UNIVERZITA KARLOVA V PRAZE

1. LÉKAŘSKÁ FAKULTA

1. INTERNÍ KLINIKA- KLINIKA HEMATOLOGIE
VŠEOBECNÉ FAKULTNÍ NEMOCNICE V PRAZE

Clinical implications of minimal residual disease evaluation by polymerase chain reaction in patients with B- cell lymphoproliferative disorders

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Praha 2008

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I hereby declare that I was working independently using the cited literature under tutorial guidance of my consultants and mentor.

I would like to thank especially to Associate Prof. Marek Trněný, MD, PhD and to RNDr. Alena Slavíčková, PhD for their great help and tremendous support. My gratitude belongs also to all the members of hematology and molecular biology laboratories at the 1st Department of Medicine, Charles University General Hospital, Prague.

I should thank to Assistant Prof. MUDr. Anna Křepelová, MD, PhD and her laboratory at The Institute of Biology, 1st Medical Faculty Charles University, Prague for their help with sequencing.

This work would be impossible without cooperation, referral of patients and diagnostic material from clinicians, namely: Associate Prof. Marek Trněný, MD, PhD; Assistant Prof. Jana Šálková, MD, PhD; Assistant Prof. Veronika Valková, MD, PhD; Assistant Prof. Robert Pytlík, MD; Assistant Prof. Josef Karban, MD and many others.

I would like to express my gratitude to my wife, my parents and my sister for their endless support during my studies and also sacrifices that they had to undergo with me.

Finally, I would like to thank to all my patients who have thought me the great art of Medicine through their victories over lymphoma or leukemia as well as their sorrows.

This doctoral work was supported by following grants: IGA MZ ČR 4822
GA UK 15/2001/C/1. LF
CEZ: J13/98:1111/00004

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1. INTRODUCTION

B- cell lymphoproliferative disorders are defined as clonal proliferations of B-lymphocytes arrested at a specific stage of differentiation. These tumors are characterized by a combination of clinical, morphological, and immunophenotypic features as well as distinctive genetic abnormalities. Despite the fact that molecular pathogenesis of B- cell lymphoproliferative disorders remains mostly unknown there are many known genetic aberrations. Some of these abnormalities such as chromosomal translocations are associated with a particular pathological entity; others include e.g. clonal immunoglobulin heavy chain gene rearrangement. These clonal markers can be detected by modern techniques of molecular biology. The most sensitive method represents polymerase chain reaction (PCR). Introduction of PCR methods into the process of initial diagnosis leads to fast and precise diagnosis, which in turn has important impact on further clinical management. The clinical significance of minimal residual disease evaluation by PCR during the follow-up after treatment has been well established in chronic myelogenous leukemia, however this has not been the case with lymphoproliferative disorders especially non-Hodgkin's lymphomas. Complete remission can be achieved in high percentage of patients, majority of those patients relapse; post-remission management of B- cell lymphoproliferative disorders takes no account of the minimal residual disease.

My doctoral studies have been conducted in molecular biology laboratory at 1st Department of Medicine Charles University General Hospital in Prague. This institution is one of the centers of the Czech Lymphoma Study Group and has always been known for interest in lymphoproliferative disorders. Patients with various types of malignant lymphomas are referred there for diagnosis, consultation and therapy that includes conventional chemotherapy,

radiotherapy, high dose therapy with autologous stem cell rescue, and recently also immunotherapy with monoclonal antibodies. The bone marrow transplantation unit was established at 1st Department of Medicine in 1993 the doctors performed more than 500 autologous stem cell transplantations within a decade. The molecular biology laboratory specializes in molecular diagnostics and monitoring of patients with B- cell lymphoproliferative disorders.

The aims of my study were: first, to introduce the use of molecular diagnostics of B-cell lymphoproliferative disorders into a broader clinical use; second, to evaluate the clinical relevance of molecular diagnostics as well as monitoring of the disease by methods of molecular biology especially PCR; and finally to assess whether there are differences among therapeutic approaches in achievement of molecular remission and whether this in turn affects prognosis.

2. OVERVIEW OF LITERATURE

2.1. THE ROLE OF DEVELOPMENTAL STEPS OF B CELL DIFFERENTIATION IN LYMPHOMA- OR LEUKEMIAGENESIS

B- cell nonhodgkins lymphomas (NHL) together with chronic lymphocytic leukemia (CLL) represent a subset of a larger group of B- cell lymphoproliferative disorders (B-LPD) that arise from a B lymphocyte. Since the vast majority of this study is dealing with patients with different subtypes of NHL or CLL of B cell origin I will refer to those as B cell lymphoproliferative disorders.

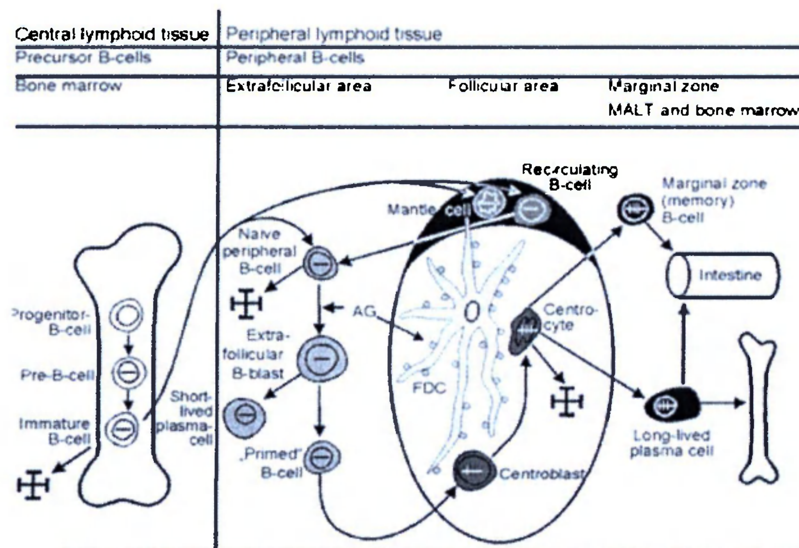
Generally lymphomas and leukemias are clonal expansions of single transformed cells from the hematopoietic system. However, a number of so-called bimorphic lymphomas (composite lymphomas) and biclonal leukemias with two phenotypically apparently unrelated malignant populations arising in a patient either synchronously or metachronously have been described (Fend et al., 1999). These neoplasms are rare, and are of therapeutic and prognostic significance (Fakan et al., 1994).

The etiology of most of the B-LPD is unknown. Exceptions are neoplasms in immunocompromised hosts where Epstein- Barr virus (EBV), Human Immunodeficiency Virus (HIV) may play an important role. Individuals on immunosuppressive therapy e.g. after a heart or liver transplant have 40-100 x higher risk of developing a NHL than normal population (Friedmann, 1994). Other factors include e.g. a prior exposition to mutagenic agents (cytostatic drugs, toxic chemical or radiation).

The cells of immune system are functionally heterogeneous and ubiquitous; therefore B-LPD may develop in lymphoid tissue at different sites throughout the body. Tumors originating in the bone marrow (BM), which is a central (primary) lymphoid organ, have been historically

called leukemias. Tumors arising from the tissue of peripheral (secondary) lymphoid organs are called lymphomas and can be divided into nodal lymphomas (coming from lymph node; LN) or extranodal lymphomas (e.g. mucosa-associated lymphoid tissue, MALT lymphoma). Tumor cells may travel from those original sites via the blood or lymph and if untreated they infiltrate other organs. In solid tumors this process is referred to as forming of metastases. The set of antigens or homing (adhesion) molecules on the cells surface dictates the actual localization of the tumor cells. This phenomenon adds to the complexity of nomenclature and may lead to a possible confusion. The term leukemisation of a lymphoma describes a situation when lymphoma cells originally from an organ localization (LN) can be found in the peripheral blood (PB). On the other hand some leukemias may present with a bulky disease, which means a large concentration of cancerous cells in a particular organ (e.g. lymph nodes).

Figure 2.1: Events in B- cell development. (Harris et al., 2001). (FDC, folicular dendritic cell; + apoptosis)



The development and maturation process of B cells begins in the bone marrow (see Figure 2.1). Here, the "pre-B cell" arises from the "progenitor (Pro) B cell" following rearrangement of the immunoglobulin heavy (IgH) and light chain genes (horizontal lines) resulting in the expression of the whole immunoglobulin (Ig) molecule on the cell surface, serving as an antigen receptor. The B- cell leaves the bone marrow, passing through the blood stream and entering the peripheral lymphoid tissue. Here, the B cell migrates to the outer region of the lymph node in the "primary" follicles and, later, to the follicle mantles. This differentiation step is associated with the additional expression of immunoglobulin D (IgD). These IgM+/IgD+ B cells are known as "naive mature B cells". When these cells come into contact with antigen (Ag), which can bind to their immunoglobulin molecules, they transform into proliferating extrafollicular B blasts, from which short-lived plasma cells and "antigen-induced" or "primed" B cells are derived. These "primed" B cells initiate and maintain the germinal center reaction, during which they transform into rapidly proliferating centroblasts. During the mitotic proliferation and differentiation of the centroblasts into centrocytes, somatic mutations in the variable region of the immunoglobulin genes are randomly inserted (vertical lines). The centrocytes with advantageous mutations (increased in the affinity) differentiate further, passing out of the germinal centre into long-lived plasma cells or into "memory" B cells. The latter remain in the marginal zone. As a result of the differentiation phases of B-cells and of the somatic mutation process, 3 major different mature forms of B-cells can be identified: Naive mature B cells (recirculating and sessile subtypes), germinal center B cells (centroblasts and centrocytes), post germinal center B cells which include memory B cells and long-lived plasma cells. From all of these different B cell forms, malignant B cell lymphomas arise, which

distinguish themselves clinically and which are characterized in their biological behavior not only by the transformation event but also by the inherent characteristics of the cell of origin.

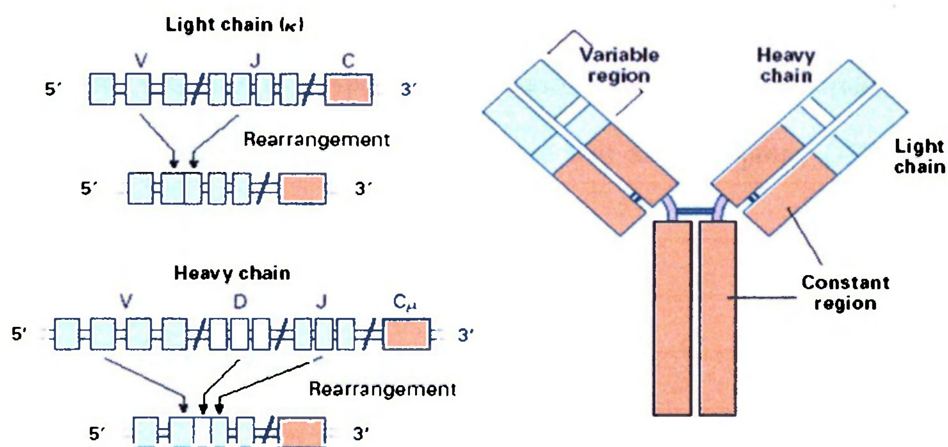
The histological and biological features have been reflected in the variety of evolving systems lymphoma classification (Rappaport, 1966; Lennert, 1967; Bennet et al., 1985; Stansfeld et al., 1988; Harris et al., 1994, 1999 and 2000). The current dogma views malignant transformation as a multistep process of accumulation of DNA damage. A particular cell loses control over regulation of growth, proliferation, cell cycle progression or apoptosis.

2.1.1 The VDJ recombination

Development of B lymphocytes is characterized by progression through a series of checkpoints defined primarily by rearrangement and expression of genes for IgH and also light chains (kappa or lambda, IgK, IgL). This process begins with assembly of variable-region (V), diversity (D), and joining (J) genes (see Figure 2.2 and 2.3). The light chains are then assembled from V and J elements. B-LPDs are caused by proliferation of a clone derived from a progenitor at specific stage of differentiation that correlates with the stage of B- cell receptor (BCR) development. The actual VDJ rearrangement results from a random recombination of germ-line segments and produces an entirely new expressed Ig gene. On one hand it is a purely stochastic process, not driven by any instructive or selective pressure and these recombination events are not inherited. On the other hand the VDJ recombination is a highly orchestrated lymphoid-specific process (Rajewsky, 1996). The many different V, D, and J segments in the germ line, and therefore each B cell generates a particular set of genes for its heavy-chain and light chain regions that differ from those of other B cells and encode a distinct antibody. These distinct rearrangements also equip each B cell with individual, molecular clonal markers - an essential

feature for the molecular analysis of B-LPDs. It has been shown that the expression of antibody as an antigen receptor on the surface of B cells is not critical only for the development, but also for the survival of B cells (Lam et al., 1997). In case the first IgH allele is rearranged out- of- frame the process moves to the second allele. If the first rearrangement is productive the process of allelic exclusion disables initiation of the recombination on the second allele through chromatin remodeling (Johnson et al., 2003).

Figure 2.2: V(D)J Recombination in B-Cell Development (Küppers et al., 1999).



Similar actions continue on alleles for the light chains where 4 attempts are possible (2 for kappa and 2 for lambda). The only surviving developing B-cells are those that acquired IgH and IgL rearrangement that can be translated into protein because they preserve the correct reading frame ("in-frame rearrangements"). Cells bearing out- of-frame VDJ rearrangements on both alleles are subjected to apoptosis (see Figure 2.1).

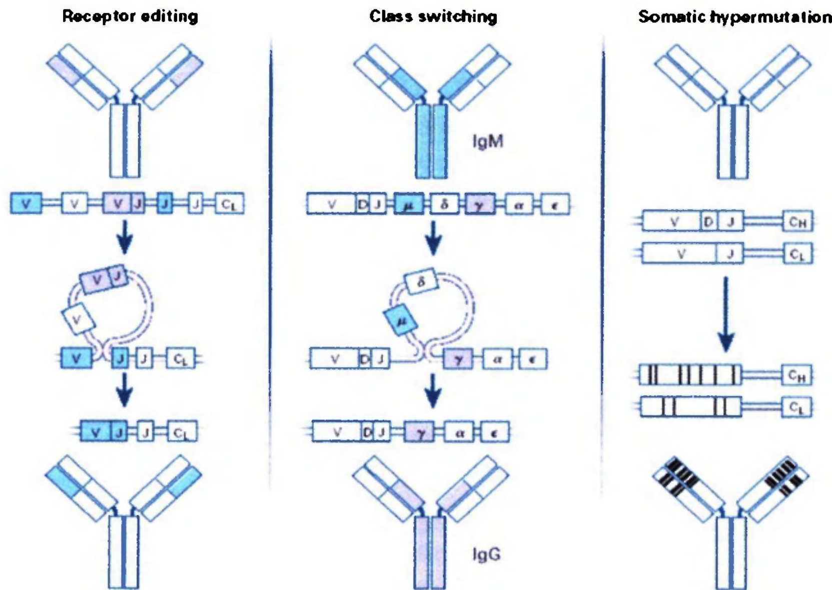
2.1.2 The somatic hypermutation (SH)

Expressing an "innocent" B-cell receptor the B cell leaves the BM to become a mature, naive (i.e., not yet exposed to any antigen) B cell. These B cells are activated through binding of cognate antigen with their antigen receptors and interaction with T helper cells, they migrate into B cell follicles of secondary lymphoid organs LN and establish so called in germinal centers (GC). In the GC the cells vigorously proliferate and activate a SH mechanism which specifically introduces mutations to the V regions of Ig genes (VH genes). During this process nucleotide substitutions are introduced into rearranged V genes. The mechanism of SH remains unknown, but recently it has been described that hypermutation is associated with double strand DNA breaks (Bross et al., 2000) The most favorable model suggest that an error-prone DNA polymerase introduces mutations in proximity of the DNA breaks.

