV4-34 patients was determined by a nanodrop instrument obtaining concentrations between 15-150 ng/µl. All DNA was of good quality as demonstrated by analysis of genomic DNA on 2 % agarose gels.

2.3 Polymerase chain reaction amplification of T-cell receptor γ rearrangements

PCR amplification of TCRγ rearrangements was performed using three sets of primers (Table 1). TCRγ rearrangements were determined in T-cells and CLL cells from 5 IGHV4-34 patients as well as CD4 and CD8 T-cells and CLL cells from 5 IGHV3-21 patients. Prior to investigation for mono- and oligoclonality with heteroduplex analysis and fragment analysis, all PCR products, except IGHV4-34 samples amplified with labeled Biomed-2 primers, were visualized on 2 % agarose gels that verified that the amplification reactions were successful.

2.3.1 Biomed-1

Using Biomed-1 primers, TCRγ rearrangements were amplified in a total of four reactions, where each reaction represented single Vγ family rearrangements (Table 2). The size of PCR fragments corresponding to TCRγ rearrangements was ~ 300 and ~ 500 bp (Table 1) which was confirmed by comparing amplified products to a 100 bp molecular size ladder (Fig 6). Analysis of CD4 and CD8 T-cells from IGHV3-21 patients revealed PCR products in all T-cell samples whereas in the corresponding CLL cells from the same group of patients PCR product were only detected in patient 5. In the group of IGHV4-34 patients PCR products were observed in four out of five patients' T-cell samples. (No amplification was observed in T-cell sample from patient 10). Amplification was also detected in CLL cells, for all patients except one (patient 9).

Table 1. Primer combinations for detection of TCR γ rearrangements[1].

Primer set	Tube	Forward primer	Reverse primer[2]	Amplicon size (bp)
Biomed-1	1	Vγ 1	Jγ 1.1 and Jγ 2.1	329
			Jγ 1.2	337
			Jγ 1.3 and Jγ 2.3	533
	2	Vγ 2	Jγ 1.1 and Jγ 2.1	318
			Jγ 1.2	326
			Jγ 1.3 and Jγ 2.3	522
	3	Vγ 3	Jγ 1.1 and Jγ 2.1	318
			Jγ 1.2	326
			Jγ 1.3/2.3	522
	4	Vγ 4	Jγ 1.1 and Jγ 2.1	354
			Jγ 1.2	362
			Jγ 1.3 and Jγ 2.3	558
Biomed-2	A	Vγ 1	Jγ 1.3 and 2.3 (green)	195-230
			Jγ 1.1 and 2.1 (blue)	230-255
		Vγ 3	Jγ 1.3 and Jγ 2.3 (blue)	145-175
				175-195
	B	Vγ 2	Jγ 1.1 and Jγ 2.1 (blue)	160-195
			Jγ 1.3 and Jγ 2.3 (green)	195-220
	C	Vγ 4	Jγ 1.1 and Jγ 2.1 (blue)	80-110
			Jγ 1.3 and Jγ 2.1 (green)	110-140
			Jγ1.1 and Jγ 2.1 (blue)	

[1] All tubes, except for tube A Biomed-2, have single Vγ family primers but several reverse primers in each tube. Only one reverse primer is used with one forward primer depending on the rearrangement to be amplified, resulting in different size of PCR products.

[2] The reverse primer in Biomed-2 set is fluorescently labelled and the colour of the dye, as seen in fragment analysis electropherogram is noted.

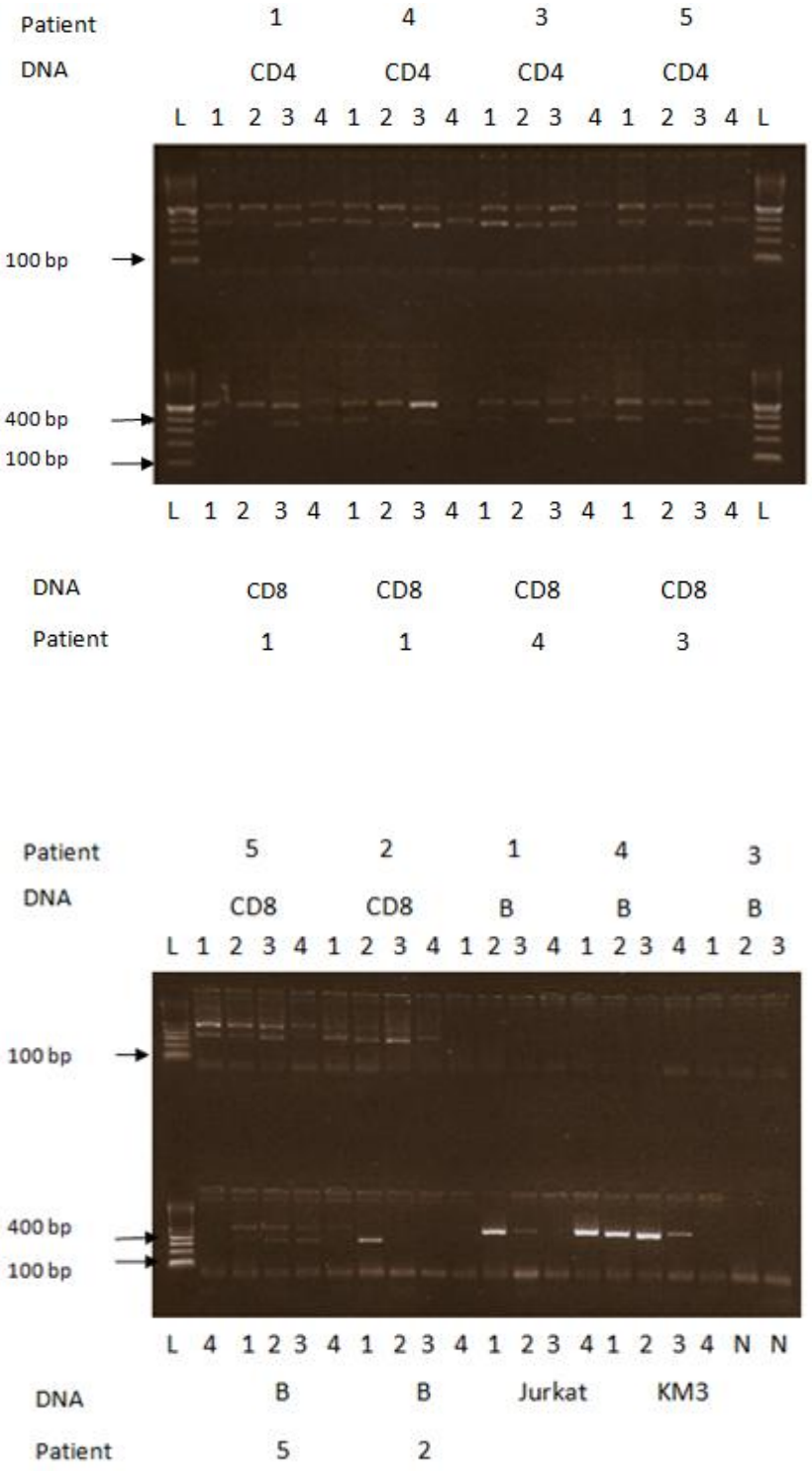


Figure 6. Agarose gel separation of PCR products from TCRγ. PCR fragments corresponding to TCRγ rearrangements in CD4 and CD8 T-cells as well as CLL cells from 5 IGHV3-21 patients are visualized on 2 % agarose gels. In each gel, applications are made on both upper and lower parts. PCR products of ~ 500 bp from Jurkat and KM3 cell lines are shown in the gel to the right as positive controls as well as two negative controls (N). The IGHV3-21 patient number is indicated as well as the DNA identity. The lanes; 1,2,3,4 refer to the four different primer tubes.

