Detection of mutation status of $\text{IgV}_H$ genes and minimal residual disease in chronic lymphocytic leukemia

Dissertation Thesis

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Preface

The aim of this work was to introduce the detection of hypermutation status of IgV\textsubscript{H} genes in chronic lymphocytic leukemia in the Czech republic, as at the time of working on this project, there was no laboratory engaged in this type of molecular investigation. Although some time has elapsed since then, nothing has changed on the fact that mutation status of IgV\textsubscript{H} genes is one of the most important independent prognostic factors in chronic lymphocytic leukemia.

Moreover, we have extended our work to the investigation of minimal residual disease in chronic lymphocytic leukemia, which at the time of starting with the project was only experimental. Nowadays, both of the investigations (detection of the hypermutation status of IgV\textsubscript{H} genes, as well as the detection of minimal residual disease) have been fully implemented in the clinical practice and help the hematologists in their clinical decision-making.

1. Introduction:

1.1. The biology of the B-cell:

The development of a B-cell starts in the bone marrow, after a committed pluripotent hematopoietic cell had “decided” to follow the fate of the lymphocytic lineage. The process of the decision is believed to be completely stochastic, influenced by many humoral factors affecting the recipient pluripotent cell. Upon starting, the developmental program of a B-cell switches on many events that end up in an irreversible change in the genome, caused by rearrangement of V, D, J and C subgenes.
**Figure 1:** Human V-region genes shuffled by gene rearrangement to generate the single heavy chain specificity characteristics of each B-cell. The VH genes can be grouped into seven different families based upon sequence homology of 80%, with VH3 being by far the largest family. Individual members of a family are interspersed throughout the locus, i.e. there is no significant grouping together of VH family genes. The additional D segment minigenes are present in the heavy chain locus (adopted from Essential Immunology, Roitt, I.M. et Delves, PJ.)

In general, there are more than 50 V (variable), 25 D (diversity), 6 J (joining) and 9 C (constant) subgenes, which present an array for very large combinatorial possibilities. Each cell uses only one subgene from the many V, D, J and C subgenes.

The choice about the particular subgene seems to be completely random again. By excision and ligation of the V, D, J and C genomic segments by specific protein machinery, the pre-B lymphocyte’s genome is created. To put even more diversity into the immunoglobulin rearrangement, at the boundaries between V and D, and D and J, completely random nucleotides are added. This task is accomplished by the terminal deoxynucleotidyl transferase (TdT), which is a unique enzyme capable of adding nucleotides to the nascent
DNA strand in a template-independent manner. In reality it means that at the boundary between the V and the D segment, and the D and J segment, cell-unique DNA stretches emerge. This non-template addition of nucleotides enlarges enormously the diversity of the already vast family of immunoglobulin genes.

**Figure 2:** The joining of V, D and J segments. Joining is masterminded by the recombination activating genes RAG-1 and RAG-2, the products of which cleave the DNA at the signal ends. RAG-1 and RAG-2 together produce several thousand times more efficient VDJ recombination than either alone. The introns adjoining the V, D and J gene segments contain specialized recombination signal sequences (RSSs), which include conserved heptamers and nonamers separated by spacers of either 12 or 23 base pairs. The two joining segments, in this example VL and JL, are brought into proximity by interaction between their respective RSSs mediated by the DNA bending and looping high mobility group-1 and –2 proteins (HMG-1 and HMG-2). RAG-1 and RAG-2 cleave the DNA to produce double-strand breaks at the border of the RSS. The excised signal sequences are ligated to form the signal joint.
resulting in a piece of circular DNA containing the excised sequences. This is probably maintained in the cell for a period of time before eventually being lost from the cell. The double strands of each coding segment form “hairpin” ends. The enzyme Ku (a dimer of Ku70 and Ku86) binds to the DNA ends and stimulates DNA-dependent protein kinase (DNA-PK), which facilitates the opening of the hairpin. Terminal deoxynucleotidyl transferase (TdT) adds nucleotides to the ends of the DNA strands in order to generate N-region diversity. Unlike the precision of the signal joint, the coding joint is variable because it can involve the addition of base pairs resulting from both the resolution of the hairpin loop (P-elements) and the TdT-mediated N-region diversity. Nucleases remove any excess nucleotides and polymerases fill in any gaps before the DNA ligase IV and XRCC4 enzymes carry out ligation of the two sequences. Since the coding elements are joined at random with respect to the reading frames, two out of three events have the coding elements out of frame. Although apparently wasteful, this is evolutionarily tolerated because it confers so much benefit in the form of antigen receptor diversity. VDJ recombination products define the major antigen-binding domains. (Adopted from Essential Immunology, Roitt, I.M. et Delves, PJ.)

The B-lymphocyte, which has had successfully rearranged its V, D, J and C subgenes, at this point resides in the bone marrow. Here, the first incomplete IgVH chain is produced and the immunoglobulin is guided to the cell membrane, where it becomes a transmembrane protein. This immature immunoglobulin reacts with a ligand (antigen), which is presented by an antigen-presenting cell (APC). Upon a productive interaction between the immature immunoglobulin and the ligand, the immunoglobulin is conformationally activated and can convey positive downstream signals into the cell.

The cell reacts by methylation of the promoter sequences of the other, yet non-transcribed allele of the immunoglobulin gene. As the result, under normal circumstances, one B-cell
produces only one type of immunoglobulin. This process is called “allelic exclusion” and is possible to happen only if the first allele had been rearranged successfully, i.e. in frame, no stop codons, no deletions.

After the other allele had been rendered non-functional, the B-cell starts to produce mature immunoglobulin. Still in the bone marrow, by the APC cells, the cell is presented auto-antigens, which are peptide fragments of proteins to be encountered in the body. For the B-cell to survive, non-reactivity with the body-self antigens is absolutely essential. If the B-cell answers to the challenge by auto-antigens, it means it does not pass the “auto-immunity” test, and it receives a strong negative signal urging the particular B-cell to die by apoptosis.

The survivors traverse to the secondary lymphatic organ (spleen, lymphatic node), where they undergo a process of so called “affinity maturation”. During this process, APC cells present the B-cells with specific antigens, but here the situation is different. The APC cells challenge the B-cells with foreign antigens that should be cleared off the body. If a particular B-lymphocyte interacts positively with the presented antigen, a process of so-called “somatic hypermutation” is fired off, which means that some additional nucleotide substitutions are created throughout the stretch of the immunoglobulin gene. The rationale behind this process is to modulate the immunoglobulin response to the presented antigen to its highest level possible. Somatic hypermutation is a very unique process in the nature; based on a protein-protein interaction, nucleotide substitutions are embedded in the genome.