2.1.3 The class switch recombination

A fraction of GC B cells undergoes class switch recombination and thereby changes the isotype of the expressed BCR, resulting in altered effector function of the antibody (Liu et al., 1996; Maizels et al., 1999). Through class switching, the original the constant (C) region genes for IgM and IgD are replaced by C genes for IgG, IgA and IgE located downstream from the V, D and J gene cluster. The antibody specificity remains unaltered, but the effector functions of the antibody are changed. The class switching takes place on both IgH alleles (Irsch et al., 1994). The putative "class switch recombinase" is still unknown, but enzymes from VDJ recombination complex are involved and double-strand DNA break repair is also needed for this mechanism.

Figure 2.3: Molecular processes modifying the genes encoding antibody molecules. (Küppers et al., 1999).

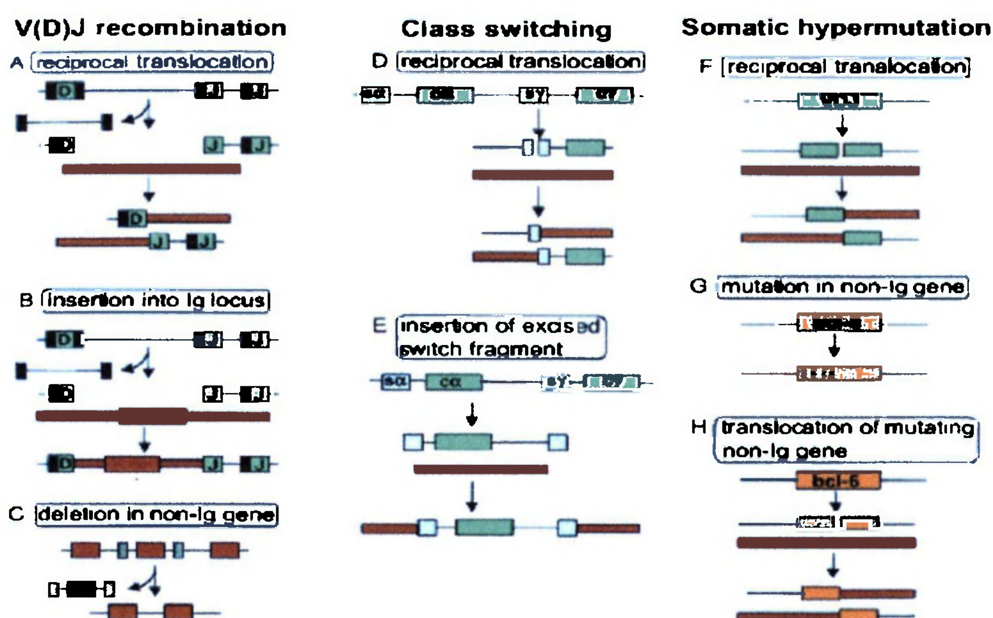


2.1.3 Receptor editing

Receptor editing is a process by which an originally expressed antibody polypeptide chain, usually the light chain, is replaced by another one. In the light-chain loci, receptor editing is mediated by secondary rearrangements of the V region gene, usually involving upstream V segments and downstream J segments. In this process, the gene encoding the originally expressed light chain is deleted from the chromosome.

Autoreactive or weakly reacting cells are either removed by apoptosis or "edit" their receptors also by means of secondary VDJ rearrangements (Wilson et al., 2000).

Figure 2.4: Ig-gene remodeling processes involved in chromosomal translocations. (Küppers and Dalla-Favera, 2001). Coding sequences of Ig genes are indicated in green. RSS sites are shown in (a) and (b) as black squares, and RSS-like sequences in non-Ig genes in (c) by grey squares. Non-Ig loci are shown in brown. In (g) bcl-6 is shown as an example of one of six known non-Ig genes (bcl-6, Fas, Pax-5, Rho/TTF, c-myc and Pim-1) that undergo SH. While bcl-6 and Fas accumulate SH in normal GC B cells, hypermutation of the four latter genes is restricted to DLCL. In (h), bcl-6 is shown as an example of one of the five known mutating non-Ig genes that undergo translocations with breakpoints located in the mutation domain.



2.2. THE REMODELING OF IMMUNOGLOBULIN GENES AND MALIGNANT TRANSFORMATION OF B LYMPHOCYTES

During the course of immunoglobulin genes recombination occasional failures of these processes occur and appear to play a decisive part in B-cell tumorigenesis through the generation of chromosomal translocations into immunoglobulin gene loci. These chromosomal aberrations recur within a specific clinico-pathologic category of lymphoma and are clonally represented in each tumor case. They involve a variety of partner genes besides the immunoglobulin genes.

Chromosomal translocations derived from all of the stages of immunoglobulin genes rearrangement (VDJ recombination, class switch and somatic hypermutation) have been described (see Figure 2.4 and Table 2.1). Typical translocations in lymphomas cause a juxtaposition of proto-oncogene next to a constitutively active promoter or enhancer. The common consequence of such translocation is the deregulated expression of a proto-oncogene by either homotopic deregulation or heterotopic deregulation. Homotopic deregulation occurs when the proto-oncogene is expressed in normal cells of the same tissue, but its regulation is changed in the tumor. Heterotopic deregulation occurs when the proto-oncogene is not physiologically expressed in the normal cells and becomes ectopically expressed as a consequence of the translocation. The exceptions to the deregulation model are represented by the translocation t(2;5) of T-cell anaplastic large cell lymphoma and the translocation t(11;18) of MALT lymphoma, which cause gene fusions coding for chimeric proteins. Fusion proteins are typical for myeloid leukemia where their presence leads to deregulation of processes including signal transduction, transcription, apoptosis signaling etc. The outcome is however similar since a particular cell with a disrupted control over the main biological processes gains and proliferative or survival advantage over normal cells.

Another possible mechanism of lymphoma development is inactivation of tumor suppressor genes such as *p53*, *p16* and *ATM* (Liggett and Sidransky, 1998; Westphal, 1997). The loss of function may happen through deletion, mutation or promoter methylation of a formerly active allele of a gene. It has been reported that in DLCL some of the genes are inactivated through a SH, which was documented to target also non Ig genes (Pasqualucci et al., 2001) (see Table 2.1).

In recent years, the process of SH has been studied quite intensively and it seems that antigen recognition plays an important role in the process of malignant transformation. And yet, the different status of V genes mutation was correlated with prognosis of patients with several types of B-LPD. Somatically hypermutated V region genes were found in follicular lymphomas (Bahler et al., 1991), Burkitt's lymphomas (Chapman et al., 1995), diffuse large-cell lymphomas (Kuppers et al., 1997), MALT lymphomas (including monocytoid B-cell lymphomas and marginal-zone lymphomas) (Qin et al., 1997), splenic lymphomas with villous lymphocytes (Zhu et al.,1995), prolymphocytic leukemias (Davi et al., 1996), hairy-cell leukemias (Wagner et al., 1994), lymphoplasmacytoid lymphomas (Aarts et al., 1998) and some B-cell chronic lymphocytic leukemias (Fais et al., 1998). In addition, multiple myeloma, a tumor of plasma cells or their precursors, consistently contains somatically mutated variable-region genes (Bakkus et al., 1992). VH gene mutational status has been described to have prognostic value in chronic lymphocytic leukemia (CLL). Patients with unmutated VH genes have worse outcome than pts with mutated VH genes (Fais et al., 1998; Hamblin et al., 1999; Damle et al., 1999). Less attention has been paid, however, to earlier steps of VDJ rearrangement including allelic exclusion and their role in lymphomagenesis. The lack of allelic exclusion has been reported to be higher in CLL than in normal cells (Rassenti and Kipps, 1997).

Table 2.1: Genetic aberrations in B cell tumors generated as mistakes of B cell-specific Ig-modifying processes (Küppers and Dalla-Favera, 2001).

Enzyme machinery	Type of molecular process	Examples	References
V(D)J recombination	1) translocation of oncogene to Ig (D)J locus;	± bcl-1/IgH in MZL	(Bakhshi et al., 1987; Cotter et al., 1990)
		± bcl-2/IgH in FL	(Jaeger et al., 2000; Tsujimoto et al., 1988)
	2) deletions in oncogenes or tumor suppressor genes;	± a(TAL 1 and MTS1 in T cell leukemias)	(Aplan et al., 1990; Brown et al., 1990; Cayuela et al., 1997)
	3) transposition of genes into the Ig locus	± bcl-2 in FL	(Vaandrager et al., 2000)
Ig class switching	1) translocation of oncogene to IgH switch regions;	± bcl-3/IgH in B-CLL	(Chesi et al., 1997; Gelmann et al., 1983;
		± bcl-6/IgH in DLCL	Ohno et al., 1993; Showe et al., 1985;
		± c-myc/IgH in BL	Baron et al., 1993; Ye et al., 1993)
		± FGFR/IgH in MM	
	2) insertion of excised switch sequences into other loci cylin D1 in MM	± insertion into	(Gabrea et al., 1999)
Somatic hypermutation	1) translocation of oncogene into rearranged V genes;	± c-myc/IgH in BL ± bcl-6/IgL in DLCL	
	2) somatic mutations in non-Ig genes;	± bcl-6, CD95/Fas in various NHL	(Migliazza et al.,1995; Mueschen et al., 2000; Pasqualucci et al., 1998: Shen et al., 1998; Pasqualucci et al., 2001)
		± Pax-5, c-myc, Pim-1, Rho/TTF in DLCL	
	3) translocation of mutating oncogene	± bcl-6, Pax-5, Pim-1, Rho/TTF	Pasqualucci et al., 2001

A
bbreviations: MCL: mantle cell lymphoma, FL: follicular lymphoma, B-CLL: B cell chronic lymphocytic leukemia, MM: multiple myeloma, BL: Burkitt's lymphoma, DLCL: diffuse large cell lymphoma, NHL: Non Hodgkin lymphoma

2.3. THE CHARACTERISTICS AND MOLECULAR PATHOGENESIS OF MOST FREQUENT B- CELL LYMPHOPROLIFERATIVE DISORDERS.

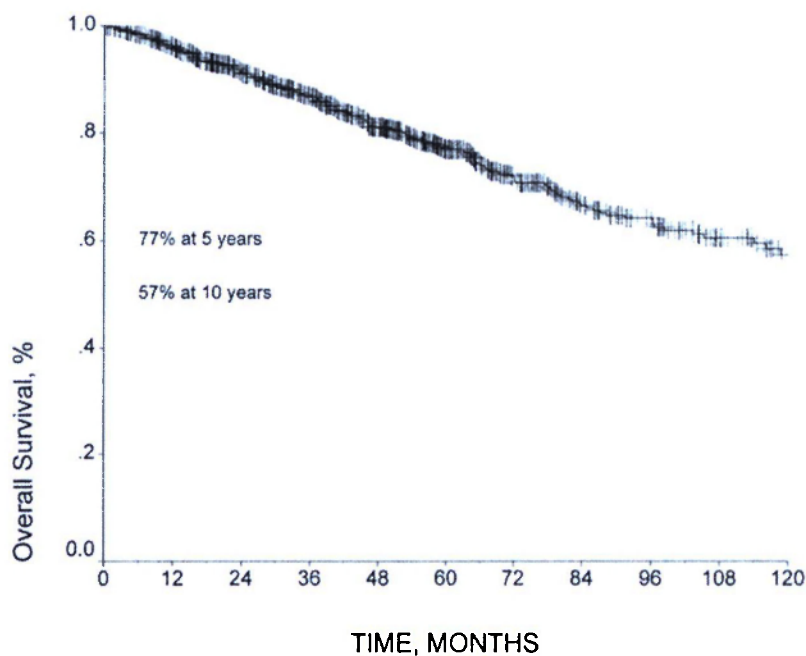
2.3.1. Follicular lymphoma (FL)

Follicular lymphoma is a typical neoplasm arising from the GC cells that have interacted with an antigen. The tumor cells are usually surface immune globulin positive, B-cell-associated antigen positive (CD 19, 20, 79a). Lack of CD5 and CD43 is useful in distinguishing follicle center lymphoma from mantle cell lymphoma and the presence of CD10 can be useful in distinguishing it from other lymphomas such as marginal zone lymphoma (Harris et al., 1994). Patients with FL represent about 30-40% of all lymphoma pts in industrialized countries with the peak of incidence in the fifth or sixth decade. This makes FL the second most frequent type of NHL. FL is a typical indolent lymphoma with approximately 57% survival at 10 years from diagnosis (see Figure 2.5). The majority of patients with FL has disseminated disease at presentation and remains incurable with conventional therapies. In addition up to 70% of FL may convert into an aggressive disease with diffuse large cell architecture over time, which is usually a deadly sentence.

The actual therapeutic approach depends on patients characteristics such as age, sex, clinical stage (CS), number of extranodal sites, presence of BM involvement, bulky disease, and so called B symptoms (unexplained high temperatures, heavy sweating at night, weight loss at least 10% of total body weight). Current management strategies include a "watch and wait", the use of oral alkylating agents (such as chlorambucil or cyclophosphamide), purine analogues (fludarabine) and combination chemotherapy (e.g. cyclophosphamide, vincristine and prednisone- CVP) with or without interferon has been used. The optimal treatment is controversial, as the vast majority of patients with advanced stages of follicular lymphoma are

not cured with the current therapeutic options. Newer approaches employing monoclonal antibodies (e.g. rituximab, anti-CD20) have shown promising results and others (e.g. reduced intensity conditioning allogeneic bone marrow transplantation) are under evaluation. However it has been to demonstrate a prolongation of overall survival (OS).

Figure 2.5: Overall survival of patients with follicular lymphoma. (Federico et al., 2000).



Chromosomal translocations that involve *BCL*-2 are the hallmark of follicular lymphoma. The *BCL*-2 gene was identified by molecular cloning of the t(14;18)(q32;q21) translocation, which is present in virtually all cases of FL (Gaidano and Dalla- Favera, 1997). The translocation joins the *BCL*-2 gene at its 3' untranslated region (UTR) to IgH sequences, resulting in deregulation of *BCL*-2 expression because of the nearby presence of Ig transcriptional regulatory elements (Tsujimoto et al., 1984). On the chromosome 14 all the breaks in the IgH gene occur

within the J segments especially JH4, JH5 a JH6 (Jäger et al., 2000). On chromosome 18, most breaks occur in the major breakpoint region (MBR), located in the 3' untranslated region of the *BCL-2* gene (Cleary and Sklar, 1985). Approximately 50% of the breakpoints on chromosome 18 are clustered within the MBR, 25% cases usually break in the more distant minor cluster region (mcr) (Bakhshi et al., 1987), about 10% of breaks occur in the intermediate cluster region (icr) (Akasaka et al., 1998; Willis and Dyer, 1997). The remaining breakpoints on chromosome 18 fall within the 5' noncoding region of the gene. These *BCL-2* breaks are found mostly, although not exclusively, in B-CLL and are involved predominantly in translocation to the Ig light chain loci (Yabumoto et al., 1996).