2.3.2 Biomed 2 (unlabeled and labeled) primers

TCRγ rearrangements were amplified using two Biomed-2 primer sets where one set was fluorescently labeled, to allow different analysis of the amplified PCR products. With Biomed-2 primers amplification of all TCRγ rearrangements was performed in three reactions (A,B,C) (Table 1) resulting in PCR fragments of ~100 and ~300 bp in size.

In the group of IGHV3-21 patients, PCR products from reactions A and B were seen in all CD4 and CD8 T-cell samples, whereas very few amplifications were detected with reaction C . Only CLL cells from patient 5 were showed positive PCR product for all TCRγ reactions (data not shown).

In T-cells from the IGHV4-34 group, amplified PCR products were visualised in all samples although very few amplifications were observed with reaction C. In addition, amplifications were observed in CLL cells from all IGHV4-34 samples (data not shown).

2.4 Heteroduplex analysis revealed mono-and oligoclonal TCRγ rearrangements both in CD8 and CD4 T-cells predominantly in IGHV3-21 patients

2.4.1 Biomed-1

PCR products amplified with Biomed-1 from all IGHV4-34 and IGHV3-21 patients were initially subjected to heteroduplex analysis. This was performed to discriminate possible true mono- and oligoclonal rearrangements from rearrangements originating from polyclonal T-cells. Following heteroduplex analysis, PCR products were separated by GenePhor polyacrylamide gel electrophoresis (PAGE) in order to discriminate between heteroduplexes with diverse junctional regions if fragments originated from polyclonal T-cell populations and homoduplexes with identical junctional regions formed if the fragments originated from monoclonal T-cell populations.

After silver staining of the gels and analysis of the PCR products, 7 potentially mono- and oligoclonal TCRγ rearrangements were identified in a total of four patients, all belonging to the IGHV3-21 subset (Figure 7). These potentially clonal TCR bands had the expected size (approximately 300 respectively 500 bp) and were not amplified in the CLL cells. The potentially clonal rearrangements are shown in Figure 7 and indicated by arrows. The observed Vγ family usages for the “clonal” TCRγ rearrangements are shown in Table 2.

Heteroduplex analysis of T-cells from IGHV4-34 patients followed by separation by PAGE revealed very weak bands. No potentially clonal TCRγ rearrangements were observed in IGHV4-34 T-cells amplified with Biomed-1 primers (data not shown).

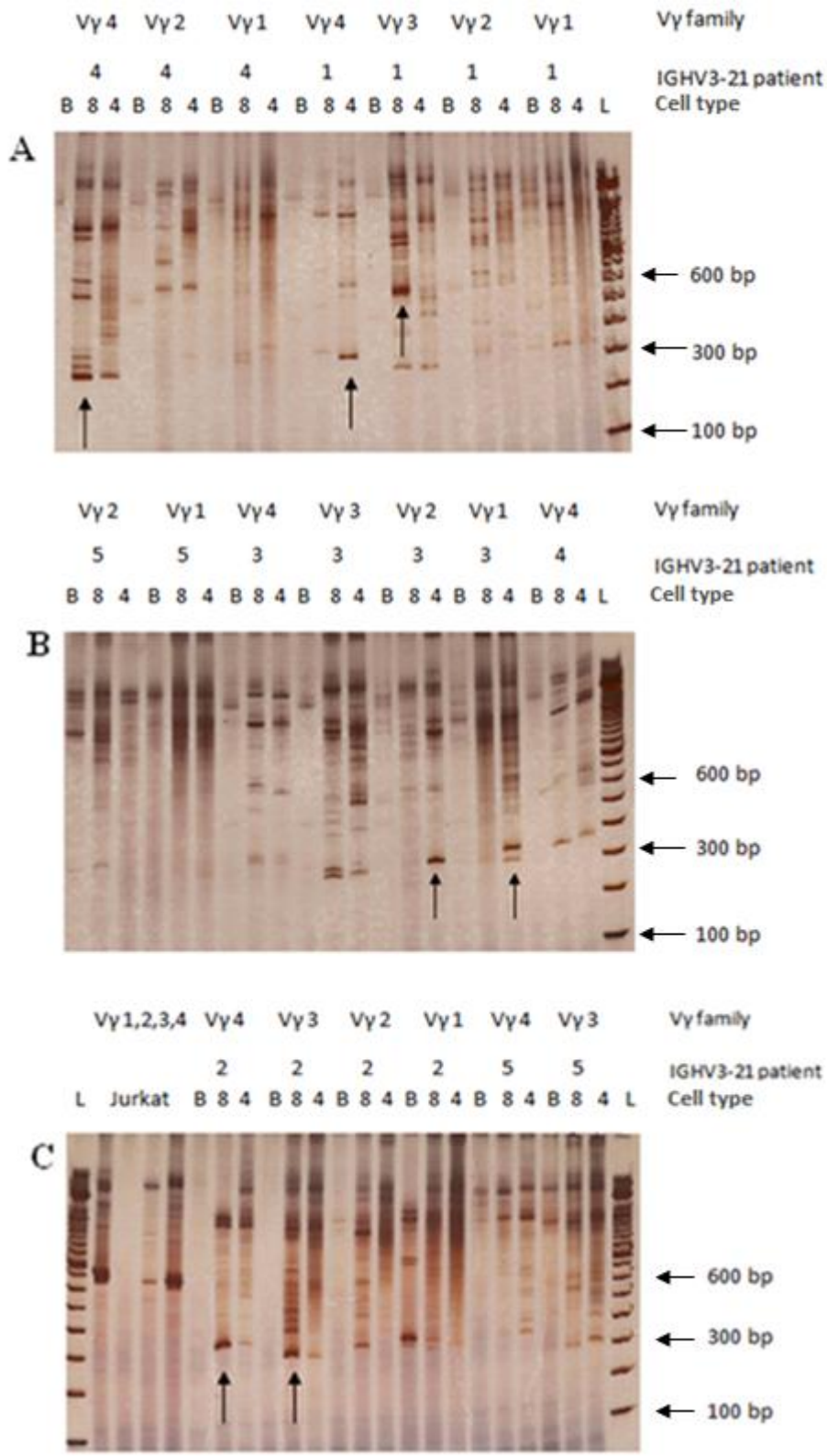


Figure 7. PAGE with silverstaining of TCRγ PCR products using Biomed-1 primers. The PCR products were subjected to heteroduplex analysis before separation by PAGE. Patient, type of DNA and Vγ family amplified are denoted. In gel A from right to left first a 100 bp molecular size ladder (L) was run followed by CD4 and CD8 as well as CLL DNA amplified with Vγ 1 primers, in patient 1. Arrows indicate potentially clonal bands from IGHV3-21 CD4 and CD8 T-cell samples in panel A, B and C. "B" is B-cells, "8" is CD 8 T-cells and "4" is CD 4 T-cells.

Table 2. Summary of observed potentially clonal TCRγ rearrangements.