The somatic hypermutation affects preferentially the variable region of the immunoglobulin gene heavy chain. The process is completely dependent on the activity and precise location of promoter and enhancer regulatory sequences of the IgV_H gene. It has been shown that shifts or deletions within these regulatory sequences might prevent the somatic hypermutation from occurring.
1.2. From the B-cell to chronic lymphocytic leukemia:

1.2.1. Disease description

Chronic lymphocytic leukemia (CLL), a malignant disorder of the B-lymphoid lineage, is the most frequent type of leukemia in the Western world and affects mainly elderly individuals. Nevertheless, about 30% of patients are less than 60 years at diagnosis[1]. CLL follows an extremely variable clinical course with overall survival times ranging from months to decades[2-4]. Some patients have no or minimal symptoms during their entire disease course and have survival times similar to age-matched controls. Other patients experience rapidly deteriorating blood counts and organomegaly and suffer from symptoms very soon, thus necessitating therapy[5-7]. Novel therapeutic modalities, such as purine analogues, monoclonal antibodies and autologous or allogeneic stem cell transplantation (SCT) are highly effective and some of them potentially curative[8, 9]. Nevertheless, given the therapy-related morbidity and mortality, it has become highly desirable to define those CLL patients who might profit from an aggressive treatment. Thus, thorough stratification and precise risk-factor assessment within the heterogeneous group of CLL patients is one of the major goals in CLL management[8, 10].

1.2.2. Staging in CLL

The standard clinical procedures to estimate prognosis are the clinical staging systems developed by Rai at al. and Binet et al[11, 12]. These systems define early (Rai 0, Binet A), intermediate (Rai I/II, Binet B) and advanced (Rai III/IV, Binet C) stage disease with median estimated survival times of more than 10, 5-7, and 1-3 years, respectively. However, there is a
marked heterogeneity in the course of the disease among individual patients within a single stage group. Importantly, the clinical staging systems do not allow to predict the probability of disease progression in an individual patient. As more than 80% of patients are diagnosed early, it is necessary to identify markers that may help to refine outcome prediction for these individuals[8, 13].

1.2.3. Prognostic markers in CLL

There has been an intensive work on clinical and biological factors of potential prognostic relevance that may add to the classic assessment provided by the staging system. Among these are i) clinical patient characteristics such as age, gender and performance status ii) laboratory parameters reflecting the tumor burden or disease activity such as lymphocyte count, lactate dehydrogenase (LDH) elevation, bone marrow infiltration pattern or lymphocyte doubling time (DLT)[14-16] iii) serum markers such as soluble CD23, β2-microglobulin (β2-MG) or thymidine kinase (TK)[17], and iv) genetic markers of tumor cells such as genomic aberrations, gene abnormalities (p53 and ATM), mutation status of the variable segments of immunoglobulin heavy chain genes (VH) or surrogate markers for these factors (CD38, ZAP-70, LPL/ADAM19)[3, 14, 18-25]. Recent research has moved toward a molecular genetics that may not only provide insight into the biology and the transforming events but may also define mechanisms directly responsible for the clinical behavior of the disease with regard to disease progression, response to treatment and overall survival.
1.2.3.1. Mutation status of IgVH genes

One of the most important molecular genetic parameters defining pathogenic and prognostic subgroups of CLL is the hypermutation status of IgVH genes[3, 18]. According to the degree of hypermutation, the heterogeneous B-CLL entity was separated into two distinct subgroups: one with unmutated IgVH genes, assumed to originate from pregerminal center cells, and the other with mutated VH genes, thought to originate from postgerminal center cells. However, genome-wide gene expression profiling studies revealed a surprisingly homogeneous pattern of gene expression in both subtypes of B-CLL, closely resembling postgerminal, memory cells[26, 27], with only a limited set of genes being differentially expressed in the B-CLL subgroups. Most importantly, it has been repeatedly shown that the VH mutation status is clinically highly relevant[3, 18]. While CLL with unmutated VH shows an unfavorable course with a rapid progression, CLL with mutated VH often showed slow progression and long survival.

![Graph A](image1.png)

**Figure 1.** Probability of survival from the date of diagnosis among patients with mutated (VH homology < 98%) and unmutated (VH homology ≥ 98%) VH status.  
A) The estimated median survival time for the VH homology ≥ 98 and < 98% groups were 79 months and 152 months, respectively.  
B) When only patients diagnosed at Binet stage A were evaluated the estimated median survival times for the VH homology ≥ 98% and VH homology < 98% groups were 75 months vs. 152 months.

Byrd JC et al., Hematology 2004
Furthermore, and independent of the mutation status, the usage of specific VH genes such as VH3-21 may be associated with an inferior outcome[28-30].

1.2.3.2. Surrogate markers for IgVH status

To make the estimation of prognosis based on the VH status accessible to the routine hematology laboratory, surrogate markers for the VH status were sought after. Originally, a correlation was observed between the VH mutation status and CD38 expression of the CLL cells pointing to CD38 expression as a prognostic marker[18]. Based on genome-wide gene expression studies, other surrogate markers such as ZAP-70 expression were identified and validated[22]. ZAP-70 expression appears to strongly correlate with the IgVH mutation status and was therefore a strong prognostic marker in a pivotal study. However, for both CD38 and ZAP-70, subsequent studies have yielded controversial results with regard to their validity as surrogate marker for VH as a prognostic indicator[14, 22, 23, 31, 32].

1.2.3.3. Genomic aberrations in CLL

Genomic aberrations are the other genetic parameter shown to be of pathogenic and clinical relevance in CLL. Genomic aberrations can be identified in about 80% of CLL cases by fluorescence in situ hybridization (FISH)[19]. Genomic aberrations provide insight into the pathogenesis of the disease since they point to loci of candidate genes (17p13: p53, 11q22-q23: ATM) and identify subgroups of patients with distinct clinical features. Specific genomic aberrations have been associated with disease characteristics such as marked lymphadenopathy (11q deletion) and resistance to treatment (17p deletion)[19].
The observation that the rate of disease progression is associated with genomic aberrations and unmutated IgVH status indicates that these factors may determine the course of the disease. The fact that overall survival was inferior for the subgroups with unmutated IgVH, 11q-, or 17p-, suggests that response to therapy may be different in genetic subgroups[19-21]. In particular, the deletion 17p- and -possibly- abnormalities of the p53 gene involved in this aberration have been associated with the failure of treatment with alkylating agents, purine analogs and rituximab[33, 34]. An interphase FISH study also showed that patients whose leukemic cells showed a 17p- deletion had significantly shorter survival times than patients without this aberration, and a relationship was found between the deletion and the response to treatment. Similarly, the monoclonal anti-CD20 antibody rituximab did not show efficacy in B-CLL with p53 deletion[35]. In contrast, it has been reported that durable therapeutic success can be achieved in B-CLL with 17p- and/or p53 mutations using the monoclonal anti-CD52 antibody alemtuzumab[36-38].

In order to further improve our understanding of molecular pathogenesis and clinical outcome prediction in CLL, microarray platforms have been developed as tools to evaluate genome wide parameters and defects. On the genomic level matrix CGH (comparative genomic hybridization against the matrix of defined DNA fragments) is a sensitive test allowing the detection of novel recurrent aberrations of potential pathogenic and prognostic importance[39]. On the level of gene expression, comprehensive profiling studies of CLL based on DNA chip technology have indicated that the global gene expression “signature” of VH mutated and unmutated CLL is very similar (resembles experienced memory cells) and that only the expression of a small number of genes discriminates between the two groups[26, 27, 40]. In addition to the characterization of expression signatures associated with the VH mutation subgroups of CLL a study of 100 CLL samples characterized for VH status and
genomic aberrations described a significant number of differentially expressed genes clustering in chromosomal regions affected by the respective genomic losses or gains[41]. Deletions affecting chromosome bands 11q22-q23 and 17p13 led to a reduced expression of the genes in the corresponding genomic region, such as ATM and p53, while trisomy 12 resulted in the upregulation of genes mapping to chromosome arm 12q. The finding that the most significantly differentially expressed genes were located in the corresponding aberrant chromosomal regions suggests that a gene dosage effect may exert a pathogenic role in CLL.