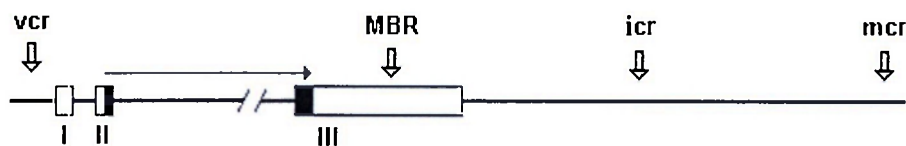
The *BCL*-2 gene encodes a 26-kDa integral membrane protein that has been localized to mitochondria, smooth endoplasmic reticulum and perinuclear membrane. *BCL*-2 has no ability to promote cell cycle progression or cell proliferation, but rather controls the cellular apoptotic threshold by preventing programmed cell death (Chao and Korsmeyer, 1998). Sequences from these breaks are used as primers for detection by PCR.

The process of V(D)J recombination was suggested to be involved in the translocation (see Figure 2.4 and Chapter 2.2), however the actual mechanism of the translocation is still unknown. The breaks on chromosome 14 and 18 occur differently. Jäger et al. observed mutations in JH and DH sequences suggestive of a secondary DH-JH recombination that most likely happens after SH in the GC. The process of SH may thus be involved in the development of t(14;18) (Jäger et al., 2000).

B cells harboring a t(14;18) translocation have been detected at a frequency of about 1 in 10^4 to 10^6 in healthy individuals (Limpens et al., 1991; Summers et al., 2001). These findings are consistent with a multistep transformation process during B cell lymphomagenesis, in which

chromosomal translocations involving Ig loci alone are not sufficient to render a B cell malignant. Malignant lymphoma develops most likely after acquisition of additional transforming events (Adams et al., 1985).

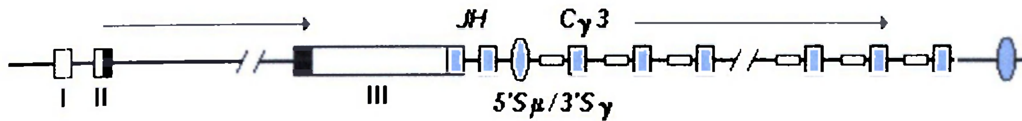
Figure 2.6: The BCL-2 gene (Willis and Dryer, 2000), which is located at chromosome 18q21.3, consists of 3 coding exons separated by an intron of about 250 kb and is involved in Ig translocations in about 80% of follicular B-NHL and 1% to 2% of all cases of CLL. The transcriptional orientation of the gene is from telomere to centromere. MBR denotes major breakpoint region; mcr, minor cluster region; vcr, variant cluster region; icr, intermediate cluster region.



2.3.2. Mantle cell lymphoma (MCL)

This type of lymphoma accounts for approximately 5-8 % of all lymphomas. The tumor consists of small or intermediate lymphoid cells with dispersed chromatin, scanty clear cytoplasm and distinct nucleoli. MCL is more frequent in those aged more than 50 years and occurs three times more frequently in men. Most patients are stage IV at diagnosis when the disease is typically widely spread with extensive lymphadenopathy with spleen, bone marrow and extranodal sites involved (e.g. gastrointestinal tract). The median survival is 3-4 years despite therapy (Weisenburger et al., 1996). The tumor cells express typical pan B-lymphocytic antigens (CD19, CD20, CD22, CD24), are CD5+ and CD10-, CD23- (in contrast with CLL), they also strongly express IgM often combined with IgD.

Figure 2.7: t(14;18)(q32;q21) translocation(Willis and Dryer, 2000). Typical IGH-BCL2 translocation of follicular B-NHL involving the BCL2 MBR within the 3' UTR of the gene and the JH segments. Note that nearly all cases of follicular B-NHL with this translocation have undergone IgH class-switch deletion.



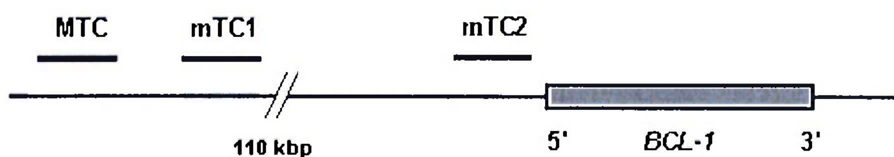
Translocation t(11;14)(q13;q32) (Figure 2.8.) is present in 70 % cases. This translocation juxtaposes the *BCL-1* locus at 11q13 with the IgH locus at 14q32, leading to heterotopic deregulation of *BCL-1* (Leroux et al., 1991; Vandenberghe et al., 1992). *BCL*-1 (also known as *CCND*1 or *PRAD*1) encodes for cyclin D1, a member of the D-type G_1 cyclins involved in cell cycle control (Raffeld and Jaffe, 1991). Cyclin D1 is not expressed in normal B cells, but is expressed and detectable by immunohistochemistry even in MCL cases lacking a cytogenetically detectable t(11;14)(q13;q32), strongly suggesting that deregulation of this gene is a critical event in the pathogenesis of MCL. The pathogenetic role of *BCL-1* activation in human neoplasia is suggested by the ability of cyclin D1 deregulation to contribute to B-cell lymphomagenesis in transgenic mice (Jiang et al., 1993). Among B-NHL, cyclin D1 overexpression is restricted to MCL and represents a useful diagnostic marker for this malignancy.

More than 80% of chromosomal breaks are clustered in 80- 110 bp region on the 11q chromosome called „major translocation cluster" (MTC) (Rimokh et al., 1990; Vandenberghe et al, 1992; Williams et al., 1993).The reported size of the breaking region is 300 bp (Rimokh et al., 1990). Further breaks were found between MTC and the coding sequence of *BCL-1* gene and were named „minor translocation cluster 1 and 2" (mTC1 a mTC2) which cover region of 120

kbp next to the 5'- end of *BCL-1* gene (closer to centromere) on 11q13 (de Boer et al., 1993; Rimokh et al., 1990 and 1993).

Molecular studies of the mechanism of translocation t(11;14) suggested that there are two types of translocations. The majority of t(11;14) occur during the primary DH-JH recombination which happens at earlier developmental of lymphocyte. A smaller number of second type of translocation occurs during the secondary recombination DH-JH after the cell went through the process of somatic hypermutation. The work of Welzel et al. suggested that the process leading to t(11;14) resembles the process involved in t(14;18) creation (Welzel et al., 2001). The mechanism of V(D)J segment recombination plays its role as well (see Figure 2.4 and Chapter 2.2).

Figure 2.8: The location of translocation clusters on the chromosome 11q13 (Rimokh et al., 1994).



The diagnosis of MCL represents often a challenge as MCL overlaps in several features with B-cell chronic lymphocytic leukemia/lymphoma (B-CLL; see Chapter 2.3.4). MCL and B-CLL are lymphoproliferative disorders of intermediate and low grades, respectively. Both malignancies are also characterized by common chromosomal aberrations. Although MCL is associated with higher complexity of the karyotype, there are striking similarities between common genetic aberrations in MCL and B-CLL: deletions on chromosome bands 13q14, 11q23, 17p13, and 6q21 and gains on chromosome bands 3q26, 12q13, and 8q24 (Bentz et al., 2000; Bea et al., 1999; Döhner et al., 2000; Monni et al., 1998; Werner et al., 1997; Leupolt et al.,

2000). For some chromosomal loci, the affected genes are identified, such as *TP53* (*p53*) on 17p13 (Hernandez et al., 1996; el Rouby et al., 1993) and *ATM* (ataxia telangiectasia mutated) on 11q23 (Stankovic et al., 1998; Bullrich et al., 1999; Schaffner et al., 1999 and 2000). Gene expression study of multiple genes involved in apoptosis and cell cycle regulation suggested 2 distinctive pathogenetic mechanisms involved in B-CLL and MCL. In B-CLL, the expression parameters are in strong favor of protection of the malignant cells from apoptosis whereas in MCL the expression data indicate an enhancement of cell proliferation (Korz et al., 2002).

The aberrations affecting genes such as *TP53* (Hernandez et al., 1996; el Rouby et al., 1993) and *ATM* (Bishop et al., 2000; Stilgenbauer et al., 1999) lead to genome instability that increases the risk of chromosomal translocation Welzel et al., 2001), however the molecular mechanisms causing MCL remain still unknown.

There is no clear consensus on treatment for newly diagnosed MCL. Current management strategies include usually a chemotherapy combination (e.g cyclophosphamide, adriamycin, vincristine, prednisone called CHOP) together with monoclonal antibody anti-CD20 (rituximab). More recently intensive chemotherapy with rituximab followed by autologous stem cell transplantation has been shown promising results, but for its toxicities it is being offered usually to younger patients (Vose et al., 2006; Geisler et al. 2007). Other approaches including reduced intensity conditioning allogeneic bone marrow transplantation are used as salvage therapy (in case of disease recurrence) in young patients who have available donor.

2.3.3. Diffuse large cell lymphoma (DLCL)

DLCL is characterized by a marked heterogeneity in phenotype and clinical behavior. DLCL cells are either GC or non-GC center derived. Consistent with this heterogeneity, the genetic lesions associated with DLCL are also heterogeneous. The immunophenotype is characterized by expression of pan B antigens (CD19, CD20, CD22, CD24, certain subtypes express CD5, or CD30). CD10 positive lymphomas originate from germinal centers and have also overexpressed BCL-6 gene (Hans et al., 2004). Approximately 25% of DLCL have transformed from FL and thus bear t(14;18)(q32;q21), they typically represent a unique subgroup of GC derived DLCL with higher expression of bcl-2 and CD10 than DLCL without t(14;18). Gene BCL-6 frequently (30- 40%) enters translocations with different chromosomal partners t(3;x)(q23:x) (Bastard et al., 1994; Horsman et al., 1995; Ye et al., 1993), 7-15% of DLCL carry translocation t(8;14)(q24;q32) involving gene *c-MYC* (Knutsen, 1998).

The standard treatment for newly diagnosed DLCL patient is usually a CHOP chemotherapy combined with rituximab. This approach alone may be curative in high percentage of patients. Autologous stem cell transplantation is typically used in case of disease recurrence or resistance.

2.3.4. Chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL)

CLL is a disease with indolent course and variable prognosis depending on the risk factors. The median of OS is approximately 6 years and 20% of patients survive 10 years.

While the median of age at diagnosis is about 65 years, up to 30% of patients are younger than 65 years.

CLL is characterized by accumulation of uniform population of small mature lymphocytes that predominantly express CD19, CD20, CD23 and CD5 antigen, in the absence of other pan-T-cell markers; the B-cell is monoclonal with regard to expression of either kappa or lambda; and surface immunoglobulin (slg) is of low density. The molecular pathogenesis of CLL/SLL is largely unknown. In particular, none among the cancer-related genes known to date has been shown to associate consistently and selectively with CLL/SLL. Among known cancer related genes, mutations of *p53* occur in 10% of the cases of CLL/SLL and the frequency of *p53* mutations increases substantially in late stages of the disease, suggesting that it may be involved in tumor progression. Since more than 80% CLL are diagnosed at early disease stages, many prognostic markers have been identified. One of the most important molecular genetic markers defining pathogenic and prognostic subgroups of CLL is the mutation status of VH gene (Damle et al., 1999; Hamblin et al., 1999). Surrogate markers for VH status are expression of CD38 and ZAP-70, but their validity is still somewhat controversial (Crespo et al., 2003; Damle et al., 1999). Patients with no VH mutations or many CD38 positive or ZAP-70 positive B cells have an aggressive course, whereas patients with mutated clones or few CD38 positive or ZAP-70 positive B cells have more indolent course (Damle et al., 1999; Kröber et al., 2002; Thunberg et al., 2001; Fais et al., 2001).

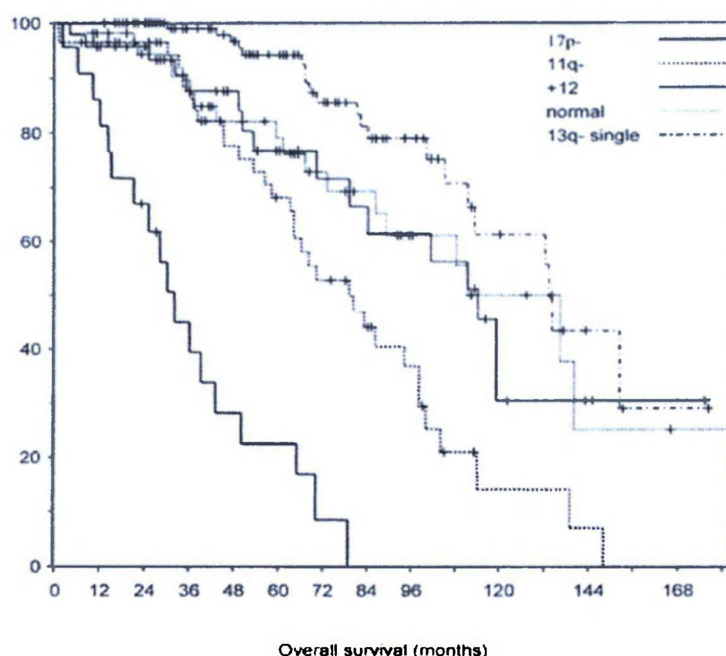
Chromosomal aberrations can be identified in about 80% of CLL by interphase FISH. The incidence of the most relevant genetic abnormalities has been reported to range from 14% to 40% for deletion 13q (del(13q)) as isolated abnormality, 10% to 32% for del(11q), 11% to 18% for addition 12q, 3% to 27% for del(17p), and 2% to 9% for del(6q), depending on the stage of the

disease and whether or not the disease was resistant to conventional therapy (Seiler et al., 2006). Patients with del(13q) as single anomaly have excellent prognosis (median survival, 133 months) whereas those with del(11q), involving *ATM*, and particularly del(17p), involving *P53*, do not respond to conventional therapy and tend to have a rapidly evolving disease (median survival, 79 and 32 months, respectively; Seiler et al., 2006). Del(11q) is associated with male gender, younger age and massive lymphadenopathy; trisomy 12 is linked to atypical morphology and immunophenotype of the leukemic cells, and del(6q) is more frequently observed in patients whose lymphocytes display plasmacytoid features and correlates with intermediate prognosis (Montillo et al., 2005). Cytogenetic abnormalities identify groups of CLL patients (Figure 2.9; Döhner et al., 2000). Broadly, two risk groups are recognized: 1) low-risk: patients with normal karyotype or isolated del(13q); and 2) high-risk: patients with del(17p) or del(11q); trisomy 12 conveys high-risk of disease progression but in contrast to patients with del(17p) or del(11q), patients with trisomy 12 usually respond to fludarabine-based therapy and their survival is better. VH mutation status and cytogenetic abnormalities are two separate prognostic parameters (Seiler et al., 2006), but they appear to correlate. Unfavorable aberrations (del(11q), del(17p)) occur more frequently in unmutated CLL, and favorable aberrations (del(13q)) occur more frequently in the mutated subgroup (Kröber et al., 2002; Oscier et al., 2002; Lin et al., 2002).

Current management strategies include a "watch and wait" in asymptomatic patients, the use of oral alkylating agents (such as chlorambucil), purine analogues (fludarabine) and combination chemotherapy (e.g. cyclophosphamide, vincristine and prednisone- CVP). The optimal treatment remains somewhat controversial, as the vast majority of patients with advanced stages of CLL/SLL are not cured with the current treatment options. Newer approaches employing monoclonal antibodies (rituximab, anti-CD20 or alemtuzumab, anti-CD52) have

shown promising results. Allogeneic stem cell transplantation and especially reduced intensity conditioning allogeneic stem cell transplantation are being investigated.

Figure 2.9: The prognostic relevance of chromosomal aberrations in CLL (Döhner et al., 2000).