Patient	T-cell type	Family rearrangement	Biomed-1 Heteroduplex analysis	Biomed-2 Heteroduplex analysis	Biomed-2 Fragment analysis
1 (IGHV3-21)	CD 8	Vγ 3	+		+
1 (IGHV3-21)	CD 4	Vγ 4	+		
2 (IGHV3-21)	CD 8	Vγ 3	+		+
2 (IGHV3-21)	CD 4	Vγ 4			+
2 (IGHV3-21)	CD 8	Vγ 4	+		+
3 (IGHV3-21)	CD 4	Vγ 1	+		
3 (IGHV3-21)	CD 4	Vγ 2	+	+	+
3 (IGHV3-21)	CD 8	Vγ 1 and Vγ 3			+
4 (IGHV3-21)	CD 8	Vγ 2		+	
4 (IGHV3-21)	CD 8	Vγ 3	+		
5 (IGHV3-21)	CD 4	Vγ 4			+
7 (IGHV4-34)	T-cells	Vγ 1 and Vγ 3		+	
8 (IGHV4-34)	T-cells	Vγ 2			+

2.4.2 Biomed-2

Amplification of IGHV3-21 DNA with unlabeled Biomed-2 primers, subjected to the same procedure as described for PCR products amplified with Biomed-1, resulted in few and weak bands, as visualized by PAGE subsequent to heteroduplex analysis (data not shown). However, two rearrangements were assessed as potentially mono- or oligoclonal, being of expected size range, approximately 200 bp, and being absent in the corresponding CLL sample (Table 2). The characteristics of the potentially clonal rearrangements are described in Table 2.

Amplifications from IGHV4-34 patients with Biomed-2 primers and PAGE analysis by the same procedure as for the other PCR products were successful. One rearrangement was assessed as potentially clonal, belonging to an IGHV4-34 patient (Table 3). Amplification with Biomed-2 primers was not successful (data not shown).

2.5 The majority of clonal T-cell receptor γ rearrangements revealed by fragment analysis belong to IGHV3-21 patients

TCRγ rearrangements in T-cells amplified with fluorescently labeled Biomed-2 primers from IGHV3-21 and IGHV4-34 patients were analyzed for mono- and oligoclonality by fragment analysis. The amplified PCR products were visualized on the electropherogram as peaks of different colours, depending on the primer used in that particular amplification. In this study primers with two fluorescent dyes were used resulting in green or blue peaks (Table 1).

The size in bp is denoted along the x-axis whereas the y-axis shows relative fluorescence units (RFUs), used as an indication of the amount of the amplified PCR product compared to the size-standard visualized in red. Multiple peaks were observed in polyclonal samples but a single or a few peaks representing one or a few types of amplicon were seen in case of potentially mono- or oligoclonal samples, respectively. A total of 10 potentially mono- and oligoclonal fragments were observed, 8 belonging to the IGHV3-21 subgroup and two to the IGHV4-34 subgroup. To indicate clonality, a distinct peak at an expected position should be observed in T-cells, but not in the corresponding CLL cells. One of the potentially clonal rearrangements is shown in Figure 8. The distinct peak at 185 bp represents a potentially clonal TCRγ rearrangement in CD8 T-cells from patient 2. In the IGHV4-34 patient group only one case, patient 8, was classified as potentially clonal (Table 2).

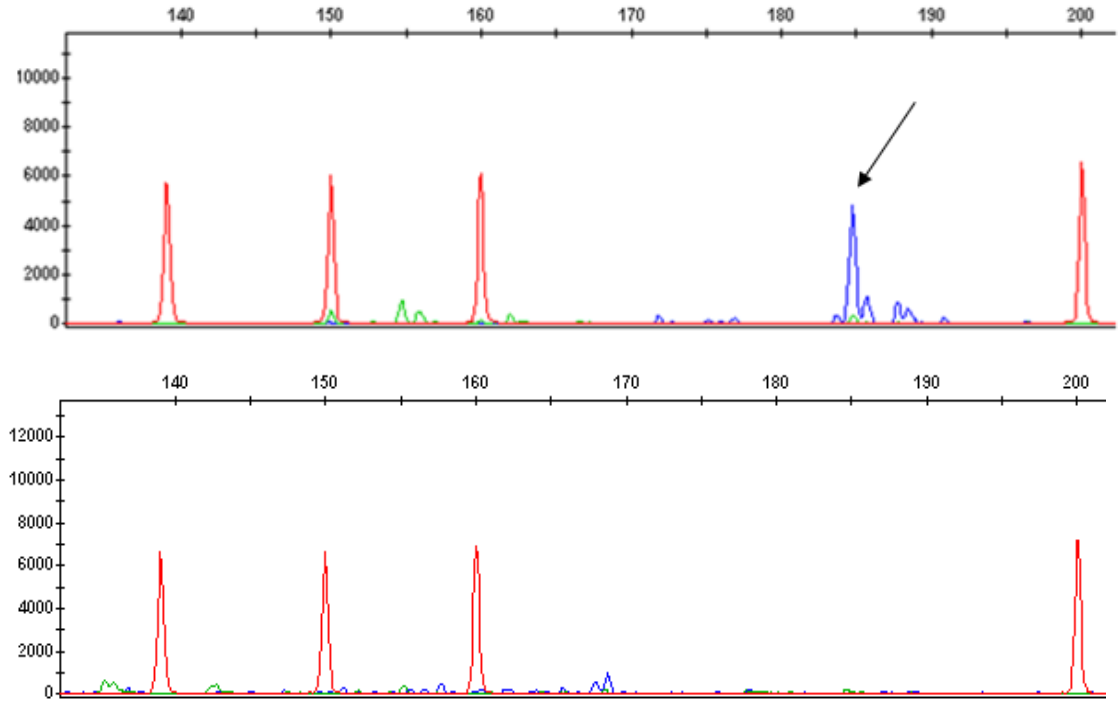


Figure 8. Fragment analysis of TCRγ in CD8 T-cells and CLL cells from patient 2. In the upper panel, a distinct blue peak represents a potentially clonal TCRγ rearrangement of 185 bp, detected in CD8 T-cells. In the lower panel the CLL sample from the same patient is shown. The size-standards, run in all reactions are shown in red. The y-axis shows RFUs and x-axis the size of the products.

2.6 Clonal T-cell receptor γ rearrangements displaying identical or similar CDR3 indicate mono-and oligoclonal T-cells

All 7 identified potential clonal TCRγ rearrangements, amplified with Biomed-1, that were observed in IGHV3-21 patients were cloned and sequenced to confirm the clonality. In addition the PCR products themselves were directly sequenced (without being cloned). Sequences from both direct sequencing and from the clones were submitted to the International Immunogenetics (IMGT) database that compared the sequence to the most similar germline genes. Analysis from IMGT confirmed that all 7 clonal TCRγ rearrangements contained TCRγ V- and J gene sequences. The exact identification of the TCRγ V and J genes for each TCRγ rearrangement as well as whether or not the rearrangements were functional are shown in Table 3. Sequenced clones from the TCRγ family 3 and 4 rearrangements observed in patient 1 were identified as non-functional by the IMGT. Non-functionality was also displayed for the clonal rearrangements in patient 2, also

involving members of Vγ3 and 4 families. Two clonal rearrangements, found in patient 3, are members of the Vγ1 and Vγ2 families. The clonal rearrangement of the Vγ1 family was also interpreted as unproductive and nonfunctional by the IMGT, due to stop codons and/or frameshift in the junctional regions. Importantly, patient 3 possessed the only rearrangement that was productive with certainty. This rearrangement, TRGV9-TRGJP, belongs to the Vγ2 family and is marked by an asterix in Table 3. The clonal rearrangement belonging to TCRγ 10 in patient 4 was also identified as productive by IMGT, but may not be functional. Codon 27 is missing from CDR1 in the sequence from the submitted clone but this codon is present in the closest germline sequence. In addition, it appears that one codon is missing at position 108 in the CDR3. However, since whole codons ares missing, the sequence is still read in frame, so this is interpreted as a productive rearrangement.