Analysis and clinical outcome prediction in CLL, microarray platforms have been developed as tools to evaluate genome wide parameters and defects. On the genomic level matrix CGH (comparative genomic hybridization against the matrix of defined DNA fragments) is a sensitive test allowing the detection of novel recurrent aberrations of potential pathogenic and prognostic importance[39]. On the level of gene expression, comprehensive profiling studies of CLL based on DNA chip technology have indicated that the global gene expression “signature” of VH mutated and unmutated CLL is very similar (resembles experienced memory cells) and that only the expression of a small number of genes discriminates between the two groups[26, 27, 40]. In addition to the characterization of expression signatures associated with the VH mutation subgroups of CLL a study of 100 CLL samples characterized for VH status and genomic aberrations described a significant number of differentially expressed genes clustering in chromosomal regions affected by the respective genomic losses or gains[41]. Deletions affecting chromosome bands 11q22-q23 and 17p13 led to a reduced expression of the genes in the corresponding genomic region, such as ATM and p53, while trisomy 12 resulted in the upregulation of genes mapping to chromosome arm 12q. The finding that the most significantly differentially expressed genes were located in the corresponding aberrant chromosomal regions suggests that a gene dosage effect may exert a pathogenic role in CLL.
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Moreover, recently a novel group of differentially expressed miRNAs has been revealed by microarray analysis. It has been shown that miRNAs might play a crucial role in CLL pathogenesis and biological behavior of the disease[42, 43].

1.2.3.4. Aberrations of the p53 gene
The observation that the rate of disease progression is associated with genomic aberrations and VH mutation status indicates that these factors may determine the course of the disease. The fact that overall survival was inferior for the subgroups with unmutated VH, 11q-, or 17p-, suggests that response to therapy may be different in genetic subgroups.

In particular, the deletion 17p- and/or abnormalities of the p53 gene involved in this aberration have been associated with the failure after treatment with alkylating agents, purine analogs and rituximab[33-35]. An interphase–FISH study also showed that patients whose leukemia cells showed a 17p- deletion had significantly shorter survival times than patients without this aberration, and a relationship was found between the deletion and the response to treatment[33]. Similarly, the monoclonal anti-CD20 antibody rituximab did not show efficacy in CLL with p53 deletion[35]. In contrast, there are anecdotal pieces of evidence that durable therapeutic success can be achieved in CLL with 17p-/p53 mutations using the monoclonal anti-CD52 antibody alemtuzumab[36].

1.2.4. Therapeutical modalities in CLL

Autologous and allogeneic stem cell transplantation (SCT) are increasingly gaining more importance as treatment options in patients with active CLL[8, 44]. These procedures may confer therapeutic benefit but are also associated with considerable toxicity and cost. The efficacy of autologous SCT relies solely on the cytotoxic therapy administered. Allogeneic SCT offers the potential additional benefit of the immune-mediated graft-versus-leukemia effect but also harbors the danger of a graft-versus-host disease. Hence, there is a need to identify prognostic factors that may help to determine whether a patient is a candidate for SCT and if an allogeneic SCT or autologous SCT should be considered.
Emerging data from prospective autologous SCT trials are demonstrating safety, improved remission rates after transplant and long survival times. In the multicenter prospective autologous SCT study of the GCLLSG3, the transplant-related mortality was 5% with a 2-year overall survival rate of 88% among 105 patients[44]. This result appears promising considering the high-risk features present in the majority of patients. However, the continuing clinical and molecular relapses observed in all series of autologous SCT in CLL are the evidence against the curative potential of the procedure in the majority of patients[45].

As compared to autologous SCT the primary therapeutic advantage of allogeneic SCT after dose-reduced conditioning is the graft-versus-leukemia effect, which may offer long-term disease control and eventual cure[8]. Indeed, a recent comparative study of minimal residual disease (MRD) as detected by consensus primer CDR3 PCR provided evidence that the graft-versus-leukemia effect is operational in CLL with unmutated IgVH genes[46]. In this study only a modest decrease in MRD levels was observed immediately after allogeneic SCT, but MRD became undetectable in 7 of 9 (78%) CLL patients with unmutated VH after discontinuing immunosuppression, chronic graft-versus-host disease or donor lymphocyte infusions[46]. After a median of 25 months, these 7 patients remain in clinical and molecular remission. In contrast, PCR negativity was achieved in only 6 of 26 (23%) control CLL cases with unmutated VH after autologous SCT after dose-reduced conditioning and was not durable[8, 46]. Therefore, allogeneic SCT appears to combine the favorable features of low transplant-related mortality with the activity of the graft-versus-leukemia effect, making the procedure a valid option when aiming at cure for high risk CLL[47].

The assessment of minimal residual disease (MRD) in CLL is becoming increasingly important in the follow-up of patients undergoing intensive treatment[48-51]. There are commonly used options for minimal residual disease evaluation, such as flow cytometry or
standard consensus PCR. Both methodologies are suitable for a routine laboratory practice, but their detection limit is $1/10^3$ up to $1/10^4$ maximum[49].

The aim of our study described below, was to introduce the methodology of IgVH mutation status detection in our CLL patients. A special emphasis was put to increase the sensitivity of the available assays, as their overall rate of positively identified IgVH clones did not surpass 70-80%. Moreover, we extended our work to the detection of minimal residual disease in CLL patients undergoing treatment, as there is increasing evidence that not only qualitative, but rather quantitative information about the patients’ disease course might be of clinical importance. Using clone-specific primers and real-time PCR technology it is feasible to increase the specificity and sensitivity of MRD detection to that extent, that it is possible to identify one malignant cell in one million of normal cells.

Using this molecular approach, we are able to monitor the clone-specific IgVH expression with a far higher specificity and sensitivity, which provides the clinician with a valuable piece of feedback information regarding the therapy response. Moreover, this methodology might also allow for predicting a molecular relapse prior to an overt clinical manifestation.

At present, the knowledge on what level of MRD is clinically relevant in CLL is far from being complete. As such, MRD investigation remains a great clinical and laboratory challenge. A lot of experimental work and clinical data is needed to shed some light on this definitely intriguing issue.

2. Results
Herein I present our paper that resulted from our work on the detection of IgVH mutation status and minimal residual disease investigation in CLL. The paper has been accepted for publication in the journal Molecular Diagnosis (impact factor 2.532).

Title of the article:

Touch-down RT-PCR detection of IgVH rearrangement and Sybr-Green-based real-time RT-PCR quantitation of minimal residual disease in patients with chronic lymphocytic leukemia

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Abstract

Background: Patients with chronic lymphocytic leukemia relapse even after aggressive therapies and autografts. We assume that to prevent relapses, the level of minimal residual disease (MRD) should be minimized as much as possible. To evaluate MRD, highly sensitive quantitative assays are needed.