Although, 14q32 involvement was described frequently in early CLL studies, t(11;14)(q13;q32) with BCL1/IgH rearrangement, is considered to be a hallmark of mantle cell lymphoma, in CLL it is associated with short survival (Reddy, 2005).

SLL is represents the lymphoma version of CLL. The cells lack expression of CD38 (different from CLL), but they do express LFA1 (CD11a), adhesive molecule, which is responsible for lymphoma like features (Zelenetz and Brodeur, 1997).

2.3.5. Lymphoplasmocytic lymphoma (LPL)

LPL consists of small lymphocytes, plasmocytic lymphocytes and plasmatic cells that express characteristic pan B antigens (CD19, CD20, CD22, and CD24). The cells also express cytoplasmatic IgM, are CD5-, CD23-, CD10- and do not express IgD. LPL derives from B-lymphocytes that differentiate into plasma cell secreting IgM (Offit et al., 1992). This disease may as any other low grade lymphoma transform into aggressive DLCL. Approximately 50% of lymphoplasmacytic lymphoma are associated with the t(9;14)(p13;q32) translocation (Offit et al., 1992). The translocation appears to display a preferential clustering with cases associated with the clinical syndrome known as Waldenström's macroglobulinemia. The chromosomal breakpoints of t(9;14)(p13;q32) involve the IgH locus on chromosome 14q32, and, on chromosome 9p13, a genomic region containing the *PAX*-5 (Paired Homeobox-5) gene (Iida et al., 1996). *PAX*-5 encodes a B-cell specific transcription factor involved in the control of B-cell proliferation and differentiation (Morrison et al., 1998). Presumably, the juxtaposition of *PAX*-5 to the IgH locus causes its deregulated expression, thus contributing to tumor development. Other genetic aberrations include abnormalities of chromosome 1, duplication of 17q and deletion of 7q32 (Iida et al, 1999; Morrison et al., 1998; Offit et al, 1995).

2.4. MINIMAL RESIDUAL DISEASE

Virtually all cancers represent a clonal process when a particular cell population (clone) has got out of physiological mechanisms that control growth and proliferation. Specific

modifications of the genome of a particular cell play the main role in this process. These changes begin a cascade of aberrations that lead into a malignant transformation.

Table 2.2: Sensitivity of different molecular biology techniques used for minimal residual disease detection (Dolken, 2001).

Method	Sensitivity	Number of tumor cells/ total cells	Features
Cytology, cytochemistry and histology	5%	5 / 100	standard clinical procedure, low sensitivity
Immunophenotyping by flow cytometry (FACS)	1-5 % (0,1%)	1-5 / 100 (to 1000)	Lack of tumor-specific antigens and antibodies
Immunophenotyping by Immunohistochemistry	1-5 % (0,1%)	1-5 / 100 (to 10 000)	Lack of tumor specific antigens and antibodies
Southern blot	1%	1 / 100	Time consuming, laborious, low sensitivity
Standard cytogenetics	5-10 %	5-10 / 100	Labor intensive, high quality metaphases
Fluorescence in situ hybridization (FISH)			Interphase FISH: false positive results, labor intensive, no need for metaphases
S-FISH („single color“)	5-10 %	5-10 / 100	
D-FISH („double color“)	1-3 %	1-3 / 100	
PCR	10^{-2}-10^{-3}	1 / 10^{4} to 10^{6}	Sensitivity depends on the total amount and quality of DNA or mRNA (cDNA)
Nested PCR	10^{-3}-10^{-5}	1 v 10^{7}	Very sensitive; false-positive results due to contamination or carry-over are serious problems

In order to cure a patient with an oncological disease it is prerequisite to destroy the malignant clone. In another words it is desirable to achieve a complete remission (CR) (Gribben et al., 1994). The quality of a CR can be assessed by different methods with different sensitivity and therefore there is a CR at the clinical, cytogenetic, immunologic, and molecular levels.

CR is defined as disappearance of the malignant clone under a detectable level when using conventional clinical tests (radiodiagnostic and or laboratory; Cheson et al., 1996; Cheson et al., 1999). Patients with a hematological malignancy have a high probability to attain CR, but there is also a high chance that the disease will recur. The residual tumor cells that are present in small number below detection level of conventional clinical tests contribute to relapses. These subclinical residua of tumor cells are called minimal residual disease (MRD). Situation when the most sensitive method usually PCR does not detect any remaining malignant cells is called a molecular complete remission (MCR). Molecular or PCR relapse is a term suggested by Lion described a situation when quantitative PCR detects expansion leukemic clone by 1 log in absence of clinical relapse (Rel; Lion, 1994). Cytogenetics, immunophenotypic studies and PCR belong to the most frequently techniques used for MRD evaluation.

2.4.1. Methods for minimal residual disease detection

2.4.1.1. Cytogenetics

Chromosomal analysis of human cells includes so-called classical cytogenetics, which studies number, shape, size and structure of metaphasic chromosomes (Heim and Mittelman, 1995). DNA probes identifying specific sequence of DNA in a gene or chromosome can be used in interphase. Initially these probes were labeled with radioactive isotopes. Introduction of

probes with various fluorochromes represented an important step forward in cancer cytogenetics. Today there are painting probes specific for each human chromosome, which allow detection of multiple chromosomal aberrations including deletions, amplifications of genetic material. This technique is called fluorescence in situ hybridization (FISH) and is also used for detection of chromosomal translocations.

Comparative genomic hybridization (CGH) compares coloring patterns of tumor and normal cells. If there is no gain or loss of DNA the region is yellow (an equal mixture between red and green). If chromosome imbalance is present, then the affected region will be red or green. A newer method is called spectral karyotyping (SKY; Schrock et al., 1996) or multicolor FISH (Speicher et al., 1996). DNA from each human chromosome is labeled with a single or multiple colors and is applied in a cocktail to the sample containing malignant cells. Using various systems to capture and analyze the image, one can detect all of the chromosome abnormalities in a single experiment. This analysis identifies the chromosomes involved, but not the specific region of the chromosome.

Cytogenetics is essential in the risk stratification process especially in myeloid leukemias (Grimwade et al., 1998; Grimwade et al., 2001). Karyotypic analysis may, however fail to identify all patients that should be assigned to the favorable risk group (Grimwade et al., 1998). Molecular screening using should be done in all patients to identify those with favorable abnormalities and other potential targets for MRD monitoring.

Measurement of response to therapy at the cytogenetic level (cytogenetic response) has been established for patients with CML. The cytogenetic response is represented by percentage of nuclei that are negative (neg) for the cytogenetic aberration, which is t(9;22) or Philadelphia chromosome (Ph). The response is then graded as no response, minimal (1% to 32% Ph neg),

minor (33% to 65 % Ph neg), major (66% to 99% Ph neg) and complete (100% Ph neg) (Italian CSG-CML, 1998). The most limiting factor of cancer cytogenetics is the low sensitivity.

2.4.1.2. Immunophenotypic analysis (Fluorescein activated cell sorting; FACS)

Flow cytometry (FACS) uses monoclonal antibodies that specifically bind to cell surface antigens. Therefore, the identification of tumor cells is mainly based on the detection of differentiation antigens (cluster differentiation, CD) or their combinations that are preferentially found on tumor cells, but rarely on normal cells. The clonality of B-LPD is assessed by detection of immunoglobulin light chain expression (Robertson et al., 1992). FACS has higher sensitivity than morphological analyses, but due to a great variability of tumor antigens this method can be reliable only in some cases (Jennings et al., 1997). Multicolor FACS represents an important improvement of MRD evaluation by FACS techniques as it enables analysis of large number of antigens and thus identification of a small subpopulation of tumor cells. This method can be applied in larger studies and has quite high sensitivity (10^{-5}) (Rawstron et al., 2001).

2.4.1.3. Polymerase chain reaction (PCR)

The most sensitive technique of MRD detection is PCR. PCR is used for amplification of a specific segment of DNA and consists of three important steps. The first step is denaturation when both strands of double stranded DNA dissociate into two single stranded DNA strands. The second is called annealing when the primers hybridize on the complementary sequence of the template. In the third step the primers are elongated (elongation) according to the template DNA

sequence. These steps are cyclically repeated and the number of copies doubles in each cycle. Therefore theoretically 1 copy of a template gives 10^9 copies after 30 cycles, but in reality the efficiency of PCR is between 80% and 90% (McPherson et al., 1996). The sequence that is amplified is surrounded by primers specifically bound to the template. Besides DNA (containing the target sequence) and primers the other components of PCR reaction are thermostable polymerase, deoxyribonucleosidtriphosphate and reaction buffer.

RT-PCR is used for amplification of a specific segment of RNA. First, the sequence in RNA is transcribed to complementary DNA (cDNA) by reverse transcriptase. This cDNA is then amplified by classical PCR as described above.

PCR technique has a lot of modifications (see Table 2.2). The reaction can be run in one round also called *one step PCR* or *nested PCR*. The second round of a *nested PCR* employs second pair of internal primers that are complementary to the sequence localized on the fragment of DNA that was amplified in the first round. This method is more sensitive, but false-positive results due to a contamination and possible carry-over constitute a necessity of intensive optimalisation (see Table 2.2). There is also so called "*touchdown PCR*" for which the cycling program was designed in order to avoid amplification of nonspecific products (Don, 1991).

2.4.1.3.1. Quantitative polymerase chain reaction (Q- PCR)

The fact that tumor markers can be detected in healthy individuals and in patients remaining in CR without relapse led to development of *quantitative PCR* (Q-PCR) (Dolken et

al., 2001; Lion, 1994; Slavickova et al., 2000 and 2002). The level of MRD can be estimated *semiquantitatively* (increase, decrease, stagnation) or *absolutely* (1 positive cell in 10^4 cells).

After a certain number of cycles the PCR reaches a plateau phase when the amount of products does not increase exponentially anymore, the efficiency of reaction drops almost to 0%. Therefore every method of Q-PCR uses the exponential phase of PCR (see Figure 2.10). Following formula can be applied calculation of the amount of the product (A_n) in exponential phase:

$A_n = A_0 (1+E)^n$

$\log A_0 = \log A_n - n \log (1+E)$

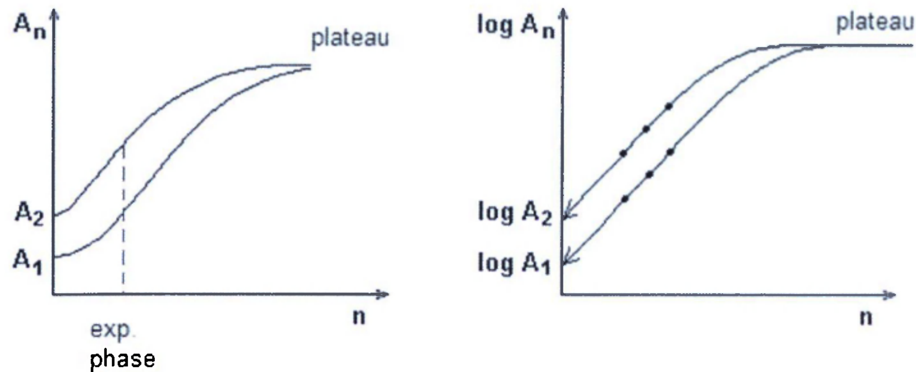
A_0 stands for number copies of a template, n number of cycles and E efficiency of amplification.

Limiting dilution is an established method of quantification of cell biology. The method can be adapted to quantitative PCR by dilution of positive samples until the target is no longer amplifiable (Ouspenskaia et al., 1995). In case of nested PCR multiple reactions at each dilution have to be performed and intensive statistical analysis must be performed to assure the reliability.

Co-amplification of a target (sample DNA) and control gene (internal standard DNA) constitutes a *comparative PCR*. The technique when two genes from the sample DNA are amplified using two different sets of primers is called *multiplex (duplex) PCR*.

Multiplex PCR belongs to semiquantitative methods (Cross et al., 1995; Slavickova et al., 2000). The conditions that must be met are as follows: similar annealing temperature for both sets of primers, similar composition of reaction mixes for both genes, the primer sequences cannot be complementary, the amounts of products of both genes must be quantified within the exponential phase of PCR (Cross et al., 1995).

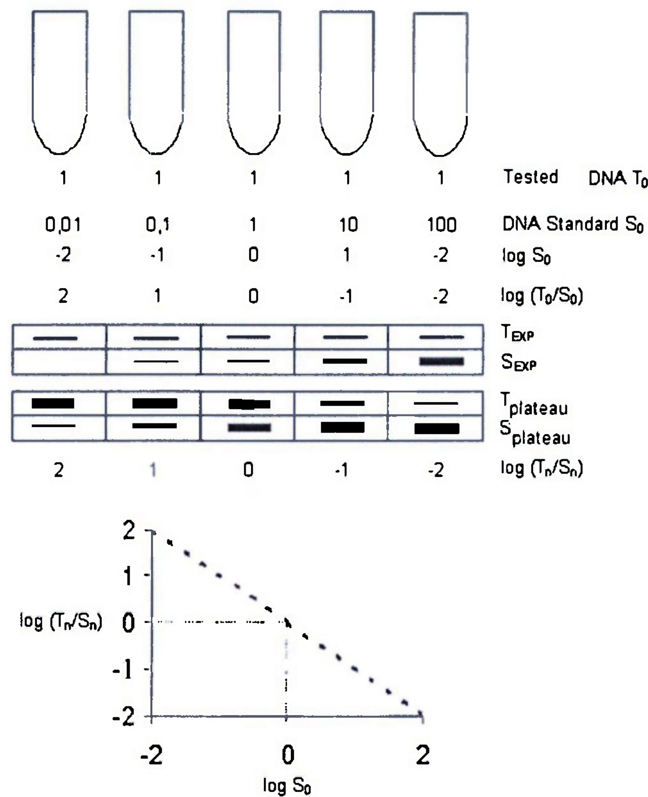
Figure 2.10. The amount of a product (A_n) in relation to a number of cycles (n) (Haskovec, 1996).



In *competitive PCR* a reference standard is co-amplified in the same reaction as the sequence of interest. The standard is usually a synthetic DNA, which is very similar to the tested DNA (Cross et al., 1995). Quantification of the target is made by comparison of the intensity of the two products. The method works well only if both the target and standard DNA are amplified with similar efficiency. Each reaction tube contains same amount of sample DNA with serially diluted standard DNA. The results from each tube are plotted in a standard curve from which the concentration of sample DNA is determined at the point of equivalence. If the amplification works with equal efficiency, the concentration of standard DNA equals the concentration of the tested DNA (Cross et al., 1995; Raeymaekers et al., 1999).

Competitive PCR controls for tube-to-tube variation in amplification efficiency. The ratio between amounts of products is constant in both exponential and plateau phase, therefore the reaction can be quantified even in plateau phase. The limitations are the necessity of standard DNA preparation, selection of suitable concentration of competitor for each sample DNA and a risk of false positive results due to contamination (Cross et al., 1995).

Figure 2.11. Ideal result of competitive PCR reaction. Each tube contains the same, but unknown amount of the tested DNA – T_0 (1 relative unit), the same amount of competitor are added (geometric series from 0,01 to 100 relative units) – S_0. The middle picture shows schematically gel results where the amounts of products are depicted in exponential phase (T_{EXP} and S_{EXP}) and the amount of product in the plateau phase ($T_{PLATEAU}$ and $S_{PLATEAU}$). The intensities of individual band fluorescence are graphed into a standard curve. The value T_0 corresponds to S_0 in the point of equivalency, the point when log (T_n/S_n) = 0 (Raeymaekers et al., 1999).