Table 3. Characteristics of clonal TCRγ rearrangements in IGHV3-21 patients.

Patient	Vγ family	Direct sequences	# Sequenced clones	# Identical clones in the CDR3	V gene	J gene	Functionality
1 (IGHV3-21)	Vγ4	Yes	15	15	TRGV11	TRGJP1	Non-functional
1 (IGHV3-21)	Vγ3	Yes	20	12	TRGV10	TRGJ1	Non-functional
				3	TRGV10	TRGJ1	Non-functional
2 (IGHV3-21)	Vγ3	Yes	15	6	TRGV10	TRGJP1	Non-functional
				2	TRGV10	TRGJP1	Non-functional
2 (IGHV3-21)	Vγ4	Yes	16	13	TRGV11	TRGJP1	Non-functional
3 (IGHV3-21)	Vγ1	Yes	22	5	TRGV8	TRGJP2	Non-functional
				5	TRGV8	TRGJP1	Non-functional
				3	TRGV3	TRGJP2	Non-functional
				2	TRGV3	TRGJP2	Non-functional
3 (IGHV3-21)	Vγ2	Yes	19	14	TRGV9	TRGJP1	Non-functional
				3	TRGV9[1]	TRGJP	Functional
4 (IGHV3-21)	Vγ3	Yes	16	13	TRGV10	TRGJP1	Non-functional
				2	TRGV10	TRGJP1	Non-functional

[1] Only the rearrangement TRGV9 – TRGJP is functional

Sequences obtained for all clones were aligned with the sequence obtained from direct sequencing of the PCR product, to analyze the number of identical clones and characterize the CDR3 in each clone. Sequences of the CDR3 in the clones and the sequences obtained directly were identical in several cases. In conclusion, the identical CDR3s observed in IGHV3-21 patients indicate clonal T-cell populations.

2.7 Fragment analysis for T-cell receptor β revealed mono- and oligoclonality in all IGHV3-21 samples investigated

TCRβ rearrangements were amplified with fluorescently labeled Biomed-2 primers in the patient samples where TCRγ mono- and oligoclonality had been detected (patient 1-5 IGHV3-21 and patient 7 IGHV4-34). All possible TCRβ rearrangements were amplified using three reaction tubes. The size of the PCR amplicons is shown in Table 4. PCR products were analyzed by fragment analysis for mono- and oligoclonality. Amplified PCR products were observed with tubes A and B at 240-285 bp for all 5 IGHV3-21 samples, but no products were observed for the IGHV4-34 sample (Table 5). With tube C peaks were observed at correct size range ranges, 170-210 bp and 285-325 bp, but a low background noise was observed as

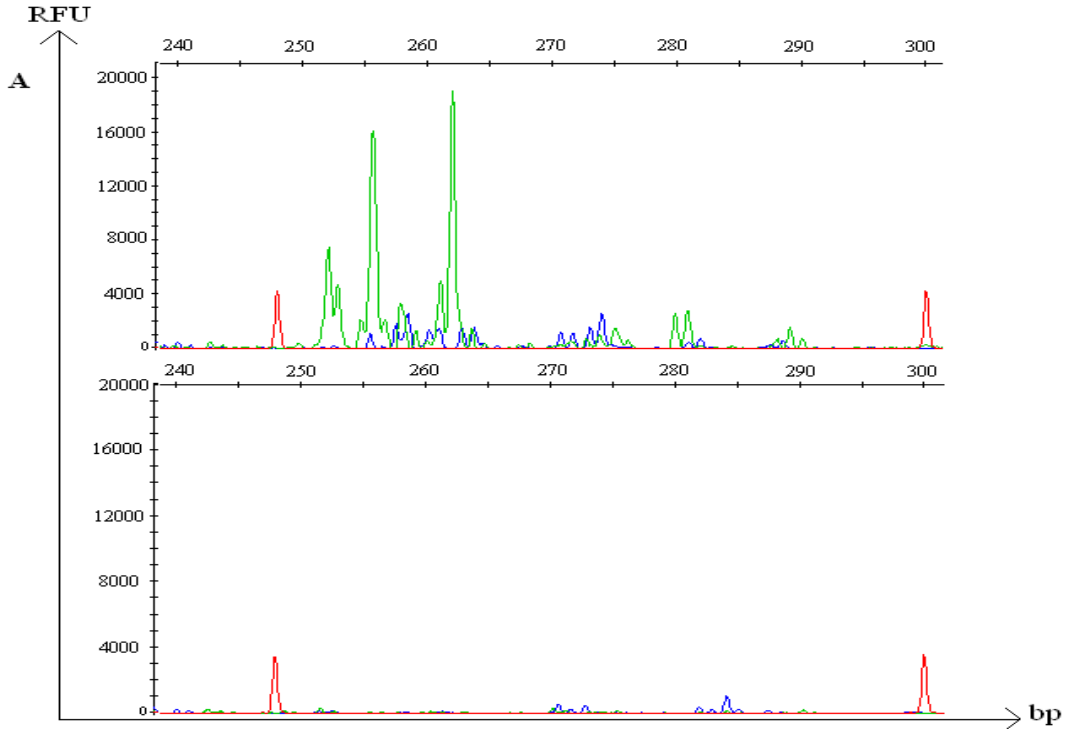
well. Potential mono- and oligoclonal TCRβ rearrangements were revealed in all IGHV3-21 analyzed samples, mostly with tubes A and B. Two of the potential oligoclonal TCRβ rearrangements are shown in Figure 9.

Table 4. Representation of the TCRβ primer tubes and corresponding PCR-product size.

Tube	#Vβ primers	#Dβ primers	#Jβ primers	PCR product size (bp)
A	23		9	240-285
B	23		4	240-285
C		2	13	170-210 ,285-325

Table 5. Potentially mono- and oligoclonal T-cell receptor β rearrangements observed in IGHV3-21 patients.

Patient	Monoclonal	Oligoclonal	Fragment size bp
1 IGHV3-21	CD8	CD4	240-285, 285-325
2 IGHV3-21	CD8		240-285
3 IGHV3-21		CD4	240-285
5 IGHV3-21	CD8		240-285