Methods: For diagnostic identification of IgVH rearrangement(s) (as a prerequisite for MRD detection), touch-down RT-PCR using degenerate primers was used. For the quantitative MRD detection in 18 patients, we have employed a real-time RT-PCR assay (RQ-PCR) making use of patient-specific primers and the cost-saving Sybr-Green reporter dye (SG). For precise calibration of RQ-PCR, patient-specific IgVH sequences were cloned.

Results: Touch-down RT-PCR with degenerate primers allowed successful detection of IgVH clonal rearrangement(s) in 252/257 (98.1%) diagnostic samples. Biallelic rearrangements were found in 27/252 (10.7%) cases. Degenerate primers used for identification of clonal expansion at diagnosis were not sensitive enough for MRD detection. In contrast, our RQ-PCR assay using patient-specific primers and SG reached the sensitivity of 10^{-6}. We demonstrated MRD in each patient tested, including 4/4 patients in complete remission following autologous hematopoietic stem cell transplantation (HSCT) and 3/3 following allogeneic "mini"-HSCT. Increments in MRD might herald relapse. Aggressive chemotherapy could induce molecular remission.

Conclusions: Our touch-down RT-PCR has higher efficiency to detect clonal IgVH rearrangements including the biallelic ones. MRD quantitation of IgVH expression using SG-based RQ-PCR represents a highly specific, sensitive and economical alternative to quantitative methods known to date.
Introduction

Until recently, treatment of patients with chronic lymphocytic leukemia (CLL) has been focused mainly on palliative management of various symptoms accompanying the disease.[1] At present, novel therapeutic modalities, such as purine analog chemotherapy, monoclonal antibodies and hematopoietic stem cell transplants (HSCT) allow more effective intervention, leading to high percentages of obtaining complete remission and possibly even cure.[2-7] CLL patients may experience quite divergent fates: CLL may present a smoldering disease not affecting survival on the one hand, or it may take an aggressive course on the other.[2,8] To distinguish between them, numerous prognostic tools in CLL have been employed.[2,3,9,10] Chromosomal aberrations (detected by FISH analyses) and recently, mutational status of $\text{IgV}_\mu$ genes (detected by PCR and sequencing), have been considered the most powerful independent prognostic markers.[11,14-17] The hypermutation degree of $\text{IgV}_\mu$ genes (mutated vs unmutated) divides the patients into favorable or unfavorable risk groups, respectively.[12,13,14] Importantly, the above described prognostic tools are operative even at the initial clinical stages of CLL,[11,15-17] such as Rai 0-1 or Binet A.[10,15] However, the widely used PCR techniques to detect $\text{IgV}$ gene rearrangements (carried out on DNA level) are sensitive enough only to identify the leukemic B cell clone in 66 up to 90% of CLL patients.[18-20]

Younger patients with poor risk CLL may be candidates for more aggressive treatment including HSCT.[5,6,21] The major pitfall of HSCT lies in the occurrence of clinical relapses. This holds true especially for the autologous setting. In the study of Ritgen et al., virtually all patients having unmutated $\text{IgV}_\mu$ genes relapsed clinically or molecularly within 4 years after autologous HSCT.[23] Facing the problem of relapses[14,23], it may only be logical to consider
the following: 1. to treat efficiently the minimal residual disease (MRD), 2. to avoid reinfusion of residual leukemic cells in the setting of autologous HSCT and to assess the MRD status before collecting the autograft and then in the autograft itself. For both tasks, precise detection of MRD seems to be mandatory. Thus, minimal residual tumor burden estimation presents a great laboratory challenge in CLL. As mere qualitative information about patient’s MRD status (positivity or negativit) might not be relevant and satisfying anymore,[14,22,24,25] quantitative analyses are given prominence in the clinical follow-up of CLL patients. Different laboratories use different approaches to this issue. The preferred methodology for MRD evaluation in CLL makes use of fluorescently labeled hybridization probes and quantitative real-time PCR technology (RQ-PCR).[24,25] Although hybridization probes are of high convenience and reliability for MRD detection in leukemias distinguished by characteristic chromosomal translocations,[26-28] they might be of limited use for MRD detection in CLL due to the pronounced target sequence heterogeneity. Other approaches in MRD detection in CLL are based on Genescan or immunophenotypic analyses.[29,30] Both of them are relatively inexpensive, high-throughput, but in comparison to PCR-based technologies, they are of limited sensitivity and specificity.[29]

Although the clinical value of quantitative MRD detection remains to be established in further studies, a real effort is being devoted to the management of technical aspects concerning MRD quantitative detection in CLL. As a prerequisite for further MRD detection, we have employed a PCR strategy, which combines the touch-down RT-PCR with the usage of degenerate primers. To improve the applicability and cost-effectiveness of the currently used methods for MRD detection in CLL,[19,24,25] we have used an approach that is based on the quantitative RQ-PCR detection of clone-specific \( \text{IgV}_{\text{H}} \) transcripts using patient-specific primers, molecular cloning of patient-specific \( \text{IgV}_{\text{H}} \) sequences, and, importantly, on
employing Sybr-Green intercalating dye in the quantitative PCR reaction. Our method meets all the above-mentioned criteria for a reasonable approach.

Materials and methods

Patients

We have studied 257 patients with CLL (96 females and 161 males) diagnosed according to the NCI criteria.\cite{31} Peripheral blood and/or bone marrow samples were taken after informed consent was obtained. Patients at varying stages of the disease, both treated and untreated, were included. For the MRD studies, 18 patients (all of them being candidates for aggressive therapy and HSCT) were recruited. The cohort comprised 17 males and 1 female, median age 53.5 (43–66) yrs. Clinical data of patients with a longer follow-up are summarized in Table 1.

As normal controls, peripheral blood samples from healthy volunteers were employed.

Table 1

<table>
<thead>
<tr>
<th>Patient Nr.</th>
<th>Date of diagnosis</th>
<th>Age at diagnosis (yrs)</th>
<th>Sex</th>
<th>Rai stage at diagnosis</th>
<th>Time F(^{-}) (date)</th>
<th>Rai stage at time F(^{+})</th>
<th>FISH(^{t})</th>
<th>IgV(_{H}) hypermutation (%)</th>
<th>IgV(_{H}) subfamily</th>
<th>CD38(^{+})</th>
<th>Therapy preceding to time F(^{+})</th>
<th>Therapy following time F(^{+})</th>
<th>Source of cells</th>
<th>Cond</th>
<th>Overall survival (mos) as of IX/03</th>
<th>Current status</th>
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<td>VIII/01</td>
<td>49</td>
<td>M</td>
<td>1</td>
<td>VIII/01</td>
<td>1</td>
<td>normal</td>
<td>0</td>
<td>1-69</td>
<td>0</td>
<td>none</td>
<td>F, RTX</td>
<td></td>
<td></td>
<td>25+</td>
<td>PR</td>
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<td>2</td>
<td>VI/99</td>
<td>46</td>
<td>F</td>
<td>0</td>
<td>XI/01</td>
<td>2(^{n})</td>
<td>17p- (3%)</td>
<td>4.5</td>
<td>3-30</td>
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<td>CLB, COP</td>
<td>FC</td>
<td>allo-HSCT</td>
<td>PB</td>
<td>F+Bu+ATG</td>
<td>51+</td>
</tr>
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<td>allo-HSCT at relapse</td>
<td>PB</td>
<td>F+Cy</td>
<td>46+</td>
<td>CR</td>
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Isolation of RNA