The newest addition to the family of PCR methods is represented by *real-time quantitative PCR (RQ_PCR)*. There are several systems of fluorescent probes that can be used. The first system called SYBR® Green employs a dye that intercalates between two strands of DNA and emits fluorescence which can be measured. Newer and more accurate technique uses a fluorogenic probe, consisting of an oligodeoxynucleotide with both reporter and quencher dyes attached (FAM™ a TAMRA™, VIC™ a JOE™, ROX™). This probe anneals between the two

standard PCR primers. When the probe is cleaved during the next PCR cycle, the reporter is separated from the quencher so that the fluorescence at the end of PCR is a direct measure of the amplicons generated throughout the reaction. This is a description of newer system TaqMan®. Another system e.g. LightCycler™ uses two hybridization probes. The first probe is labeled with flurochrome (fluorescein) at 3'end and the second has the flurochrome attached at the 5'end. The probes are designed in order to hybridize next to each other. After excitation by a laser the first flurochrome emits light that excites the second flurochrome. This is called Fluorescence Resonance Energy Transfer (FRET). The emission of light can be accurately measured. The first reports of *real-time PCR* monitoring of residual disease were represented by Donovan et al. in patients with acute lymphoblastic leukemia (ALL), (2000), Ladetto et al. in multiple myeloma (2000) and in follicular lymphoma (2001).

This method eliminates the need for post-PCR sample processing and thereby greatly increases throughput. *Real-time PCR* also reduces the potential for false positive results by adding the additional level of specificity provided by the hybridization of a probe to sequences internal to amplification primers and offering a closed tube assay system. Real-time PCR instruments are still relatively expensive making this promising technology less feasible for larger clinical studies.

2.4.2. The source of tissue for minimal residual disease detection

Monitoring of MRD from samples of BM or PB has been well established especially in patients with myeloid leukemias. The situation is however a slightly more complicated in ALL patients. In patients with T- cell ALL (T- ALL), leukemic cells can be consistently found in the

peripheral blood when they are detected in the bone marrow. In sharp contrast, patients with B-ALL often had no detectable leukemic cells in peripheral blood, despite their presence in bone marrow. However, the presence of MRD in peripheral blood denoted more aggressive leukemia with an extremely high risk for recurrence. Peripheral blood thus can be used for MRD studies in patients with T- ALL. It allows frequent monitoring of leukemia while sparing patients the discomfort of bone marrow aspirations. Peripheral blood is not as reliable for MRD evaluation in B- ALL patients (Coustan-Smith et al., 2002). This could be explained by the fact that ALL of T-cell origin may well derive from progenitor cells that naturally reside in the thymus rather than in the bone marrow. If leukemic T- lymphoblasts migrate to the bone marrow through the circulating blood, studies of peripheral blood might be as informative as studies of bone marrow. In patients with B- ALL (originating from bone marrow progenitor cells), the presence of circulating lymphoblasts at the time of clinical remission might indicate a propensity of the malignant cells to exit the bone marrow prematurely (Greaves et al., 1986), but negative PB result does not necessarily correlate with BM status.

The situation is even more complicated in NHL. The heterogeneous involvement of blood or bone marrow in all but the leukemic forms of NHL leads to sampling inaccuracies. The homing capacities of the different lymphomas and their tendency to disseminate to blood and bone marrow are highly variable. Thus it is not clear what tissue is the most reliable for MRD monitoring.

Using non-quantitative PCR monitoring, both paired PB and BM samples some authors see a BM as a superior source for MRD monitoring as they have shown its correlation with better disease prognosis (Gribben et al., 1994; Zwicky et al., 1996). Others prefer monitoring of PB as it they observed no difference when compared with BM. PB sampling is also more convenient

(Bendandi et al., 1999; Lopez-Guilermo et al., 1998). Lopez Guilermo et al. tried to draw a relation between prognosis of patients with FL and PCR results either from BM or PB in post therapy follow up. Seventy percent of PCR findings from PB correlate with BM results and vice versa (Lopez-Guilermo et al., 1998; see Table 2.3).

A nested semi-quantitative competitive PCR assay showed that the number of cells with the rearrangement was lower in PB than in BM but the majority were within one log difference (Leonard et al., 1998). A prospective quantitative monitoring study of 34 FL patients treated with chemotherapy combination CVP and interferon- α- 2b for first line treatment found that the copy number of BCL-2/IgH positive cells pre-treatment did not influence progression free survival (PFS). Furthermore, because they were easily cleared from the PB after first line therapy, BM rather than PB may be more informative for establishing the relationship between molecular remission and clinical outcome (Mandigers et al., 2001). One explanation for the lower sensitivity of PB for the MRD detection is the shortened lymphoma cell survival in the circulation due to the absence of stromal cells, which provide survival signals.

Table 2.3: The correlation between MRD results in BM and PC at different intervals from therapy. (BM- bone marrow; PB- peripheral blood; n- number of patients)

	3-5 months (n= 57)	6-8 months (n= 41)	9-14 months (n= 35)
PB(+)/BM(+) or PB(-)/BM(-)	70%	66%	77%
PB(+)/BM(-)	13%	5%	9%
PB(-)/BM(+)	17%	24%	14%

There are reports that even patients who cleared lymphoma cells their PB or BM can still relapse from residual disease in their lymph nodes (LN; Gupta et al., 1998; Cerny et al., 2003a). Patients with bulky disease have less tumor cells circulating in their blood stream as their tumor cells have higher expression of adhesion molecules (e.g. L-selectin) on the cell surface and tend to reside within the lymphoid tissue rather than home to BM (Drillenburg et al., 2001).

The increasing use of peripheral blood progenitor cells (PBPC) for autologous stem cell transplantation (ASCT) together with suspicion that residual tumor cells in PBPC harvests may contribute to disease recurrences (Brenner et al., 1994; Pilarski et al., 2000) opened another dimension for PCR evaluation of MRD. PCR monitoring started to be use for evaluation of efficiency of different in vitro (Valkova et al., 2002) and in vivo (Magni et al., 2000) purging approaches. There are more reports suggesting that patients with tumor contamination of PBPC have worse prognosis than patients with tumor free grafts (Sharp JG et al., 1996; Corradini et al., 1997; Freedman et al., 1999; Ladetto et al., 2002) than reports where autografting with lymphoma cells did not influence outcome (Hardingham et al., 1995).

There is a common agreement that a LN tissue should be tested by PCR at diagnosis and that MRD monitoring is performed from both BM and PB. Other tissues e.g. PBPC, spleen, pleural effusion, cerebrospinal fluid, skin, mucosa (gastric biopsy) can be used in special situations (diagnosis, local relapse), but they cannot be used for routine monitoring (Bendandi et al., 1999; Lopez-Guilermo et al., 1998).

2.5. MINIMAL RESIDUAL DISEASE IN SELECTED LYMPHOPROLIFERATIVE DISORDERS

2.5.1. Chronic lymphocytic leukemia (CLL)

CLL is a disease with indolent course and variable prognosis depending on the risk factors. The median of OS is approximately 6 years and 20% of patients survive 10 years. While the median of age at diagnosis is about 65 years, up to 30% of patients are younger than 65 years. CLL is incurable with conventional therapy and in case risk factors are present the survival is shorter than 3 years. This is not acceptable especially for younger patients and therefore new therapeutic approaches are being studied in order to eradicate the disease and achieve a long lasting remission.

Minimal residual disease is commonly measured by PCR and/or flow cytometry. PCR analysis for clonal IgH rearrangement recognizing the complementarity determining region III (CDRIII) detection is reported to detect 70- 80% when consensus primers are employed. Consensus IgH PCR has a sensitivity of 2 x 10^{-1}- 10^{-3} and is a technically less cumbersome. The main limitations are decreased sensitivity and inapplicability in occasional patients carrying mutations in the VH gene (Botcher et al., 2004; Moreno et al., 2006). In allele-specific oligonucleotide (ASO- PCR), patient's hypervariable immunoglobulin genes are sequenced, based on which >=1 oligonucleotide primers specific to the individual patients are designed (Botcher et al., 2004). Both patient-specific and consensus primers are then used in the PCR reaction. This sensitivity of this technique

is improved (10^{-4}-10^{-5}). However, ASO-PCR is time consuming, labor intensive, and expensive. It is difficult to generate results in a timely fashion using this approach.

Flow cytometry techniques have become more sophisticated. Initial studies that measured MRD used 2-color (CD19, CD5) classic flow cytometric techniques. Robertson observed that CLL patients can achieve MCR after fludarabine as assessed by bone marrow biopsy, dual-color flow cytometry with CD19 and CD5 markers and Ig gene analysis using Southern-blot method (Robertson et al., 1992). Vuillier combined flow cytometry and CDRIII PCR for evaluation of molecular remission in CLL patients (Vuillier et al., 1992). The quality of the remission was predictive of the response duration (Robertson et al., 1992; Vuillier et al., 1992). Flow cytometry is a very efficient method for MRD evaluation, but sensitivity is not optimal. Therefore, current measurements with 4-color methods have been further improved by using a sequential gating strategy to assess the expression of CD19, CD5, CD20, and CD79b. This approach is able to detect 1 CLL cell in 10^4-10^5 normal cells (Rawstron et al., 2001). A similar- level of sensitivity can be obtained by utilizing quadruple antigenic combinations (Moreno et al., 2006).

Provan et al demonstrated that persistent PCR positivity after ASCT is connected with high risk of relapse. At the same time significant number of patients treated with intensive therapy including ASCT is PCR negative and remain disease free. This suggests that HDT may be curative in selected patients with CLL. MRD detection by using molecular methods should be thus employed to determine whether complete remitters, are indeed molecular remitters (Provan et al., 1996). Magnac described results of MRD monitoring in 12 patients after achieving CR. (follow up: 17- 60 months; see Table 2.4; Magnac et al., 1999).

Table 2.4: Results of MRD monitoring in 12 patients with CLL who reached CR. Standard- 8 x fludarabine (4-12 cycles) a 1x CHOP, 3 x salvage (refractory to standard chemotherapy)- ESHAP (3-6 cycles).

No. patients	Therapy	PCR- vs. PCR+	Relapse (PCR- vs. PCR+)	Death (PCR- vs. PCR+)
9	Standard	1/8	1/2	0/2
3	Salvage+ ASCT	3/0	0/0	0/0

As the number of monitored patients is relatively small these results must be interpreted with caution. Nevertheless, HDT proved that it has a potential to induce MCR and in turn leads to a better prognosis of patients who achieve MCR (Magnac e al., 1999). Interestingly, MCR can be induced even if unmanipulated PBPC with possible CLL contamination is used. In the study of Italian authors 20 CLL patients in CR (after fludarabine) were given an unmanipulated PBPC transplant and were longitudinally monitored for MRD. Seventy five percent of them attained MCR (15/20) and those patients had in prolonged response. The study also showed that conversion from PCR negative to positive during the clinical follow-up anticipates immunophenotypic recurrence of disease with an interval from molecular to immunophenotypic relapse which ranges between 6 and 10 months. Furthermore, the data indicate that monitoring of MRD in CLL patients after PBPC autografting is important to verify the disappearance of the leukemic clone or to identify early recurrence of disease. Persistence or reappearance of the molecular signal after engraftment predicted a subsequent immunophenotypic and clinical CLL recurrence (Meloni et al., 2000).

Table 2.5: Eradication of minimal residual disease (MRD) in chronic lymphocytic leukemia (CLL). (SC- subcutaneous, IV- intravenous, TIW- three times a week, MCR- molecular complete remission.)

Author	Regimen	MCR rate
Montillo et al. (2006)	alemtuzumab 10 mg SC TIW x 6 weeks	19/34 (56%)
O'Brien et al. (2003)	alemtuzumab 10- 30 mg IV TIW x 4 weeks	11/29 (38%)
Moreton et al. (2005)	alemtuzumab 30 mg IV TIW	18/91 (20%)
Keating et al. (2005)	fludarabine/cyclophosphamide/ rituximab	138/207 (67%)
Wierda et al. (2005)	fludarabine/cyclophosphamide/ rituximab	12/37 (32%)
Bosch et al. (2005)	fludarabine/cyclophosphamide/ mitoxanthrone	19/69 (28%)
Faderl et al. (2006)	fludarabine/cyclophosphamide/ mitoxanthrone/rituximab	15/26 (58%)
Kay et al. (2007)	fludarabine/alemtuzumab	6/24 (25%)

In recent years, various combinations of purine analogues, alkylating agents, and monoclonal antibodies such as rituximab (anti-CD20), and alemtuzumab (anti-CD52) have yielded high response rates in treatment-naive patients as well as patients with relapsed/refractory CLL (Zent et al., 2006; Keating et al., 2005; Wierda et al., 2005). Alemtuzumab is effective in obtaining clearance of the peripheral blood and bone marrow involvement of CLL and has been shown to improve the quality of response and achieve eradication of MRD. When CLL patients received fludarabine followed either by ASCT or alemtuzumab followed by ASCT 76% (19/25) patients became MRD negative. Patients with detectable MRD had significantly worse event free survival (EFS) than MRD free patients (p= 0.0001). Similarly the OS was shorter in MRD positive group (p= 0.007). Another important observation was that in all patients the number of detectable CLL cells increased over time (Rawstron et al., 2001).

In a phase II trial conducted by Montillo and colleagues, patients were initially treated with fludarabine with or without cyclophosphamide; responding patients were consolidated with alemtuzumab (Table 2.5; Montillo et al., 2006). Of the 34 evaluable patients, 19 (56%) achieved an MRD negative CR. In a trial conducted by O'Brien and colleagues, patients with residual disease following response to chemotherapy received alemtuzumab (Table 2.5; O'Brien et al., 2003). Of the 29 patients assessed by ASO- PCR, 11 (38%) achieved a molecular remission. A phase II trial evaluated alemtuzumab consolidation therapy following induction therapy with oral fludarabine/ cyclophosphamide in 36 patients with previously untreated CLL (Delmer et al., 2006). Of the 19 patients evaluable by flow cytornetry, 15 had undetectable MRD at the time of completing 8 weeks of alemtuzumab. In a trial conducted by Moreton and colleagues of single-agent alemtuzumab in patients with relapsed/refractory CLL, 18 (20%) of the 91 evaluable patients achieved an MRD negative remission (Table 2.5; Moreton et al., 2005). Both time to next treatment ($p< 0.0001$) and median OS ($p= 0.0007$) were significantly prolonged in the 18 patients with an MRD negative CR compared to the 73 patients with an MRD positive CR, partial remission (PR), or no response.

Patients with CLL who received initial treatment with the chemoimmunotherapy regimen fludarabine/cyclophosphamidc/rituximab (FCR) and were found to have 1% residual CLL cells as measured by flow cytometry assay had long-lasting remissions (Table 2.5; Keating et al., 2005). In a trial conducted by Wierda and colleagues, FCR also showed activity in patients with relapsed/refractory CLL (Table 2.5; Wierda et al., 2005). Of the 37 patients evaluated for MRD status who achieved a CR, 12 (32%) achieved MRD negativity as assessed by ASO-PCR. In a trial reported by Bosch, which included 69 patients, MRD eradication was attained by 19 patients (28%) in CR after fludarabine, cyclophosphamide/mitoxantrone (FCM). Responses were more

durable in the MRD negative subgroup (Table 2.5; Bosch et al., 2005). A phase II trial evaluated the addition of rituximab to FCM in patients with previously untreated CLL (Table 2.5; Faderl et al., 2006). Of the 27 evaluable patients, 11 (41%) achieved a CR, and 16 (59%) achieved a PR/near PR, for an overall response rate of 100% (Table 2.5). Of these patients, < 1% CD5/CD19 cells was seen in 15 (58%) of the 26 evaluable patients, and MCR was seen in 12 (63%) of the 19 evaluable patients. A phase II trial evaluating chemoimmunotherapy with pentostatin/cyclophosphamide/rituximab in 64 evaluable patients with previously untreated CLL also demonstrated a significant correlation between flow cytometry negative status (< 1% CD5+/CD19+ B cells as measured by 2-color flow cytometry) and prolonged PFS ($p < 0.001$; Kay et al., 2007). The final results of the UKCLL02 trial reported MRD negative remissions with subcutaneous alemtuzumab in 6 of the 24 responding patients with fludarabine-refractory CLL (Table 2.5; Sayala et al., 2006).