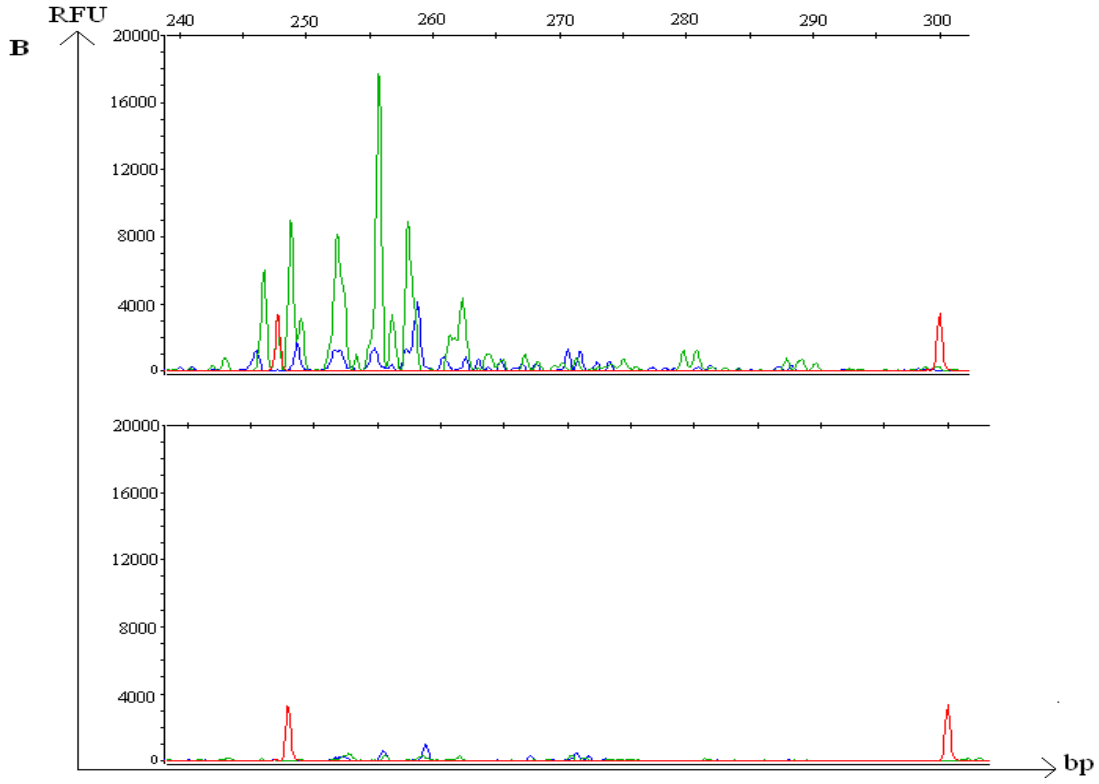


Figure 9. Fragment analysis for TCRβ in two IGHV3-21 patient samples. Green: fragments corresponding TCRβ rearrangements. Read: the size-standard. Panel A shows TCRβ rearrangements in patient 5 (IGHV3-21) from tube A. CLL cells, in the lower part of panel A. Panel B shows rearrangements amplified with tube A in patient 2 (IGHV3-21), and in the corresponding CLL cells, in the lower part of panel B. The y-axis represents RFUs and x-axis shows the size of the products.

3. Discussion

3.1 Analysis of amplified T-cell receptor γ rearrangements using two sensitive techniques

The focus of this study was on mono-and oligoclonal TCRγ rearrangements, possibly originating from one or a few expanded T-cell populations that had encountered the same antigen(s) as the CLL cells, or perhaps expanded in response to the CLL clones. This is in contrast to polyclonal rearrangements which come from many different small T-cell expansions as seen in healthy individuals. Potentially clonal TCRγ rearrangements were observed mainly in the IGHV3-21 subset, although some were identified in IGHV4-34 using patients. In a total of 13 potentially mono- and oligoclonal TCRγ rearrangements (from all three TCRγ primers set used), only two were found in IGHV4-34 patients.

Seven of the potentially mono-oligoclonal rearrangements observed in IGHV3-21 patients were amplified using Biomed-1 primers. However, the same rearrangement pattern was not observed when the same patient material was amplified with unlabeled Biomed-2 primers and the PCR products analyzed in the same way. Only one rearrangement was observed both with the unlabeled Biomed-2 primers, and Biomed-1 primers. As few or very faint amplifications were observed in heteroduplex analysis performed with unlabeled Biomed-2 primers, compared to Biomed-1 primers, it is likely that the analysis of PCR-products amplified with unlabeled Biomed-2 was not optimal.

Fluorescently labeled Biomed-2 primers, which allowed the amplified PCR products to be analyzed by fragment analysis, revealed three of the potentially clonal TCRγ rearrangements from IGHV3-21 patients. These were also detected using heteroduplex analysis of Biomed-1 PCR products.

Although some dissimilarity exist among the two Biomed-primer sets, inconsistency in identification of mono- and oligoclonal rearrangements is nevertheless surprising as both sets of primers should target the same Vγ-Jγ rearrangements. In addition, different results in identification of clonal TCRγ rearrangements were observed when using the same primer set, Biomed-2, but analyzing the PCR products with different techniques. The two sets of Biomed-2 primers differ only in fluorescent labeling and the reason for dissimilarity in detection of clonal TCRγ rearrangements when using this same primer set is unknown. However, these diverse results strengthen the importance of using different, complementary techniques when studying mono- and oligoclonality.

From heteroduplex and fragment analysis it can be concluded that the identified potential mono and- oligoclonal TCR clonality indeed was restricted to T-cells, as the TCRγ rearrangements were not observed in the corresponding CLL cells. As previous studies have shown that amplification of TCR genes might occur in CLL cells due to crosslineage rearrangements[30], this issue was particularly considered in this study. In addition, the T-cell clonality was more frequently observed in the IGHV3-21 patients. Interestingly, IGHV3-21 patients have a more aggressive form of CLL than the IGHV4-34 patients. This indicates that T-cells might have different impact in different CLL subgroups and may possibly be more involved in patients with a more severe disease. However, a larger cohort has to be analyzed to corroborate this proposal.

3.2 Clonal T-cell receptor γ rearrangements

There was no uncertainty concerning the 7 clonal TCRγ rearrangements found in samples from IGHV3-21 patients amplified with Biomed-1 primers, as subcloning followed by nucleotide sequencing confirmed the observation of mono- and oligoclonality. In each of the

cloned samples a significant number of clones with identical CDR3s were found, indicating that these rearrangements originated from mono- or oligoclonal T-cells. Despite this observation of identical CDR3s among the clones, the pathological role of the identified TCRγ clonality in these CLL patients is nonetheless uncertain.

T-cell recognition of antigen might result in a clonal T-cell population in which the TCR V genes and CDR3s are identical[24]. The identified clonal TCRγ rearrangements do not seem to result from expanded T-cell populations having encountered antigen by their γδTCRs since all except one rearrangement were non-functional. Of the 7 clonal TCRγ rearrangements, 5 are members of the families Vγ3 and Vγ4. These clonal rearrangements produced non-functional TCRγ rearrangements and are highly unlikely to be part of T-cells that express γδTCRs, even if they are encoded from the TCRγ locus. However, amplified TCRγ rearrangements do not necessarily have to originate from T-cells expressing the γδTCR. They can be amplified from T-cells that express the other TCR type, αβTCR. One explanation of the results of this study is the rearrangement process of the TCR genes that takes place during T-cell differentiation. In general, this process occurs in a hierarchical order; TCRδ genes are the first to rearrange, followed by TCRγ genes. If the γδ rearrangement is functional, TCRγδ protein can be produced and expressed on T-cells[5]. However, T-cells with non-functional TCRγδ rearrangement do not express any γδTCR but continue the rearrangement process of the TCRβ locus followed by deletion of the TCRδ rearrangement and a final rearrangement in the TCRα locus[5].

If the TCRαβ rearrangement is functional, it results in expression of TCRαβ at the surface of T-cells[5]. As non-functional TCRγδ rearrangements results in deletion of TCRδ rearrangements but not of TCRγ rearrangements, TCRγ rearrangements are present in αβTCR expressing T-cells. The fact that TCRγ rearrangements identified in this study were non-functional suggests that they originate from T-cells expressing αβTCRs rather than from T-cells expressing γδ TCRs.

Even the observed clonal rearrangements that are members of functional families, Vγ 1 and Vγ 2, were non-productive, with one exception. However, the functional rearrangement found in patient 3 (IGHV3-21) is reported to be frequent in older individuals[5]. As CLL patients are elderly, the significance of this clonal rearrangement is uncertain as it may be the result from a normal ageing process[5].

Another important factor that supports the origin assessment of the identified clonal rearrangements is the actual number of T-cells expressing γδTCRs in humans. Only 1-10% of T-cells in peripheral blood are estimated to express γδTCRs. Thus, the majority of T-cells express αβTCRs. Furthermore, less than 1 % of the T-cells that express γδTCRs express CD4 while approximately 30 % express CD8[13]. Consequently, the majority of γδTCRs are negative for both of these cell surface markers and no unique cell surface molecule has been observed for T-cells expressing γδTCRs[13].