Mononuclear cells from CLL bone marrow or peripheral blood samples were separated using Ficoll-Paque (Sigma) density gradient. 5x10^6 cells were lysed in TriZol reagent (Gibco BRL). Both RNA and DNA were isolated according to the manufacturer’s recommendations.

cDNA preparation

Reverse transcription was carried out as follows: 25 pmol of random hexamers (Perkin-Elmer) were incubated with 1 μg of total RNA at 65 °C for 10 min. After denaturation, the sample was cooled down to 4 °C and the master mix consisting of 200 μM dNTPs (Promega), 10 mM dithiothreitol (Gibco BRL), 1x first strand buffer (Gibco BRL), 10 U RNasin (Promega), 100 U Superscript II (Gibco BRL) and sterile water to a final volume of 10 μl was added. The reaction mixture was incubated for 1.5 hr at 42 °C. As the final step, reaction mixture was heated to 94 °C for 2 min in order to terminate the reverse transcription. cDNA samples were stored at –20 °C.
Identification of IgV_H family expression using touch-down RT-PCR and degenerate primers

Seven IgV_H families were identified using primers described elsewhere, with the exception for primer VH1. Its sequence has been slightly modified to match better its recognition site (Table 2). The seven IgV_H families were amplified in six individual PCRs, with VH1 and VH7 families being amplified by the same set of primers. Using the PTC-200 thermal cycler (MJ-Research), touchdown RT-PCR was carried out in a final volume of 25 µl in the reaction mixture consisting of 50 pmol of forward and reverse primer (Invitrogen), 200 µM dNTPs, 1x PCR reaction buffer (Perkin-Elmer), 2 mM MgCl₂ and 1 U AmpliTaq Gold polymerase (Perkin-Elmer). The touch-down PCR amplification program was designed as follows: initial denaturation at 94 °C for 5 min and subsequent cycling: in each step, 15 sec denaturation at 94 °C and polymerization at 72 °C for 30 sec, with annealing steps in between, ramping down from 65 °C to 50 °C, with a ramping rate 1 °C/1 step. At 50 °C, additional 30 cycles with profile 94 °C, 15 sec; 50 °C, 30 sec; 72 °C 30 sec were added. PCR products were separated on 2% agarose stained with ethidium bromide.

Table 2
Sequences of the primers and the abl hybridization probe
Adopted from Souto-Carneiro et al. except for primer VH1, the sequence of which was slightly modified. The abl probe was designed according to Visani et al.

Sequencing of the predominant IgV\textsubscript{H} clone(s) and establishing the mutational status of IgV\textsubscript{H} genes

Bands of approximately 300 bp (obtained as described above) were cut out, phenol : chloroform purified and directly sequenced using the Big Dye Terminator kit v. 3 and ABI Prism 310 Genetic Analyzer (Perkin-Elmer). Sequences were analyzed using the program Chromas 1.5 (Technelysium) and aligned to the nearest IgV\textsubscript{H} germline sequences using the IgV\textsubscript{H} BLAST program. The percentage of hypermutation was calculated. As recommended, a threshold of 2% was set to distinguish between “mutated” and “unmutated” IgV\textsubscript{H} sequences.

Design of patient-specific primers

For 18 patients, patient-specific primers were designed, based on the previous detection of their IgV\textsubscript{H} sequences. The forward primers were designed to match precisely either the FWR1

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<td>AAAAGCCCGGGAGTCTCTGARGA</td>
</tr>
<tr>
<td>VH6</td>
<td>ACCTGTCAGTTCTCCGGGACAGTG</td>
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<td>GGTGACCAGCTBCCYTGGCCAGC</td>
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<td>JH6</td>
<td>GGTGACCAGTGGCTCCCTGTCCAG</td>
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<tr>
<td>5’ FAM- abl- BHQ 3’</td>
<td>CCAGTAGCATCTGACTTTGAGCCTCA</td>
</tr>
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</table>
or CDR1 regions of the patient’s particular \textit{IgV} gene, preferably the most diverse stretches within these regions. The individual reverse primers were designed to be complementary to the CDR3 unique sequences, with the nontemplated “fingerprint” nucleotides matching the very 3’ end of the primer. The amplicons sized approximately 200 bp. All primers were purchased from Invitrogen.

To check the suitability of patient-specific primers for SG-based assay, control PCR was carried out. In those instances, when only one specific PCR product was observed, patient-specific primers were accepted for the SG-based assay.

**Cloning of patient-specific \textit{IgV} sequences and abl gene**

For RQ-PCR \textit{IgV} quantitation experiments, as external standards, patient-specific \textit{IgV} sequences as well as a fragment of human \textit{abl} gene were cloned. \textit{IgV} sequences were amplified using patient-specific primers as described above. The sequences of primers used for amplification of the \textit{abl} gene are listed in Table 2. PCR products were gel-purified using phenol : chloroform method.\textsuperscript{[33]} Purified products were T/A ligated into pCR2.1 vector (Invitrogen) according to the manufacturer’s recommendations. Competent cells DH5-\(\alpha\) (Invitrogen) were transformed by ligation mixture and grown on LB-plates with ampicillin overnight.

To detect positive clones harboring desired plasmid constructs, colony PCR was carried out, with each single \textit{E. coli} colony used as a template for PCR. For each construct, one positive clone was selected and grown in LB medium with ampicillin at 37 °C overnight. Plasmids were prepared using alkaline lysis followed by phenol : chloroform extraction. The
concentration and purity of plasmid preparations were determined using Beckman spectrophotometer. The correctness of all constructs was verified by sequencing.

RQ-PCR quantitation of IgVH messages in CLL samples

The RQ-PCR assay: To quantify the amount of clone-specific IgVH transcription, cDNAs from CLL patients were prepared as described above. Plasmids harboring individual patient-specific IgVH sequences were serially diluted by a factor of 10, starting from 1 ng/µl and ending at 1 pg/µl. Using the Rotor-Gene 2000 RQ-PCR cycler (Corbett Research, Mortlake, N.S.W., Australia) equipped with a software for PCR product quantitation, CLL cDNA samples as well as diluted plasmids were subjected to PCR amplification with patient-specific primers. To control the reproducibility of pipetting, sample handling and reaction chemistry on its own, each reaction was run in duplicate. To enable measurement of the increasing amount of PCR product, as a reporter dye, the dsDNA binding dye Sybr-Green (SG; Biosearch Technologies) was added to the reaction mixture. PCR master mix for RQ-PCR consisted of 5 pmol of forward and reverse patient-specific primer, 200 µM dNTPs, 1 x PCR reaction buffer, 2 mM MgCl₂, 1 : 60 000 finally diluted SG and 2 U Ampli Taq Gold Polymerase in a final volume of 20 µl. PCR amplification program was as follows: 94 °C, 5 min; 45 x (94 °C, 15 sec; 60 °C, 30 sec; 72 °C, 30 sec, fluorescence acquisition). Emitted fluorescence was measured using Rotor-Gene 2000 quantitation software.