2.5.2. Nonhodgkin's lymphomas (NHL)

Specific interchromosomal translocations serve as a molecular marker for molecular monitoring of the subclinical residual disease in NHL patients. The most frequently studied molecular markers include well known *BCL-2* oncogene, which is involved in translocation t(14;18) in FL. *BCL-1* oncogene is involved in t(11;14) in mantle cell lymphoma (MCL). PCR amplification of clonal rearrangement of CDRIII within the IgH was reported to detect up to 90% malignant B cell lymphoproliferative disorders (Zwicky et al., 1996). Similarly in T cell derived diseases the clonal TCR rearrangement serves as a molecular marker of clonality.

2.5.2.1 Minimal residual disease after standard chemotherapy.

The data from early observations stated that MRD is detectable virtually in all patients who were treated by conventional therapy e.g. CHOP (Gribben et al., 1991). On the other hand intensified standard chemotherapeutic regimen CHOD-Bleo/ESHAP/NOPP was successful in converting 68% (13/19) formerly *BCL-2* positive patients with a low grade (LG) lymphoma into PCR negativity (McLaughlin et al., 1994). The long term follow up of these patient indicated that 88% achieved CR and 72% MCR. The 5- year DFS was 73% for the PCR negative versus 28% for the PCR positive group of patients respectively, but neither curve of survival plateaued (Cabanillas et al., 1999). Newer chemotherapeutic regimen FMD (fludarabine, mitoxanthrone, dexamethasone) gave 68% molecular responses (17/25) and 32 % (8/25) of patients reached PCR negativity (Crawley et al., 2000). In long term follow-up of patients with FL in CR, evidence of PCR detectable MRD was associated with recurrent disease in several studies (Gribben et al., 1994; Lopez-Guillermo et al., 1998; Haas et al., 1994; Ha et al., 2001). In other studies, long term remissions were achieved in patients despite being persistently PCR positive in PB and/or BM (Price et al., 1991; Lambrechts et al., 1994) suggesting that MRD can be cleared perhaps by immune mechanism at later point.

In addition, some patients may relapse despite clearance of tumor cells from PB or BM (Gupta et al., 1998). How can we explain these discrepant results? The explanations include: 1) the timing and frequency of molecular assessment, 2) the sensitivity of the PCR assay, 3) the samples being tested (PB, BM or both) and 4) the sample size of the reported trial (inadequate power to detect a difference). It has been also shown that it may take significant time for some

patients to clear MRD from their PB or BM (Lopez-Guillermo et al., 1998). The results are shown in Table 2.6.

Table 2.6: Increasing clinical and molecular remission rates at different time-points after treatment. (CCR- complete clinical remission; MCR- molecular complete remission.)

Time points	N	CCR rate	MCR rate
3–5 months	118	29	37
6–8 months	86	63	53
9–14 months	101	86	56
15–19 months	74	97	66

In the same study MCR was higher in clinical CRs, but one-third did not achieve MCR and, furthermore, one-third of partial responders at 3- 5 months also achieved a MCR. Patients who achieved a MCR during the first year of treatment had significantly longer failure free survival (FFS) than those who did not (4 year FFS=76 versus 38%; $p<0.001$). By multivariate analysis, β2 microglobulin and molecular response were independent factors predictive of outcome.

The prognostic value of MCR was also validated in the CR patients alone. Patients who achieved a MCR and sustained it had a better FFS than those who either reverted back to PCR positivity or never achieved a MCR. No correlation was observed between treatment regimen and MCR. The concordance between molecular results in PB and BM was 66–77%. More than 75% of the patients who achieved a PCR negative state at any of the tested time points were expected to be alive and in clinical CR 5 years after starting therapy (Lopez-Guillermo et al., 1998). This study demonstrated that: 1) MCR can be achieved using standard-dose chemotherapy, 2) MCRs within the first year of therapy correlate with durable CRs, 3) the

degree or extent (CR, PR) of clinical and molecular remission can improve with time and suggests that the predictive value of MCR may vary depending with the time of measurement, 4) the lack of a clear-cut plateau in FFS curves for MCR versus no MCR suggests that most patients with FL eventually progress.

2.5.2.2 Minimal residual disease after chemoimmunotherapy.

The addition of monoclonal antibodies anti-CD20 (rituximab, R) to chemotherapy represents a significant milestone in treatment of CD20 positive lymphomas in the last decade (McLaughlin et al., 1998; Maloney et al., 1997; Coiffier et al., 1998). Rituximab has been first tested in low grade and particularly follicular lymphoma. Molecular analyses of BM and/or PB were performed on subsets of FL patients in various trials and are shown in Table 2.7.

Molecular analyses have been also performed in patients with FL treated with anti-idiotypic monoclonal antibodies (Brown et al., 1989). A 66% overall response rate including 13% CR were seen in 45 patients. Of five patients with prolonged CR (from 3- 8 years) PCR detectable lymphoma was found in the PB of all five patients using methods for specific IgH rearrangement.

MCRs have been seen in patients treated with idiotype vaccines (Bendandi et al., 1999). Out of 20 patients treated, 11 were found to have an amplifiable molecular marker. Patients were first debulked using combination chemotherapy, but despite this the 11 patients remained PCR positive in their PB. However, after treatment with idiotypic vaccine together with granulocyte macrophage colony stimulating factor (GM-CSF) as an adjuvant, MCR occurred in all 11. In addition 18/20 patients remained in CR after a median follow-up of 42 months. These data

showed that molecular analyses for occult lymphoma can be a useful surrogate endpoint to assess the clinical activity of new therapies in MRD states.

Table 2.7: Molecular remission in FL patients treated with single agent or combination therapy with rituximab. (FL- follicular lymphoma; OR- overall response; CR- clinical response; BM- bone marrow; PB- peripheral blood; TTP- time to progression; FFR- failure free rate; PCR- polymerase chain reaction.)

Author	Patient population	*N*	Clinical OR/CR	Duration of response med. TTP	*N*	Molecular result
McLauglin et al. (1996)	Recurrent/ refractory FL	166	48%/6%	13 months	52	mol. remission PB=26/45 (62%); BM=9/16 (56%) at 3 months
Foran et al. (2000)	Recurrent/ refractory FL	50	46%/4%	11months	21	13/21 (62%)
Colombat et al. (2001)	Untreated FL	50	73%/20%	Not reported 10/36 relapsed at 1 year	33	7/30 (57%), 9/29 (31%) at 50 days; 16/26 (62%) at 12 months; PCR negative had improved time to progression (TTP)
Mandigers et al. (2001)	Untreated FL	34	78%/44%	23 months	28	13/28; no correlation between TTP and PCR status
Czuczman et al. (1999)	Untreated or previously treated FL	40	95%/55%	Not treated	8	7/8; 5/7 molecular remission for over two years
Rambaldi et al. (2002)	Untreated FL	128	94%/57%		77	59% (12 wks), 74% (20 wks), 63% (44 wks), improved FFR in PCR negative patients

MRD detection has also been used to evaluate other biological therapies such as interferon therapy. Thirty-five patients with previously treated FL were treated with CVP and a 3-month course of interferon-α- 2b at 3 MU/m^2 (Fernandez-Ruiz et al., 2003). Maintenance therapy with interferon was administered to 10 patients. Clinical CRs occurred in 28/35 patients (80%) and PFS at 2 years and 5 years was 91% and 78% respectively. Molecular markers were detected in 29/35 patients and of these 29 patients, 86% experienced a MCR in their BM. Of

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these 29 patients, 20 patients were in a clinical CR. Four out of five patients with a PR and MCR did not have progression. Eight out of the 20 patients with a CR had molecular relapses but no evidence of clinical relapse. Thus although this trial has shown that combination therapy with chemotherapy and interferon can produce a relatively high MCR the results of molecular assays did not correlate well with clinical status.

Rituximab induces high rates of MCR both in monotherapy and in combination with chemotherapy in other types of lymphomas including aggressive types. MCR is associated with better prognosis (Table 2.8; Solal-Celigny et al., 1999; Emmanouilides et al., 1999; Vose et al., 1999; Foran et al., 2000).

For its high efficiency in eradicating lymphoma from BM tissue rituximab has been used in monotherapy or in combination with chemotherapy for so called in vivo purging in patients who are getting mobilized prior to peripheral stem cell harvest (Gianni et al., 1998; Buckstein et al., 1999; Voso et al., 2000; Ladetto et al., 2001; Hess et al., 2002; Flohr et al., 2002; Galimberti et al., 2003). Chemoimmunotherapy leads to 93% PCR negative PBSC grafts in patients with FL or MCL while chemotherapy alone achieves 40% ($p<0.007$; Magni et al., 2000). These results are even better than results after so called high dose sequential chemotherapy, which can attain 12% of PCR negative PBPC harvests in MCL and 42% in FL. The feasibility, safety and efficacy of in vivo purging will likely result in replacing in vitro purging in the near future. Galimberti et al. compared in their small study (23 patients with FL) rituximab versus no rituximab as an in vivo purging agent prior to stem cell collection. MRD was assayed by both real-time and qualitative PCR. A total of 86% of harvests treated with rituximab were PCR negative compared with 14.3% of controls. Real-time PCR was more sensitive, although MRD negativity assessed by clasical PCR correlated with CR rate and 5 year PFS. Quantitative analysis revealed a correlation

between the amount of contamination in the harvests and relapse after transplantation, suggesting that a very low amount of contamination does not appear to negatively affect outcome. According to Buckstein 3 cycles of rituximab prior to stem cell mobilization did not eliminate PCR detectable disease in most grafts (assessed by qualitative nested PCR), but rather several log reductions in lymphoma contamination were achieved. Despite low levels of contamination, 80% have achieved and maintained MCR in serial PB and BM samples post-transplant (Buckstein et al., 1999). In addition to purging, rituximab has been also used as consolidation post-HDT and showed great potential for eradicating MRD (Horwitz et al., 2004).

Table 2.8: Induction of molecular remission in NHL patients after treatment with monoclonal antibody anti-CD20.

(LG- low grade; IG- intermediate grade; HG- high grade; FL- follicular lymphoma; MCL- mantle cell lymphoma; R- rituximab (anti-CD20); CHOP- cyclophosphamide, vincristine, Adriamycin, prednisone; Mit- mitoxantrone; Cy- cyclophosphamide; DFS- disease free survival.)

Author	NHL	Therapy	MCR	No. of relapses	Note
Solal-Celigni et al. (1999)	FL	R	57% (17/30)	6% (1/17)	In 1st year
Emmanouilides et al. (1999)	LG	Mit+ Cy+ R	71% (5/7)	Na	
Vose et al. (1999)	IG + HG	R-CHOP	85% (11/13)	Na	Expected longer DFS and OS
Foran et al. (2000)	MCL	R-CHOP	48% (11/23)	Na	Median DFS 16 months

A retrospective comparison from the former International Bone Marrow Transplant Registry (IBMTR) included 904 patients undergoing autologous and allogeneic SCT for FL found that purged autotransplants had a 26% lower recurrence risk than unpurged autotransplantation. Five year treatment related mortalities were 30, 14 and 8% and 5- year recurrence rates were 21, 43 and 58% for allogeneic, autologous purged and autologous unpurged stem cell transplants, respectively (van Besien et al., 2003). A case control study using European Bone Marrow Transplantation (EBMT) Registry data in 448 patients (100 low grade) found no significant difference in PFS for patients whose BMs were purged compared with unpurged BMs, although in a sub-group analysis patients with indolent NHL (n=100) and purged BM transplants at large transplant centers had improved OS but not PFS compared with unpurged BM recipients (p=0.0016; Williams et al., 1996).

In one retrospective study of low, intermediate and high grade lymphomas (n=65 total) treated with autologous peripheral stem cell or bone marrow cell transplantation the actuarial relapse free survival (RFS) at 5 years for patients who received tumor negative grafts (Southern Blot BCL-2/J, sensitivity 0.01%) was 64 and 57% compared with 17% if the BM harvest was histologically negative but MRD contaminated (Sharp et al., 1996).

2.5.2.3 Minimal residual disease after high dose chemotherapy.

The clinical situation in which MCR has been most extensively investigated and validated for prognostic importance is post- HDT. HDT followed by ASCT has been successful in attainment of PCR negativity with a broad range of efficiency between 42- 70% (Zwicky et al., 1996; Corradini et al., 1997; Horning et al., 2001; Gribben et al., 1993; Andersen et al.,

1997). It has been observed that patients with FL who had myeloablative chemotherapy and autologous transplant had improved DFS if their PB and BM became PCR negative. Ongoing MCR is strongly predictive of continued CR (Ladetto et al., 2002 and 2008; Apostolidis et al., 2000; Sharp et al., 1996; Hardingham et al., 1995; Corradini et al., 1997). Several prospective studies are listed in Table 2.9.

Probably the longest experience with MRD monitoring in the ASCT setting comes from the Dana Farber Cancer Institute (DFCI). Gribben et al. were one of the first to publish that immunological purging of autografts in addition to high dose therapy leads to high rates of MCR in patients with low and intermediate grade lymphomas (Table 2.10; Gribben et al., 1993). This was followed by reports from Freedman et al (1999 and 2000) who transplanted 153 patients with relapsed and heavily pretreated FL with ASCT following Total Body Irradiation (TBI) conditioning and in vitro purging with monoclonal antibody and complement. Only 30% were in clinical CR at BM harvest and overt BM infiltration was present in 47%. Actuarial DFS and OS at 8 years were 42% and 66%, respectively and 12 year survival from diagnosis was 69% suggesting a prolongation of survival with HDT and ASCT compared with historical controls. Patients with successfully purged BM grafts as measured by PCR for bcl-2/IgH rearrangement experienced longer freedom from recurrence than those whose BM remained PCR positive. PCR positivity in the graft increased the risk of failure to 11.7 times that of patients who were PCR negative. Continued PCR negativity in follow-up samples was also strongly predictive of continued CR. Virtually all relapses occurred in the first 4 years after ASCT, suggesting that a subset of patients was cured (Freedman et al., 1999).

Table 2.9: Significance of MRD in patients undergoing ASCT. Abbreviations used: MCR, molecular remission; CR, complete remission; PFS, progression free survival; PR, partial remission; DFS, disease free survival; BM, bone marrow; OS, overall survival; ASCT, autologous stem cell transplant; HDT, high dose sequential chemotherapy; MAb+C, monoclonal antibody+complement; Cy/TBI, high dose cyclophosphamide and total body irradiation; f/up, follow-up; PBSC, peripheral blood stem cells, pts- patients.