Taken together, it is likely that the observed non-functional clonal TCRγ rearrangements originate from clonal T-cell populations expressing αβTCRs.

3.3 Clonality in T-cell receptor β genes

In previous analysis of TCRβ, clonal populations of T-cells in CLL patients have been observed[7,23,25]. These clonal T-cell populations are assumed to result from recognition of epitopes on the leukemia cells. An observation that supports this assumption is the ability of T-cells in CLL patients to recognize CLL cells specifically and stimulate a response against them[24]. As the majority of T-cells recognize antigens by αβTCRs, the clonal TCRβ rearrangements might result from clonal expansion of αβT-cells after their response with the CLL cells, or some other antigens. Therefore such clonal T-cell populations might be

involved in the pathogenesis of CLL.

Fragment analysis revealed presence of potentially mono- and oligoclonal TCRβ rearrangements in the IGHV3-21 patient samples that already displayed TCRγ mono- and oligo clonality. This could be an indication of possible mono-oligoclonal expansion of αβ T-cells taking place in patients with CLL. Whether the clonality is a result from interaction of the tumor cells or triggered by other antigens remains unresolved. The observed TCRβ clonality gives a strong indication that these T-cell expansions have a functional relevance in perspective to the CLL pathogenesis. Antigen recognition or an immune response to CLL cells is a possible scenario resulting in mono and oligoclonal expansions of T-cells in CLL patients.

3.4 Future perspective

The findings of mono- and oligoclonal T-cell populations in the IGHV3-21 subgroup with restricted CDR3 features suggest that T-cells in these patients have participated in development of CLL. In this study mono- and oligoclonality was observed for both TCRβ- and TCRγ genes, but only verified in the latter. Whether T-cell clonality is restricted to certain CLL subgroups with worse prognosis, such as IGHV3-21, needs to be verified by analyzing a larger cohort of patients. By performing subcloning followed by nucleotide sequencing of the observed TCRβ mono- and oligoclonal rearrangements in IGHV3-21 patients, this could verify if these expansions originate from functional TCR rearrangements. If so, the support for T-cell clonality in CLL would be unambiguous.

4. Materials and Methods

4.1 Patient samples

Tumor samples from patients diagnosed with CLL were obtained from the Department of Pathology at the Uppsala University Hospital. The diagnosis was based on morphologic and immunophenotypic features according to the World Health Organization (WHO) classification, and tumour cells typically expressed CD5, CD19, CD23 and low levels of IG.

Patient samples came from two different subgroups of CLL, IGHV3-21 and IGHV4-34 (Table 6). Four IGHV3-21 patients display identical CDR3 classified as stereotyped and one patient display non-identical CDR3 classified as non-stereotyped. From the IGHV4-34 subgroup, four patients displayed non-identical CDR3 while one displayed restricted CDR3 (Table 6). All tumour samples were collected from peripheral blood. As positive controls the cell lines Jurkat (DSMZ) and melanoma KM3 (DSMZ) were used, as well as one ALL sample (Uppsala University Hospital), since they display known TCR gene rearrangements.

Table 6. Characteristics of the CLL patient samples used in this study.

Samples	IGHV group	% similarity[1]	CDR3 length	Stereotyped or Non-stereotyped[2]
1	IGHV3-21	99.6	9	Stereotyped
2	IGHV3-21	96.4	9	Stereotyped
3	IGHV3-21	96.9	9	Stereotyped
4	IGHV3-21	96.8	9	Stereotyped
5	IGHV3-21	97.3	12	Non-stereotyped
6	IGHV4-34	94.7	15	Non-stereotyped
7	IGHV4-34	92.1	17	Non-stereotyped
8	IGHV4-34	91.6	14	Non-stereotyped
9	IGHV4-34	94.9	17	Non-stereotyped
10	IGHV4-34	92.9	20	Stereotyped

[1] % similarity to the most similar germline sequence. IGHV sequence displaying ≥ 2 % mutation to the germline sequence is considered as mutated whereas < 2 % mutation to the germline sequence considered unmutated.

[2] The stereotyped cases have highly similar (almost 100 % similarity) CDR3 whereas the non-stereotyped cases have CRD3 sequences that deviate from the stereotyped CDR3 sequences.

4.2 Isolation of lymphocytes using Ficoll-Paque density gradient

Ten ml blood sample was diluted 1:5 times in 1 × phosphate buffered saline (PBS; 137 mM

NaCl, 2.7 mM KCl, 10 mM $NaHPO_4$, pH 7.4 without Ca^{2+} and Mg^{2+}, provided by Medieberedningen at Rudbeck Laboratory), in a 50 ml centrifuge tube. To a new 50 ml centrifuge tube, 10 ml Ficoll-Paque^{TM}-PLUS (GE Healthcare) was added and the diluted blood was carefully layered on the Ficoll-Paque using a pasteur pipette. The samples were centrifuged at 650 x g at room temperature for 30 min. The layer containing lymphocytes was removed using a pasteur pipette.

Lymphocytes were washed three times in cold 1 × PBS and centrifuged at 300 x g for 10 min. The supernatant was discarded and the cell pellet resuspended thoroughly in 40 ml ice-cold 1 × PBS at RT. The number of lymphocytes was counted in a small volume of cells using a Coulter counter. The remaining cells were centrifuged at 300 x g for 10 min and the cell pellet resuspended in foetal bovine serum (FBS, Gibco, Invitrogen) supplemented with 10 % dimethyl sulfoxide (DMSO). Cryotubes containing 3 x 10^7 cells in 1 ml of FBS+DMSO were stored at -80 C° and after 24 h placed in liquid nitrogen tanks.

4.3 Thawing of lymphocytes

Samples were quickly thawed at 37 °C in a water bath and then added to a 15 ml falcon tube. RPMI1640 + GlutaMAX (Invitrogen) cell medium supplemented with 10 % FBS, 0.5 % gentamycin (Invitrogen) and 1 % penicillin-streptomycin (Invitrogen) was warmed to 37 ºC and added to the samples in increasing volumes: 5 × 50 µl, 5 × 100 µl, 5 × 200 µl, 5 × 500 µl and 5 × 1000 µl, to prevent osmolysis. Samples were centrifuged at 230 x g for 5 min, followed by removal of the supernatant. The cells were resuspended in 1 ml fresh medium containing 1 × PBS supplemented with 2 mM EDTA and 1 % FBS. Five µl was removed from the sample for use as isotype control in fluorescence activated cell sorting (FACS) analysis.

4.4 Cell sorting in IGHV4-34 tumour samples

Samples were sorted by Dynal® B Cell Negative Isolation Kit[11] (Invitrogen) where CLL cells expressing CD5 and CD19 were sorted out from the rest of the lymphocytes by using the Dynal® B Cell Negative Isolation Kit´s Depletion Dynalbeads and an Antibody Mix that targets non-B cells expressing CD2, CD14, CD16, CD36, CD43, and CD235a. For one lymphocyte sample, 200 µl Depletion Dynalbeads were mixed in a tube together with 1 ml Buffer 1 (1 × PBS, 2 mM EDTA, 1% FBS). The tubes were placed in a magnetic holder (Invitrogen) for 1 min and the supernatant was discarded. The tubes were removed from the magnet and the washed Dynalbeads were resuspended in 200 µl Buffer 1.