The “threshold line” and calculation of Cₐ values: Using the Rotor-Gene 2000 quantitation software, the so-called threshold line was set up. This line was positioned to cross the plasmid amplification curves in their exponential phase, whereby each amplicon of the diluted plasmid was assigned its Cₐ value. The Cₐ value was defined as the cycle number at which exponential
amplification started. The $C_t$ value for each cDNA sample was calculated from the intersection of the threshold line and the sample curve. By plotting the $C_t$ values against dilution factors of plasmids, a calibration curve was constructed. The software-generated R-values of about 0.99 and more, characterizing the quality of plasmid calibration, were accepted for further patient-specific $\text{IgV}_H$ transcript evaluation.

**RQ-PCR for the abl housekeeping gene:** cDNA sample form each CLL patient was quantitatively analyzed for abl gene expression, as a housekeeping gene. The calibration of the assay was, in principle, carried out precisely the same as the calibration of the $\text{IgV}_H$ RQ-PCR assay described above, with the only exception that instead of SG dye a Taq-Man hybridization probe (Table 2) was used to detect the PCR product.

Master mix for RQ-PCR consisted of 5 pmol of forward and reverse abl - specific primers, 200 $\mu$M dNTPs, 1x PCR reaction buffer, 2 mM MgCl$_2$, 8 pmol of 5’JOE- 3’BHQ1 labeled abl hybridization probe (Biosearch Technologies) and 1 U Ampli Taq Gold polymerase in a final volume of 20 μl. PCR amplification program was: 94 °C, 5 min; 50x (94 °C, 15 sec; 60 °C, 30 sec, fluorescence acquisition; 72 °C, 30 sec). By setting the threshold value, each amplicon was assigned its $C_t$ value and the calibration curve was constructed.

The “relative $\text{IgV}_H$ expression”:

The relative content of $\text{IgV}_H$ transcripts in the patient’s sample assayed by RQ-PCR was given as the "relative $\text{IgV}_H$ expression." This parameter was expressed as a ratio of the results of $\text{IgV}_H$ and the housekeeping abl gene quantitations. The amount of the detected cDNA at the start of the assay inversely correlates with the $C_t$ value detected by the Rotor-Gene 2000 software. In the exponential phase of the assay, the number of transcripts doubles during each cycle. Thus, the amount of the cDNA detected at the point $C_{t+1}$ is double the amount of cDNA detected at the point $C_t$. Then, the relative $\text{IgV}_H : abl$
expression is the ratio \(2^{Ct_{\text{abl}}} : 2^{Ct_{\text{IgVH}}}, \) i.e. \(2^{(Ct_{\text{abl}} - Ct_{\text{IgVH}})}\). In samples with no effective IgV\(\text{H}\) amplification (the theoretical Ct value of which lies in infinity), the values of 0 are given.

**Statistical analyzes**

The relative frequencies of patients with mutated and unmutated IgV\(\text{H}\) genes in a particular subfamily were analyzed using the two-tailed Fisher's exact test for contingency tables (employing the GraphPad Prism 3.0 software).

**Results**

**Predominant IgV\(\text{H}\) family detection in CLL patients**

Using degenerate primers, all of the 7 IgV\(\text{H}\) families were amplified using 6 touch-down RT-PCRs for each sample. For each IgV\(\text{H}\) family tested, healthy donors gave a smear-like pattern of PCR products on the agarose gel (Figure 1a). This physiological type of expression was regarded as polyclonal. On the other hand, CLL patients, though for some families still polyclonal, were usually distinguished by a single, predominant band in one of the IgV\(\text{H}\) families tested (Figure 1b). We were able to find a clonal expansion in 252 out of 257 (98.1%) diagnostic CLL samples. In 27 out of 252 (10.7%) patients, two major bands were detected, representing biallelic (or biclonal) cell population (Figure 1c).

**Figure 1**
PCR amplification of 7 IgV<sub>H</sub> families at the cDNA level using degenerate primers. In a healthy donor (a), all IgV<sub>H</sub> families gave a smeared (polyclonal) pattern (emerging due to the uneven length of CDR3 regions within the particular family). (b) In CLL patient No. 3., a major sharp band was detected in IgV<sub>H</sub>3 family. Direct sequencing of this preponderant PCR product revealed the IgV<sub>H</sub>3-23 clonal expansion. (c) CLL patient with a biallelic disease. Sequence analysis confirmed clonality in IgV<sub>H</sub> subfamilies VH1-8 and VH4-59. 2% agarose gel electrophoresis stained with ethidium bromide.

**Bias of IgV<sub>H</sub> subfamilies to either mutated or unmutated status**

Sequencing analysis of successfully identified clonal expansion(s) in 252 CLL patients revealed that 116/252 (46%) of them had unmutated and 141/252 (56%) mutated IgV<sub>H</sub> genes. Moreover, based on the sequencing data we have found out that the usage of some IgV<sub>H</sub> subfamilies by CLL cell clones was uneven. The most preferred subfamilies found were VH4-34 (26 cases), VH3-30 (23 cases), VH1-69 (22 cases), VH3-23 (19 cases), VH5-51 (18 cases), VH3-7 (13 cases) and VH3-21 (13 cases). Furthermore, sequencing analyses of patient’s IgV<sub>H</sub> genes showed a remarkable tendency of certain IgV<sub>H</sub> subfamilies to either mutated or unmutated IgV<sub>H</sub> gene status (Table 3). CLLs with a clonal expansion of VH3-7 subfamily
were usually distinguished by mutated $\text{IgV}_H$ gene status ($P=0.0429$), whereas the majority of CLLs using $\text{IgV}_H$ subfamilies VH1-69 ($P<0.0001$) and VH5-51 ($P=0.0247$) had unmutated genes.

**Table 3**

Tendency of certain $\text{IgVH}$ subfamilies to either mutated or unmutated $\text{IgVH}$ gene status

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Qualitative detection of minimal residual disease: comparison of patient-specific primers and degenerate primers

We have designed patient-IgV\textsubscript{H}-specific primers for MRD detection in 18 CLL patients. Using these primers, we were able to demonstrate MRD in each of them (data not shown) in the course of their follow-up (see below). The PCR products gave single, specific, sharp band on agarose gel (Figure 2a). In contrast, MRD detection was obscured, when degenerate primers were used. The result was a smear-like product with no apparent specific band (Figure 2b), mimicking thus a result of a healthy donor.