Reference	Brice et al (2000)	Moos et al (1998)	Freedman et al (2000)	Ladetto et al (2002)	Hardingham et al (1995)
N	153 relapsed	99 relapsed	92 first remission	47 (25 first remission)	28 relapsed or progressed
Stem cell source	BM	BM	PBSC	PBSC	PBSC
Purging	In vitro MAb+C	In vitro MAb+C	In vivo HDT	No	No
Pts in CR at ASCT (%)	36	38	88	46	14
Conditioning	Cy/TBI	Cy/TBI	Mitoxantrone+ melphalan	Cy/TBI	BEAM
DFS % (years)	42 (8)	63 (5)	67 (4)	40 (4)	33 (2)
OS % (years)	66 (8)	69 (5)	84 (4)	86 (10)	54 (2)
CR status prognostic?	No	No	Yes 24/81 in CR and 5/6 in PR relapsed	NA	NA
MCR (%):					
Graft	43	31	47	28	48
BM f/up	54	64	65	56	68
MCR prognostic for PFS in:					
Graft	Yes	Yes	No	No	No
BM f/up	Yes	Yes	Yes	Yes	Yes

Apostolidis and colleagues transplanted 99 patients with recurrent FL as consolidation of second or subsequent remission. 5- year FFS and OS were 63% and 69%, respectively. For patients with measurable disease, the absence of a PCR detectable bcl-2/IgH rearrangement

during follow-up was associated with a significantly lower risk of recurrence (hazard ratio, HR= 0.13, p<0.001) and death (HR= 0.25; p= 0.02). PCR status of reinfused BM did not correlate with outcome. These results, in keeping with those from the DFCI following HDT and from the MD Anderson Center after conventional chemotherapy lends support to the concept that MCR is a goal worth achieving since it correlates with prolonged remission (Apostolidis et al., 2000).

Table 2.10: Analysis of MRD in patients with low grade (LG) and intermediate grade (IG) NHL treated by ASCT (Gribben et al., 1993). (cont. PCR- continuous PCR negativity, cont. PCR+ continuous PCR positivity, PCR+ → PCR- patients who converted to PCR negative, PCR- → PCR+ patients who converted to PCR positive, PCR+/PCR- patients with mixed results during follow up)

After ASCT	Cont. PCR-	Cont. PCR+	PCR+ → PCR-	PCR- → PCR+	PCR+ / PCR-
Patients	43% (58)	26% (35)	14% (19)	10% (14)	6% (8)
Relapses	0%	71% (25)	0% (19)	47% (6)	25% (2)

In one Australian study the DFS of high risk NHL patients (46% low grade) was significantly shortened in patients in whom lymphoma cells were detected by PCR in serial BM samples post-transplant and these patients had a 24-fold higher risk of relapse (p=0.03). Presence of PCR positive cells in the graft did not influence OS (Hardingham et al., 1995). Ladetto and colleagues, in a prospective multicentre study, evaluated intensified purging-free high-dose sequential chemotherapy with peripheral autografting in 92 previously untreated patients aged <60 years with advanced stage FL. A total of 87% completed the planned treatment schedule. CR was 88%, while OS and DFS at 4 years were 84% and 67%, respectively. PCR negative

harvests were collected in 47% of cases (Ladetto et al., 2002, Corradini et al., 1997). Following autograft, 65% of the evaluable patients achieved CR and MCR. The DFS of this group was 85%. The DFS for PCR negativity versus positivity in graft or in follow-up showed statistically different curves. Only 12% of those achieving PCR negativity post-ASCT relapsed compared with a relapse rate of 77% in those who failed to achieve molecular remission.

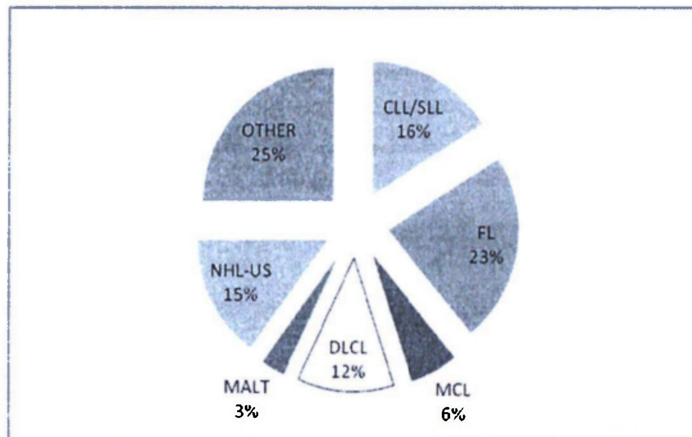
Recently Coiffier et al. presented retrospective data from two trials (GELF-86 and GELF-94) showing that treatment of relapsed FL represented by a combination of salvage chemotherapy and rituximab followed by HDT was superior to rituximab-containing chemotherapy alone (Coiffier et al., 2007). These observations were recently expanded by publication of the first randomized trial using PCR results in FL patients (Italian GITMO ILL trial), in which the molecular data were evaluated prospectively. The authors reported that achieving MCR is critical for effective disease control, regardless of which treatment is used, but they also stated that the high dose sequential therapy with rituximab ensures superior disease and molecular outcome over standard chemoimmunotherapy (Ladetto et al. 2008).

3. MOLECULAR EVALUATION OF PATIENTS WITH B-CELL LYMPHOPROLIFERATIVE DISORDERS TREATED AT OUR INSTITUTION.

3.1. SAMPLES OF PATIENTS SUBMITTED FOR MOLECULAR EVALUATION

The study was performed using samples from patients who were diagnosed and treated for indolent B-cell lymphoproliferative disorders at the 1st Department of Internal Medicine at Charles University General Faculty Hospital in Prague, Czech Republic.

Figure 3.1: Overview of diagnoses from samples submitted for PCR evaluation. CLL/SLL- chronic lymphocytic leukemia or small lymphocytic lymphoma; FL- follicular lymphoma, MCL- mantle cell lymphoma; DLCL- diffuse large cell lymphoma; MALT- mucosa associated lymphoid tissue lymphoma; NHL-US- nonhodgkins lymphoma, unspecified; other- other diagnosis.



Between November 1999 and July 2002 there were 945 samples submitted for PCR detection of molecular markers (translocation t(14;18) and translocation t(11;14) and also

CDRIII rearrangement) from 432 patients, 350 were new patients. The actual histological subtypes and molecular markers are summarized in Figure 3.1, Tables 3.1 and 3.2

Table 3.1: The number of PCR positive and negative results in submitted material. (LN- lymphnode; BM- bone marrow; PB- peripheral blood; PBPC- peripheral blood progenitor cells; IgH-CDRIII- immunoglobulin heavy chain gene- complementarity determining region III; MBR- major breakpoint region; MTC_ major translocation cluster; other tissues such as spleen, skin or gastric biopsies are not included).

Positive result	Diagnostic material			
	LN	BM	PB	PBPC
IgH-CDRIII	28	183	74	26
t(14;18)-MBR	3	44	9	1
t(11;14)-MTC	3	14	7	2
Negative	27	362	117	42
Total tested	61	603	207	71

Our institution has a large referral base and these results include all submitted samples. They include patients who were suspected to have a lymphoproliferative disorder, but then were diagnosed with either different malignancy or malignancy may have been ruled out completely. These results also include referred patients whose samples were sent only one time and they were lost to follow up. This explains the relatively low percentage of detected molecular markers.

Table 3.2: Detection of molecular markers in particular diseases. (CLL/SLL- chronic lymphocytic leukemia or small lymphocytic lymphoma; FL- follicular lymphoma, MCL- mantle cell lymphoma; DLCL- diffuse large cell lymphoma; NHL-US- nonhodgkins lymphoma, unspecified; MALT- mucosa associated lymphoid tissue lymphoma; other- other diagnosis; CDRIII- complementarity determining region III; MBR- major breakpoint region; MTC- major translocation cluster).

Disease	Percentage of detected molecular markers		
	CDRIII	t(14;18)-MBR	t(11;14)-MTC
CLL/SLL	88.7	0	0
FL	42.2	20.6	0
MCL	65.5	0	26.0
DLCL	34	0	0
NHL-US	12.5	3.1	0
MALT	7.1	0	0
Other	19.6	0.9	0

We have noticed a significant increase of diagnostic samples submitted for PCR evaluation. The molecular biology laboratory received 243 samples in the year 1999, 295 samples in the year 2000 and 553 in the year 2001. It was projected that in the year 2002 there would be approximately 350 samples sent for PCR testing. This together with the fact that the molecular biology laboratory was internationally accredited (namely by the Royal College of Pathologists of Australasia Quality Assurance Programs Pty Ltd) I see as one of positive outcome of work related to my thesis. The awareness of molecular testing and monitoring of MRD increased rapidly among the clinicians and thus I feel that aim one of my PhD work was accomplished.

3.2. CORRELATION OF MINIMAL RESIDUAL DISEASE AND CLINICAL OUTCOME

Because of the difficulties mentioned above we were able to follow longitudinally 57 patients with indolent lymphoproliferative disorders who had molecular marker detectable by PCR. These patients represent the core of the first manuscript in the Appendix, which is presented in its entirety (see Appendix I). Main points will be discussed here briefly.

We have evaluated the impact of molecular remission on clinical outcome. We have also assessed the effect of treatment approach on patients' outcome. For the purpose of this study we have therefore excluded also all patients who received treatment with rituximab as a salvage therapy after they failed previous ASCT. This was another reason why we had only 57 patients available for our evaluation. The first group of patients was treated with rituximab based chemoimmunotherapy and the second group received rituximab based chemoimmunotherapy followed by ASCT. The treatment groups were not randomized and the decision whether ASCT was pursued was made by treating physician. Since PBPC were available for MRD evaluation, we also assessed the implications of MRD contaminated autografts on outcome.

Failure to achieve molecular remission was identified as independent risk factor regardless of treatment modality (HR for PFS 3.489 (1.7071-7.1309), p= 0.001, and HR for OS 4.6848 [1.6071-13.6567], p= 0.005; see Appendix I- Table 4). Patients with disease detectable by PCR had shorter PFS in contrast with patients in MCR after rituximab (median 0.75 and 2.5 years respectively; p=0.006; see Appendix I- Figure 3A) or patients in MCR after rituximab followed by ASCT (median 3.3 years; p=0.0032). PCR positive patients had a 5-year OS of only 40% compared to a 5-year OS of 76% for PCR negative patients after rituximab (p=0.0186) and 86% PCR negative patients after rituximab followed by ASCT (p=0.003; see Appendix I- Figure 3B).

All 9 patients transplanted with PCR positive grafts relapsed (p=0.0023) with shorter PFS, but had still excellent OS (p=0.0008; see Appendix I- Figure 5). Rituximab based therapy induced MCR in 25 (64%) compared to 18 (100%) patients after rituximab followed by ASCT (p =0.0025). We observed no difference in PFS between transplant group (3.3 years) and rituximab based treatment (1.9 years), but the 5- year OS of patients with transplant was 86% and 62% respectively (p= 0.041; see Appendix I- Figure 1).

The study conclusions were: patients with indolent B-LPDs who achieve MCR have better prognosis; not attaining MCR was a strong predictor for relapse and shorter OS. Rituximab based therapy induces MCR in high number of patients, which can be further improved by ASCT and those patients then have an excellent outcome. Patients with PCR positive PBPC harvest are at high risk of relapse after ASCT.

3.3. OBSERVATIONS FROM SEQUENCING OF MOLECULAR MARKERS

We have reported the use of comparative duplex PCR for monitoring of MRD is feasible (Slavickova et al. 2005; see Appendix II). We planned to employ real-time PCR eventually. It became clear that in order to do so we needed to know the specific sequences of each patient's molecular marker. Otherwise the sensitivity of our MRD evaluation would decrease. The sequencing project was not completed before the end of my presence at the institution; however we were able to report some interesting observations from sequencing several patients with so called biallelic rearrangements (Cerny et al. 2004) and also a few case reports using sequencing results (Cerny et al. 2003a, 2003b and 2003c). These articles are in Appendix III-VI.

In the study with biallelic rearrangements we have reported a group of patients with CLL/SLL who had both alleles for the immunoglobulin heavy chain genes rearranged (bilgH) and detectable by PCR. Although we have observed some clinical differences such as increase incidence of autoimmune disorders in patients with bilgH the presence of bilgH had no impact on patients' outcome as measured by PFS or OS. This study confirmed that true biclonalities are very rare, but biclonalities should be ruled out as they may have prognostic and therapeutic implications. The presence of additional PCR band significantly affects the sensitivity of PCR and this should be taken into consideration for purposes of quantitative MRD monitoring. We have also confirmed that biclonality ought to be ruled out by a combination of at least two methods.

4. CONCLUSIONS

The achievement of molecular remission is a realistic goal in patients with B-LPDs and should be the aim of treatment especially for young patients and patients that have unfavorable prognostic factors. Our observations suggest that molecular emission is a strong predictor of prolonged survival. We have also seen that more intensive therapy ensures superior outcome. Similar observations were recently reported by a randomized, multicenter phase III trial in patients with FL, which was the first trial to prospectively explore the value of MCR as a prognosis predictor and to determine whether treatment intensity showed any difference in patients' outcome (Ladetto et al., 2008). While awaiting the data from other ongoing studies to mature and quantify the relevance of MRD and OS, efforts should be focused on making flow cytometry and/or molecular monitoring of MRD routinely available to most centers and further optimizing and standardizing these sensitive and sophisticated methods. An upcoming challenge will be to revise the current response criteria accordingly. The evaluation of MRD and its physiologic relevance will need to be incorporated in the guidelines for the management of lymphoproliferative disorders.

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APPENDICES:

RELATED MANUSCRIPT:

I. Rituximab based therapy followed by autologous stem cell transplantation leads to high rates of polymerase chain reaction (PCR) negativity and superior outcome in patients with indolent B-cell lymphoproliferative disorders.

RELATED PUBLICATIONS:

II. The use of comparative duplex PCR in monitoring of patients with non-Hodgkin´s lymphoma (NHL) and chronic lymphocytic leukemia (CLL). Folia Biologica 2005; 51: 177-179.

III. Biallelic IgH rearrangements in patients with indolent lymphoproliferative disorders: molecular and practical implications. Journal of Cell Physiol 2004; 199: 217-226.

IV. Familial chronic lymphocytic leukemia. Haematologica 2003; 88: 1190-1191.

V. Unusual sequence of VDJ rearrangement revealed by molecular analysis in a patient with indolent lymphoma. Haematologica 2003; 88: (05) ECR15.

VI. Chemotherapy alone may lead to a PCR negative stem cell harvest in transformed lymphoma refractory to rituximab. Haematologica 2003; 88: (05) ECR06.

VII. The significance of minimal residual disease and methods of its detection in patients with hematological malignancies. (Czech), review in The Journal of the Czech and Slovak Oncological Societies (Klinicka Onkologie), 2003; 16: 2: 43-50.

VIII. Clinical importance of semi-quantitative monitoring of lymphomas using the comparative polymerase chain reaction. (Czech) Cas Lek Ces, 2002; 141 (23): 735-738.

Rituximab based therapy followed by autologous stem cell transplantation leads to better outcome and high rates of PCR negativity in patients with indolent B-cell lymphoproliferative disorders.