Twenty µl Antibody Mix were added to each lymphocyte sample in Buffer 1. Samples were mixed and incubated for 20 min. The cells were then washed by adding 2 ml Buffer 1. The samples were mixed by tilting the tubes several times followed by centrifugation at 230 x g for 8 min at 4 °C. The supernatant was discarded and the cells were resuspended in 800 µl Buffer 1 and 200 µl pre-washed Depletion Dynalbeads were added to the samples. Thereafter the samples were incubated for 15 min at RT with gentle tilting and rotation. The bead-bound cells were resuspended by turning the tubes up-side down five times followed by addition of 1 ml Buffer 1 and mixing the samples. Sample tubes were placed in a magnetic holder for 2 min and the supernatant, which contained the negatively isolated CLL cells, was removed. The beads were again washed with 1 ml Buffer followed by placing the tube in a magnet for 2 min and removal of the supernatant. The remaining suspension containing T-cells was centrifuged at 230 x g for 10 min and cells resuspended in 10 ml Buffer 1.

4.5 Cell sorting in IGHV3-21 tumour samples

4.5.1 Isolation of chronic lymphocytic leukemia cells by magnetic beads

To the resuspended cells, 400 µl Buffer 1 was added. Following addition of 100 µl anti-CD19 magnetic beads (Miltenyi Biotec), samples were gently rotated for 15 min at 4 ° C, washed with 50 ml Buffer 1 and centrifuged at 300 x g for 10 min followed by removal of supernatant and re-suspension of the samples in 1 ml Buffer 1. CLL cells were isolated from the suspension using LS separation columns and a MidiMACS separation unit (Miltenyi Biotec). The magnetic beads in the LS columns were washed with 3 ml Buffer 1 whereafter the samples were applied into corresponding LS columns. The flowthrough was collected as it contained the non-anti-CD19 expressing cells, which includes T cells. Three times three ml Buffer 1 were rinsed through the LS columns and the flowthrough collected to obtain remaining T-cells. The LS columns were removed from the magnet and placed on collection tubes. Using a plunger, CLL cells were separated from the LS column and eluted with 5 ml of Buffer 1. Falcon tubes containing CLL cells were then centrifuged at 300 x g for 10 min and the pellet resuspended in 200 µl Buffer 1.

4.5.2 Separation of CD4 and CD8 T cells using magnetic beads

Collected T-cells were centrifuged at 300 x g and resuspended in 1 ml Buffer 2 containing 500 ml RPMI1640 and 1% FBS. Dynalbeads (Invitrogen, 25µl per sample) coated with a monoclonal antibody targeting the CD4 molecule, was washed before use by re-suspension in 1 ml Buffer 1 and mixing for 1 min. The tubes were then placed in a magnetic holder (Invitrogen) for 1 min and supernatant discarded. Subsequently, the Dynalbeads were resuspended in 25 µl Buffer1. The Dynalbeads were added to each sample and incubated for 20 min at 4° C, with gentle rotation of the beads to bind to the target cells. Thereafter the tubes were placed in a magnetic holder for 2 min, followed by collecting the supernatant containing remaining cells. Bead-bound cells were washed 3 times by re-suspension in 1 ml of buffer 1. The supernatant was collected for each sample, since it contained remaining cells including $CD8^+$ T-cells, which were used to go through anti-$CD8^+$ selection using the same Dynalbead procedure as explained for $CD4^+$.

4.5.3 Detachment of Dynalbeads

Bead-bound cells were detached by adding 10 µl DETACHaBEAD (Invitrogen) solution to each sample and incubating for 60 min at room temperature with gentle mixing. The tubes were placed in the magnetic holder for 1 min and the supernatants containing the released cells were transferred to new tubes. To obtain remaining cells, the beads were washed 3 times in 500 µl Buffer 2 and the supernatant collected. The cells were washed thoroughly by re-suspension in a volume of 1 ml of Buffer 2. The cells were then centrifuged at 400 x g for 6 min to remove DETACHaBEAD and then resuspended in 1 ml of Buffer 2.

4.6 Fluorescence activated cell sorting

The cell sorted samples containing T-cells and CLL-cells from 5 CLL patients belonging to the subgroup IGHV4-34 as well as samples containing unsorted cells were fluorescence-labeled for analysis by fluorescence activated cell sorting (FACS). Approximately 5 µl of each cell sample was used. Ten µl Premixed Antibodies (Invitrogen) containing R-phycoerythrin dye (R-PE)-labelled anti-CD8 antibodies and fluoroisothiocyanate dye (FITC)-labelled anti-CD3 antibodies, were added to the cell suspension with T-cells. In addition, 10 µl of Premixed Antibodies (Invitrogen) containing R-PE-labelled anti-CD4 antibodies and

FITC-labelled anti-CD3 antibodies were added (Table 7). The anti-CD3 and CD8 antibodies permit the enumeration of the CD8 T-cells, and the anti-CD3 and CD4 antibodies enumerates CD4 T-cells. Thus both T-cell subsets can be detected.

The isotype control was provided with 10 µl Mouse IgG2a FITC + Mouse IgG2a RPE (Invitrogen), to estimate and correct for nonspecific binding to lymphocytes. Ten µl of the BD Simultest (BD Biosciences) containing R-phycoerythrin dye (R-PE) directed against the CD19 molecule and Fluoroisothiocyanate dye (FITC) directed against the CD5 molecule was added to the samples with isolated CLL (Table 7). These two antibodies detect B-cells expressing CD5 and CD19 molecules. The isotype control for CLL cells was provided with 10 µl of Simultest control γ2/ γ1 (BD Biosciences). Samples provided with fluorescent dyes were mixed and incubated for 15 min at RT in dark and then resuspended in 1 × PBS. FACS analysis was performed (BD LSR II SORP) on isolated CLL cells and the cell suspension containing T-cells, for each patient. The data was analyzed using BD FACSDiVa Software 6.0. FACS analysis was similarly performed on CD4, CD8 and CLL-cells[17].

Table 7. Monoclonal antibodies used in FACS for detection of B-cells, CD4-and CD8 T-cells.

Fluorescent dye	Monoclonal antibody	Targets for the antibody-combination
FITC	Anti-CD3	
R-PE	Anti-CD4	CD4 T-cells
FITC	Anti-CD3	
R-PE	Anti-CD8	CD8 T-cells
FITC	Anti-CD5	
R-PE	Anti-CD19	CD5/ CD19 B-cells

4.7 DNA isolation

DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen)[20] according to the instructions provided in QIAamp DNA Blood Mini Kit Handbook[21] (Qiagen). DNA concentration was measured with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies). DNA with OD_{260} / OD_{280} 1.7-1.9 was considered good quality. Samples with low DNA concentrations were concentrated using vacuum centrifugation (CentriVap DNA Concentrator, Labconco).

4.8 Polymerase chain reactions (PCR)

TCRγ rearrangements were amplified from IGHV3-21 and IGHV4-34 patients using three primer sets, Biomed-1 (unlabeled, In VivoScribe Technologies), Biomed-2 (unlabeled, InVivoScribe Technologies) and Biomed-2 (fluorescent-labeled, InVivoScribe Technologies) (Table 9). With Biomed-1, the PCR amplifications was carried out in 50 µl volume reactions containing 200-300 ng DNA, 10 × PCR Rxn Buffer (Invitrogen), 5 µM forward primer (InVivoScribe Technologies), 15 µM reverse primers (InVivoScribe Technologies, IVS), 5 U Platinum Taq (Invitrogen), ddH_2O, 2.5 mM dNTP and 50 mM $MgCl_2$. As negative control H_20 was used and as positive control, Jurkat and KM3 cell lines. The PCR program was carried out using cycling conditions 95 °C 10 min, 40 × (94 °C 30 sec, 60 °C 30 sec, 72 °C 45 sec), 72 °C 7 min.