**Figure 2**

Comparison of PCR amplification of IgV\textsubscript{H} sequences using patient-specific primers (a) and degenerate primers (b) for qualitative detection of MRD in a CLL patient (No. 2). (a): lane 1 - IgV\textsubscript{H} PCR amplification on cDNA using patient-specific primers; lane 2 - IgV\textsubscript{H} PCR amplification using patient-specific primers on a plasmid harboring patient-specific IgV\textsubscript{H} gene; lane 3 - negative control. (b): IgV\textsubscript{H} PCR amplification on the same cDNA (as in figure 2a – lane 1) using degenerate primers for IgV\textsubscript{H}3 family. 2% agarose gel electrophoresis stained with ethidium bromide.
Quantitative IgV\textsubscript{H} detection using RQ-PCR and Sybr-Green as the reporter dye – a model of serial dilutions

To test the applicability and sensitivity of the Sybr-Green (SG)-based RQ-PCR technique in MRD detection in CLL, we have designed a model, in which serially diluted cDNA from a CLL patient was used as a template for quantitative RQ-PCR detection of the patients' clone-specific IgV\textsubscript{H} transcript. cDNA from patient No. 4 was serially diluted into cDNA from a healthy donor by a factor of 10, starting at dilution of 10\textsuperscript{0} and ending at dilution of 10\textsuperscript{-5} (Figure 3). These diluted templates were subjected to quantitative RQ-PCR using patient-specific primers, SG dye and reaction chemistry based on Ampli Taq Gold polymerase. For Ampli Taq Gold polymerase, 1 : 60000 final SG dilution and 2 mM final Mg\textsuperscript{2+} concentration gave the best results with steep curves in RQ-PCR and single bands on agarose gel electrophoresis without any traces of non-specific products or primer dimers. In this patient with a mild absolute lymphocytosis\textsuperscript{35} (WBC 13.2 x 10\textsuperscript{9}/l with 62% of lymphocytes in peripheral blood), the sensitivity of the SG-based RQ-PCR assay was only 10\textsuperscript{-4} (Figure 4). On the other hand, the same dilution experiments performed on cDNA from a patient with leukemia with marked lymphocytosis (his WBC was 153 x 10\textsuperscript{9}/l with 83% of lymphocytes in peripheral blood) showed the limiting sensitivity of the assay being 10\textsuperscript{-6} (data not shown). Though from
practical purposes the dilution experiments were done on cDNA into cDNA diluted samples, we verified the results using cells to cells dilution assay as well (data not shown). In our hands, results obtained by both assays were precisely the same.

**Figure 3**

Sensitivity of the SG-based quantitative RQ-PCR assay for MRD detection in a CLL patient (No. 4) using patient-specific primers. Patient’s cDNA was serially diluted into cDNA from a healthy donor by a factor of 10 ($10^0$ down to $10^5$) and PCR amplified using the patient-specific primers. The insertion depicts the same products on 2% agarose gel. cDNA dilutions: Lane 1: $10^0$; lane 2: no cDNA (negative control); lanes 3 and 4: $10^1$; lanes 5 and 6: $10^2$; lanes 7 and 8: $10^3$; lanes 9 and 10: $10^4$; lane 11: $10^5$. The final dilution of $10^{-5}$ gave no product.

**Figure 4**
Quantitative MRD detection in CLL samples using SG-based RQ-PCR assay and patient-specific primers

For 18 patients (including one with a biallelic rearrangement), we have cloned their patient-specific IgV\textsubscript{H} genes, designed their patient-specific primers and confirmed them to be suitable for the SG-based quantitative RQ-PCR assay (see Materials and Methods). Since all 18 primer sets gave single, specific PCR products on agarose gel electrophoresis, they could be used for longitudinal follow-up of MRD in these patients. All of the 18 patients were high-risk: all but one (patient No. 2) had unmutated IgV\textsubscript{H} genes. Patient No. 2 had a prolymphocytic transformation of a previously typical CLL. Since the time-point when the patient-specific primers were designed, complete or partial remissions (CR or PR) with normalization of blood counts have been achieved (following various treatments) in 13 out of the 18 patients. These patients were chosen for MRD monitoring. The remaining 5 of the 18 patients did not achieve CR or PR, so that MRD monitoring was considered useless (all these patients eventually died). Using our RQ-PCR assay with patient-specific primers, we were able to demonstrate MRD in each of the 13 patients followed up for MRD. Some clinically interesting findings were noted. In 3/3 patients in CR after allogeneic "mini"-HSCT, MRD
was detected (days 43 to 1156 post-transplant). Despite this fact, they still remain in CR (patient No. 2 even in molecular remission). In 4 out of 4 patients after auto-HSCT performed 393 to 551 days before starting the molecular follow-up, MRD persisted. Each of the 4 patients later relapsed (1 of whom died). Figure 5 depicts some typical shapes of $\text{IgV}_H$ quantitation curves during the follow-up. More intensive treatments, such as FC or FCR (see Table 1) in patients, who had not been heavily pretreated, produced relatively rapid declines in MRD (Figures 5a and 5b), with a molecular remission in one case. On the other hand, monotherapy with fludarabine or rituximab produced only mild decrease in MRD quantity (Figure 5c). Figure 5d represents the typical situation in post-auto-HSCT patients: an ongoing increase in MRD heralded a clinical relapse. Another important point is that negativity in our sensitive RQ-PCR assay ($10^{-6}$ as shown in Figure 5a) did not preclude the risk of an eventual relapse. Figure 5b records the course of MRD that may be typical for allogeneic "mini"-HSCT performed after a short but intensive pretreatment: the initial decrease in the relative $\text{IgV}_H$ expression followed by MRD (irrespective of the 100% engraftment according to the chimerism studies), which disappeared only after immunosuppression with cyclosporin A was stopped.

**Figure 5**
Time-course follow-up of MRD detected using RQ-PCR with patient-specific primers and SG. (a): Achievement of molecular remission in patient No. 4 following FC and FCR chemotherapy, and a subsequent molecular relapse. (b): Decreases in the relative $\text{IgV}_H$ expression following FC chemotherapy and allogeneic "mini"-transplant in patient No. 2. Molecular remission was achieved only after discontinuing cyclosporine A immunosuppression. (c): Relatively small decreases in the relative $\text{IgV}_H$ expression in patient No. 1 following monotherapy with fludarabin, and later, after a severe hepatitis, decrease in MRD following rituximab treatment. (d): Increase in the relative $\text{IgV}_H$ expression preceding an overt relapse in a post-auto-HSCT patient No. 3.

**Discussion**
In the present study, we deal with the technical aspects concerning detection of \( \text{IgV}_{\mu} \) gene mutational status in CLL at diagnosis. Moreover, we introduce here a highly specific, sensitive, widely applicable and economical RQ-PCR method as an alternative for hybridization probes-based MRD detection in CLL.

We have studied the hypermutational pattern of \( \text{IgV}_{\mu} \) genes in 257 CLL patients. When screening for the predominant \( \text{IgV}_{\mu} \) expression at the time of diagnosis, the primers used for the detection of the 7 \( \text{IgV}_{\mu} \) families were to some extent degenerate.\(^{32}\) The usage of degenerate primers has one major advantage: although the primer site was variable, we were able to amplify the majority of \( \text{IgV}_{\mu} \) fragments. To boost the successful amplification of \( \text{IgV}_{\mu} \) families even more, we have employed the touch-down RT-PCR technique. This approach allowed amplification of target sequences even in cases of low primer complementarity, which might have been otherwise critical to a standard PCR. Moreover, when working with cDNA instead of DNA, the chance of a successful clonal detection is even more pronounced, since the amount of \( \text{IgV}_{\mu} \) mRNAs in a B-cell is abundant. As a rule of thumb, \( \text{IgV}_{\mu} \) family detection at genomic level is not so powerful; the number of successfully detected clones does not exceed 90\%.\(^{20}\) In contrast, in our hands, using RT-PCR technique we were able to detect clonal \( \text{IgV}_{\mu} \) rearrangement in 98.1\% of the patients tested. From these reasons, we strongly recommend detection of \( \text{IgV}_{\mu} \) genes at cDNA level, by employing the RT-PCR method. Moreover, we would like to stress that for MRD detection in CLL, which is even more demanding than screening for the expression of \( \text{IgV}_{\mu} \) family at the time of CLL diagnosis, the RT-PCR technique seems to be the methodology of choice.
Nevertheless, it might happen that even RT-PCR fails to amplify any specific IgV_H product: Five out of our 257 patients showed a polyclonal healthy donor-like pattern without any preponderant PCR product. The explanation for this phenomenon might be that (i) the disease was just at its beginning (or perhaps it was prevalently lymphomatous\textsuperscript{[35]}), (ii) the patient responded successively to chemotherapy and thus the clonal band was indistinguishable, or (iii) the particular IgV_H gene priming site had suboptimal primer complementarity. All of the above mentioned pitfalls might have prevented effective PCR reaction from occurring.

In accord with other groups,\textsuperscript{[13,37]} we came across several intriguing points. The first apparent feature of CLL cells was the uneven usage of IgV_H subfamilies. It seemed to be not random that some IgV_H subfamilies were found in CLL more often than others. Interestingly, some IgV_H families even showed a propensity to either mutated or unmutated IgV_H status. Using microarray technology, Klein \textit{et al.}\textsuperscript{[38]} showed that the expression profile of a CLL cell resembles very closely the expression profile of a memory B cell. In the light of Klein’s data, it is tempting to hypothesize that it might have been some inherent defect to the CLL cell that allows it to traverse the germinal center without losing its naive IgV_H gene configuration. Another interesting point was the relatively high occurrence (10.7% of cases) of CLLs with two detectable IgV_H families. It is believed that biallelic CLLs emerge due to the lack of allelic exclusion during the B-cell development.\textsuperscript{[39]} Nevertheless, it cannot be ruled out that some of the biallelic CLLs represent a truly biclonal disease. Further study is warranted to clarify this issue.

To our knowledge, available data on MRD detection in CLL refer to either qualitative analysis only (earlier papers\textsuperscript{[19,40]} or quantitative analysis using gene-specific hybridization probes, which require absolute sequence complementarity (recent publications\textsuperscript{[24,25]}). There
are reports on employing flow cytometry in CLL MRD detection as well, but this approach may not allow to reach the sensitivity and specificity needed.\cite{29,41} Since the sequence variability of IgV\textsubscript{H} genes in CLL might be extremely vast, we find designing hybridization probes for MRD detection by RQ-PCR in each individual patient with CLL cost consuming, little versatile and hence ineffective. These obstacles inspired us to address MRD detection in CLL in more economical and applicable way. Our assay is based on the fluorescent dye SG, which is distinguished by its dsDNA binding activity. SG intercalates into any double-stranded DNA independently of the nucleotide sequence of the target molecule. This promiscuous activity of SG gives ease to the RQ-PCR MRD detection in CLL, since this feature allows the methodology to be applied on each CLL patient, irrespective of the nucleotide sequence of their IgV\textsubscript{H} genes. However, it should be borne in mind that a PCR reaction without non-specific products is essential, as SG tends to intercalate into any dsDNA it encounters (primer dimers, non-specific products). These unwanted products are responsible for false increase in the measured fluorescence. However, by careful primer design and Mg\textsuperscript{2+} concentration optimization, this requirement is easy to meet.

In an RQ-PCR, there may be a great variability in the commencement of an effective PCR amplification caused by priming hindrance, when degenerate, instead of the patient-specific primers are used. Nevertheless, this serious obstacle in quantitative PCR technology is rather easy to overcome. Based on sequence analysis of the particular patient’s IgV\textsubscript{H} gene, we have designed patient-specific forward and reverse primers. The priming sites were preferably situated within the hypermutation hot spots and/or those regions of the particular IgV\textsubscript{H} gene, which were sequentially unique and could serve as the patient’s “fingerprint”. Using these sets of primers, the PCR reaction turned to be very specific and sensitive (the sensitivity being
10^6), able to capture the specific product on a background of many other subfamilies of an IgV_H family.

Various investigators use different graphical outputs of RQ-PCR quantitation, \[^{25,26,28}\] but generally, they relate the exact copy number of the gene of interest (GOI) to the exact copy number of a housekeeping gene (HG). As the ratio of the exact copy number of GOI and HG is a figure without any unit of measurement, we did not bother with calculations of exact copy numbers of plasmids used for calibrations of our RQ-PCR assays. Instead, we used nanograms down to picograms of plasmids to calibrate the assay. This approach is not only more practical, but it is very precise as well, as the concentration of each of the constructs was measured spectrophotometrically.

In our study, we used the so-called “relative IgV_H expression”, showing the ratio of C, IgV_H and the housekeeping abl gene. There is a common consent that quantifying the amount of GOI without quantitation of HG might be misleading. Moreover, when detecting IgV_H transcript at cDNA level, there is no clear relationship between transcript copy number and the cell number. Thus, we suggest that it is the dynamics of the quantitatively detected MRD level (the trend of MRD) that might provide the clinician with relevant information.

From the clinical point of view, our study using a highly sensitive test for MRD detection has revealed several interesting observations: 1. Following auto-HSCT, MRD could be consistently demonstrated in each patient (in spite of being considered "negative" in another laboratory, which employed the same primers for IgV_H family identification at diagnosis as well as for MRD detection). All these 4 patients had an unmutated IgV_H gene status, and have already relapsed – similar to the observations of Ritgen et al.\[^{23}\] 2. MRD apparent after the nonmyeloablative allo-HSCT\[^{36}\] in one CLL patient disappeared only after cessation of
immunosuppression. 3. The combination of fludarabine-cyclophosphamide-rituximab may induce a molecular remission even at a high sensitivity MRD detection level. 4. Increments in the relative IgVH expression may herald clinical relapse.

Due to the rapid development of molecular biology, we have all the tools necessary for MRD detection and quantitation in CLL. However, the clinical impact of the precise estimation of MRD is yet to be established in larger studies. We believe, anyway, that in the future, precise MRD detection and quantitative evaluation in CLL patients will lead to tailored treatment\[^{[5]}\] of the disease.

References


3. Conclusion

The thesis contains four publications. The results described here in the most detail deal with the project aimed at the detection of hypermutation status of IgVH genes in chronic lymphocytic leukemia and molecular monitoring of minimal residual disease.

The methodology presented here has been successfully launched into a routine clinical practice and to our greatest pleasure the number of CLL patients investigated using our methods is still growing.

4. References