Table 8. Oligonucleotides.

Primer set	Primer name	Sequence	Used for
Biomed-1	Vγ 1-8	CAGGCCGACTGGGTCATCTGC	PCR
Biomed-1	Vγ 9	CAGCCCGCCTGGAATGTGTGG	PCR
Biomed-1	Vγ 10	GACATACCTTGCAAGATATCGAGC	PCR
Biomed-1	Vγ11	CTGAAATATCTATTTCCAGACCAGC	PCR
Biomed-1	J γ 1.1& 2.1	TTACCAGGTGAAGTTACTATGAGC	PCR
Biomed-1	J γ 1.2	AAGAAAACTTACCTGTAATGATAAGC	PCR
Biomed-1	J γ 1.3 & 2.3	CCGTATATGCACAAAGCCAAATC	PCR
Biomed-2	Vγ 1-8	GGAAGGCCCCACAGCR(G/T)TCTT	PCR
Biomed-2	Vγ 9	CGGCACTGTCAGAAAGGAATC	PCR
Biomed-2	Vγ 10	AGCATGGGTAAGACAAGCAA	PCR
Biomed-2	Vγ11	CTTCCACTTCCACTTGAAA	PCR
Biomed-2	J γ 1.1& 2.1	TTACCAGGTGAAGTTACTATGAGC	PCR
Biomed-2	J γ 1.3 & 2.3	GTGTTGTTCCACTGCCAAAGAG	PCR
M13	M13 F	GTAAAACGACGGCCAG	Sequencing
M13	M13 R	CAGGAAACAGCTATGAC	Sequencing

Amplification by Biomed-2 primers was performed in 25 µl reactions containing 200-300 ng DNA, 1.1 X Mastermix Abgene (ABgene) 5-10 µM forward primer and 10 mM reverse primer. The PCR program was carried out using cycling conditions 95 °C 5 min, 35 × (94 °C 30 sec, 60 °C 30 sec, 72 °C 45 sec), 72 °C 7 min.

The same reaction conditions and PCR program was used for labeled Biomed-2 primers with the difference that the reverse primers were labeled with fluorescent dyes FAM-6 and HEX and the reaction mixtures were protected from light. H_2O was used as negative control while Jurkat and KM3 DNA was used as positive control.

To detect TCRβ gene rearrangement multiplex PCR was performed using Biomed-2 primers labeled with fluorescent dyes HEX and FAM-6 (InVivoScribe Technologies). Three multiplex primers mixes (Tube A with 23 Vβ + 6 Jβ1 +3 Jβ2 primers, tube B with 23 Vβ + 4 Jβ2 primers and tube C with 2 Dβ + 13 Jβ primers, InVivoScribe Technologies) were employed. PCR amplifications was carried out in 25 µl volume reactions containing 200-300 ng DNA, 10 pmol of each primers, 200 µM dNTP, PCR buffer (IVS), 1.5 mM $MgCl_2$, 2U Taq gold (Applied Biosystems). PCR was carried out using thermal cycler conditions 95 °C 7 min, 35 × (95 °C 45 sec, 60 °C 45 sec, 72 °C 90 sec), 72 °C 10 min.

PCR products were cloned prior to sequencing. Gel purified PCR products were cloned using TOPO TA Cloning Kit® (Invitrogen)[12] as described by the manufacturer, followed by transformation into chemically competent *Escherichia coli* (TOP 10). After incubation the

mix was spread out on Luria-Bertani + agar plates (1% Tryptone, 0.5 % yeast extract, 1 % NaCl, pH 7,0, provided by Medieberedningen at Rudbecklaboratory) supplemented with 2.5 µg/ml kanamycin (Sigma), using a sterile loop. Plates were incubated over night and at least 15 colonies were picked the day after using a sterile loop.

Cloning PCR was performed in 50 µl containing, 50 mM $MgCl_2$, 10 mM dNTPs, 5 U Taq (Invitrogen), ddH_20, 10 X PCR Rxn Buffer 5 mM (Invitrogen), M13 forward primer, 5 mM M13 reverse primer (Table 8) and one bacterial colony. PCR was carried out using thermal cycler program 95 °C 2 min, 40 × (94 °C 30 sec, 55 °C 1 min, 72 °C 1 min), 72 °C 7 min. These PCR products were purified using 20,0 U Exonuclease I (Exo) and 1,0 U Shrimp Alkaline Phospahase (SAP) in a thermocycler at 37 °C for 30 min followed by 85 °C for 15 min.

The amplicons were sequenced with the BigDye® Terminator Cycle Sequencing Reaction Kit[3] (Applied Biosystems, Foster City, CA) in both directions using the same forward and reverse M13 primers (Table 8).

PCR products that indicated clonality were also sequenced directly, without cloning. For one 10 µl sequencing reaction BigDye® Terminator v3.1 Cycle, BigDye® Terminator v3.1 Cycle 5 X Sequencing Buffer, ddH_20, 5 mM M13 forward primer or 5 mM M13 reverse primer and PCR-product were included. The PCR program was carried out using the thermal cycler program 95 °C 2 min, 40 × (94 °C 30 sec, 55 °C 1 min, 72 °C 1 min), 72 °C 7 min.

The sequencing reaction was followed by standard DNA precipitation protocol. The pellets were dissolved in HiDi Formamide followed by the analysis using an automated DNA sequencer (Applied Biosystems 3730xl, Foster City, CA). Sequences were aligned and analyzed using Vector NTI® 10 software and subsequently sent to the IMGT (http://imgt.cines.fr/IMGT_vquest/share/textes/) and database NCBI-BLAST (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen.cgi?taxid=9606) to verify the sequence. Sequences from each clone were aligned and compared to each other using BioEdit version 7.0.0. A clonal rearrangement was confirmed with a minimum of 3 identical sequences.

4.9 Gel electrophoresis

PCR products were analyzed to a 100 bp molecular size ladder (Invitrogen), on 2 % agarose gel in 1 x Tris-Borate- EDTA buffer (TBE; 1mM EDTA, 5 mM Boric acid, 40 mM TRIS, pH 8.3, provided by Medieberedningen at Rudbecklaboratory) with 0.01 µg/ml ethidium bromide at 100 V.

DNA was purified from agarose gels using QIAquick® GelExtraction Kit (Qiagen)[22].

4.10 Heteroduplex analysis

Heteroduplex analysis was performed by heating the PCR-products for 5 min in 95 °C in order to denature the DNA duplex followed by rapidly cooling down the PCR-products for 1 h in 4° C, to renature the DNA-strands. Samples were separated on 12.5 % polyacrylamide gels (GeneGel ™ Excel Kit[8], GE Healthcare) run on a GenePhor ™ Electrophoresis Unit. The running conditions were 600 V, 25 mA and 15 W at 15 °C for 100 min. Following separation gels were silver-stained using Hoefer Automated Gel Stainer according to the manufacturer´s instructions (Pharmacia LKB Biotechnology).

4.11 Fragment analysis

PCR products amplified using Biomed-2 primers labeled with fluorescent dyes FAM-6 and HEX were separated on 2 % agarose gels and subsequently diluted in ddH_20 1:20 times and 1:10, as determined by the amount of PCR product visualized on the agarose gel. Diluted

PCR products from TCRγ and TCRβ were added to a 96 well plate and sent to Uppsala Genome Center for fragment analysis, which was carried out on an ABI3700 genetic analyser (Applied Biosystems). The results were analyzed using GeneMapper Software v 3.7 (Applied Biosystems).

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