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Running title: PCR negativity after R-ASCT

Funded by: Grants: MSMT CEZ: J13/98:1111/00004 and GAUK 203580

Keywords: **Follicular lymphoma, mantle cell lymphoma, Chronic lymphocytic leukemia, prognosis, molecular remission, PCR**

ABSTRACT:

Autologous stem cell transplantation and rituximab based therapy represent effective treatments of indolent B-cell lymphoproliferative disorders that often induce molecular remission. We assessed the impact of molecular remission after treatment on prognosis of 57 patients with indolent B-cell lymphoproliferative disorders. We also evaluated the impact of therapy on patients' outcome. Failure to achieve molecular remission was identified as independent risk factor regardless of treatment modality. Patients with disease detectable by PCR had shorter progression free survival in contrast with patients in molecular remission after rituximab (median 0.75 and 2.5 years respectively; p=0.006) or patients in molecular remission after rituximab followed by autologous stem cell transplantation (median 3.3 years; p=0.0032). PCR positive patients had a 5-year overall survival of only 40% compared to a 5-year survival of 76% for PCR negative patients after rituximab (p=0.0186) and 86% PCR negative patients after rituximab followed by autologous stem cell transplantation (p=0.003). All 9 patients transplanted with PCR positive graft relapsed (p=0.0023) with shorter progression free survival (p=0.0008). Rituximab based therapy induced molecular remission in 25 (64%) compared to 18 (100%) patients after rituximab followed by autologous stem cell transplantation (p =0.0025). We observed no difference in progression free survival between transplant group (3.3 years) and rituximab based treatment (1.9 years), but the 5- year overall survival of patients with transplant was 86% and 62% respectively (p= 0.041). Patients with indolent lymphoproliferative disorders who achieve molecular remission have better prognosis. Rituximab based therapy induces molecular remission in high number of patients, which can be further improved by autologous stem cell therapy and patients have an excellent outcome. Patients with PCR positive harvest are at high risk of relapse after autotransplantation.

INTRODUCTION

Follicular lymphoma (FL), mantle cell lymphoma (MCL), small cell lymphocytic lymphoma (SLL) together with chronic lymphocytic leukemia (CLL) represent most frequent subtypes of indolent B-cell lymphoproliferative disorders (B-LPD). Indolent B-LPDs are characterized typically with widespread disease at the time of diagnosis. A high percentage of patients achieve complete remission (CR) after treatment of this disease, however it is currently incurable by conventional chemotherapy. Relapses are the primary reason for treatment failure. It is generally accepted that minimal residual disease (MRD) contributes to relapses of hematological malignancies. [1] The situation with no detectable MRD is called molecular complete remission (MCR), and is defined as disappearance of previously detectable molecular marker. MCR correlates with better clinical outcome. Only a limited number of studies have shown that MCR (or PCR negativity) can be achieved by a standard chemotherapy alone. [2] On the other hand, intensive sequential chemotherapy or high dose therapy (HDT) with autologous stem cell transplantation (ASCT) can induce PCR negativity in a significant number of patients with indolent lymphomas. [3-5] In these studies, PCR negative patients have significantly better disease free survival than PCR positive patients (at 12 years: 85% vs 20%). [6,7] It has been also demonstrated that monoclonal antibody anti-CD20 (rituximab) can induce MCR in significant number of patients with lymphomas (both indolent and aggressive). [8-11] The patients who achieved MCR after first line therapy had a better clinical outcome that those patients that did not. [12] Because of reported high efficiency in eradicating lymphoma from BM, rituximab has been used in monotherapy or in combination with chemotherapy for vivo purging of patients getting mobilized prior to peripheral stem cell harvest. [13,14] This raises the following question. Is molecular remission an independent prognostic factor for patients with indolent B-LPD, or does a

particular type of therapy influence the patient's outcome? Our study assessed the prognostic impact of PCR status after treatment in patients with indolent B-LPDs. We have compared the efficacy of rituximab based therapy (with or without standard chemotherapy) versus rituximab based therapy followed by HDT and ASCT in achieving MCR. We have also evaluated the impact of contamination of autografts by tumor cells as detected by PCR. MRD was evaluated by a combination of molecular markers: translocation t(14;18) for FL patients [15] and translocation t(11;14) in patients with MCL [16] and complementarity determining region III (CDRIII) rearrangement for heavy chain immunoglobulin gene (IgH) in patient with CLL and in all patients who do not have t(14;18) or t(11;14) detectable by PCR. [17]

DESIGN AND METHODS

Patients

Data was collected on 57 patients diagnosed with an indolent B-LPD between November 1999 and July 2002, who had PCR detectable molecular markers. The patients received treatment at 1st Department of Internal Medicine, Charles University, General Faculty Hospital, Prague, Czech Republic. The first group of patients was treated with either rituximab based therapy (rituximab alone and/or rituximab with chemotherapy) deemed treatment group R. The second group of patients received rituximab based therapy followed by autologous stem cell transplantation (ASCT). Patients were treated based on the decision of treating physician. Patients with chronic lymphocytic leukemia were grouped together with patients that had small cell lymphoma and will further be referred to as CLL. CLL patients were not part of comparisons of lymphoma specific characteristics (e.g. International prognostic index).

Clinical Monitoring

Baseline evaluation included clinical examination, radiography of the chest, computed tomography (CT) of the chest, abdomen, and pelvis, and unilateral bone marrow biopsy. Laboratory testing included routine hematology, serum chemistries, serum immunoglobulin levels, lactate dehydrogenase, and beta-2-microglobulin assays of blood and urinalysis. The patients had hematology and serum chemistry evaluations together with full tumor restaging after completion of treatment every 3 months within the first year and every 6-12 months thereafter. The clinical and molecular monitoring was performed before therapy and during the follow up period until relapse or progression occurred.

Therapy:

Monoclonal antibody anti-CD20 (rituximab, Mabthera®) was given at a dose of 375 mg/m^2 by intravenous infusion for a total of 4 dosages (days 1, 8, 15, 22) on an outpatient basis. The drug was administered according to standard manufacturer's guidelines. In cases when rituximab was given together in combination with a standard chemotherapeutic regimen, it was administered on the first day of the combination. The chemotherapeutic regimens used were ESAP (methylprednisolon, etoposide, cisplatin, cytarabine; n=18), Flu-Cy (fludarabine, cyclophosphamide; n=8), Flu (fludarabine; n=2), IVE (ifosphamide, etoposide, epirubicin; n=2), CHOP (cyclophosphamide, doxorubicin, vincristin, prednisone; n=2), CVP (cyclophosphamide, vincristin, prednisone; n=2), ICE (ifosphamide, etoposide, carboplatin; n=1) or PACEBO (ifosphamide, etoposide, epirubicin; n=1). Groups were not randomized and treatment decisions were made based on patient's clinical situation by the treating physician or the patient.

High dose therapy

The is defined as the following preparative regimens: BEAM 200 (BCNU, etoposide 200mg/m^2, cytarabine, melphalan) in 6 patients or BEAM 400 (BCNU, etoposide 400mg/m^2, cytarabine, melphalan) in 12 patients.

Molecular detection of oncogene-JH gene or complementary determining region (CDRIII) rearrangements before during and after therapy.

The molecular studies were performed on lymph node samples (LN), bone marrow samples (BM) and/or, peripheral blood samples (PB) at the time when disease was active (diagnosis, progression, relapse detected by FACS or histology). BM and PB samples were tested during the follow up period and peripheral blood progenitor cells (PBPC) were tested prior to transplantation. All samples were collected and DNA was extracted using standard procedures with precautions to avoid cross-contamination. For detection of t(14;18), approximately 1 mg of DNA was amplified using MBRA-(TATGGTGGTTTGACCTTTAG) specific nucleotide together with JH-(ACCTGAGGAGACGGTGACC) oligonucleotide and RedTaq polymerase (Sigma, Prague, Czech Republic). For detection of t(11;14), approximately 1 mg of DNA was amplified using MTC C-(CTACTGAAGGACTTGTGGGTTGCT) specific nucleotide together with JH-(ACCTGAGGAGACGGTGACC) oligonucleotide and RedTaq polymerase (Sigma, Prague, Czech Republic). We used a touchdown PCR technique that has been described previously. [18]

Samples in which the PCR did not detect oncogene-JH rearrangement were further assayed for the presence of CDRIII rearrangement. For each CDRIII detection reaction, approximately 1 mg DNA was amplified using FR3A-(ACACGGCYSTGTATTACTGT) oligonucleotide,

together with JH-(ACCTGAGGAGACGGTGACC) oligonucleotide and RedTaq polymerase (Sigma, Prague, Czech Republic). Each reaction included: 5 min at 94°C followed by 30 cycles of 94°C 45 sec, 58°C for 1 min 30 sec, 72°C for 1 min 30 sec, followed by 72°C for 7 min.

Each reaction contained positive (CDRIII, MBRA, or MTCC positive DNA) and negative control (normal DNA isolated from PB of healthy individuals). PCR products were visualized on agar gel stained with ethidium bromide. Under these conditions, the sensitivity of CDRIII detection was 10 positive cells in 10^5 normal cells, the PCR detection of t(14;18) or t(11;14) had higher sensitivity (10 or less positive cells in 10^6 normal cells).

Definitions

Complete remission (CR) and/or partial response (PR) in lymphoma patients were scored according to the criteria reported by Cheson et al. [19] The National Cancer Institute (NCI)-sponsored Working Group guidelines were applied for the definition of response and time to progression in patients with CLL (with 2 minor modifications: To be considered a complete remission (CR) or partial remission (PR), the patient must maintain the response criteria for at least 1 month, and down-staging of a stage must occur to define a remission). [20]

Disease status at the time of rituximab therapy was as defined as follows: 1- previously untreated or 1st partial remission; 2- 1st progression or 1st relapse; 3- 2nd partial remission or 2nd relapse; 4- 2nd and higher progression or resistant disease (Tables 1-3).

Statistical analysis:

Comparisons of clinical characteristics between individual groups of patients were performed with non-parametric Wilcoxon and Mann- Whitney tests. The differences were tested at the level

of significance 0.05 (*p*-value). We have also performed a Cox regression or stepwise Cox regression analysis (multivariate analysis) of clinical characteristics and tested whether they had an impact on patients' prognosis (overall survival or progression free survival). The survival curves were estimated by the method of Kaplan and Meier and compared by the log rank test.[21]

RESULTS:

Baseline descriptive values

57 patients with B-LPD had detectable molecular marker by PCR. Their median age was 55 years (23 to 76 years). There were 28 females (49%). Eighteen (32%) patients were diagnosed with CLL, 27 (47%) had FL and 12 (21%) patients were treated for MCL. The most frequent molecular marker was CDRIII in 30 patients (53%), followed by t(14;18) in 20 patients (35%) and in 7 (12%) patients t(11;14) was used. The majority (87%) of patients was diagnosed with advanced disease (stage IV: 76% and stage III: 11%). B symptoms were present in 42% cases at the time of diagnosis. When calculating international prognostic index (IPI) for patients with lymphoma, we found that IPI was 1,2 and 3 in 53%, 36% and 11% of patients respectively. Approximately two thirds (69%) of patients had untreated disease, 1st partial remission (PR) or 1st progression (Prog) or relapse (Rel) at the time of study entry. Remaining patients (31%) had 2nd PR (or 2nd Rel) or 2nd and higher Prog or resistant disease (RD). Consequently 61% patients received less than 2 lines of previous therapy. Thirty nine (68%) patients received R based therapy and were then monitored and eighteen (32%) patients completed treatment with R +/- chemotherapy followed by ASCT. Forty five (79%) patients achieved complete remission (CR) as best response to therapy. The total

of 43 patients (75%) attained MCR after treatment. We have observed 38 (67%) progressions or relapses and 16 (28%) deaths during the follow up period (media 4.7 years). See alsoTable 1.

Comparison of baseline clinical characteristics risk factor analysis based on PCR status.

The impact of different variables was assessed using univariate (see Table 2) and multivariate analysis (Table). Cox regression analysis showed that only no MCR had an adverse impact on OS. Also each additional line of pretreatment had a stepwise impact on shorter OS (see Table 4). When analysis was performed without considering PCR results then no CR was a strong predictor for adverse outcome. The stepwise impact on shorter OS by each additional line of pretreatment was similar (see Table 4). All these risk factors were even stronger predictors for shorter PFS (see Table 4). Taken together, these data suggest that successful induction of MCR early in the course of disease correlates with longer survival.

The group of 43 patients who became PCR negative after treatment did not differ from patients who remained PCR positive with regards to gender, and age (see Table 2). There were also no statistical differences in clinical stage, number of previous treatments, and disease status at the time of rituximab between both groups of patients (PCR negative vs PCR positive). A univariate analysis showed that majority of PCR negative patients had FL (36; 60.5%), CLL (9; 64.3%) patients represented majority of PCR positive patients (p=0.00141). Attainment of MCR was easiest among patients with t(14;18) (95%) followed by patients with CDRIII marker (66%) and then patients with t(11;14) (57%; p=0.0361). Only 13 (34.2%) PCR negative patients experienced B symptoms when compared to 7 (70%) of PCR positive patients at the time of therapy initiation (p=0.047). All 43 (100%) PCR negative patients achieved CR as a best result of therapy compared to only 2 PCR positive patients (14.3%; p<0.00001). The total of 25 (58.1%) PCR

negative patients experienced disease relapse/progression while 13 (92.9%; p=0.0216) patients with PCR positivity developed disease relapse/progression. The total of 8 (18.6%) PCR negative patients died whereas there were 8 (57.1%) deaths among the PCR positive patients during follow up period. Taken together, these data show that patients with MCL and or CLL are less likely to achieve MCR when standard chemoimmunotherapy is used. Not surprisingly patients with detectable disease recur faster, more often and eventually succumb to their disease more likely than patients who achieved MCR.

Comparison of baseline clinical characteristics risk factor analysis based on the type of therapy.

Despite no randomization there were no statistical differences between the R and R-ASCT in following patients' clinical characteristics: age, sex, histological diagnoses, molecular markers, clinical stage, presence of B symptoms, international prognostic score, number of previous treatments, disease status at time of therapy, relapse/progression rate or number of deaths. We did not observe any difference in outcome whether rituximab was given alone or together with chemotherapy, which further justified our plan to group these patients together in one group.

The group of patients treated with R based therapy included 39 patients (18 women, 46.2%). They were 23 to 76 years of age (median 57). The group of patients treated with R therapy followed by ASCT included 18 patients (10 women, 55.6%). They were 38 to 62 years of age (median 53). R-ASCT induced complete remission in all 18 (100%) patients; all 18 (100%) patients were also PCR negative. Rituximab based therapy induced 27 (69.2 %; p< 0.0214) complete remissions, and 25 patients were PCR negative (64.1%; p= 0.025). Further details are in a Table 3. In vivo purging achieved 8 (47%) PCR negative PBPC harvest prior ASCT. These data suggest that

chemoimmunotherapy induces high rates of MCR, which can be further improved when followed by ASCT.

Survival analysis based on PCR status after therapy and on type of therapy.

The median follow up of living patients was 4.7 years (0.3-6.3 years). There were 38 (67%) relapses/progressions and 16 deaths (28%) recorded. The PFS was not affected by histological diagnosis, molecular marker, age at time of treatment, sex, clinical stage and IPI. There was no difference in PFS between R-ASCT (3.3 years) and R (1.9 years; Figure 1A). The 5- year overall survival was 86% and 62% for R-ASCT patients and R patients respectively (p= 0.041; Figure 1B). We did not observe difference in OS based on histological diagnoses, molecular marker, number of previous therapies, age at time of treatment, sex, clinical stage and IPI.

Patients that became PCR negative had significantly better PFS (median 3.1 years) than patient with PCR positivity (median 0.75 years; p=0.0006; Figure 2A). This resulted in an improved 5-year survival 80% and 40% for PCR negative and PCR positive patients respectively (p= 0.001; Figure 2B). Survival analysis based on both PCR status and type of therapy showed that patients who remained PCR positive did poorly in contrast with PCR negative pts after R (median PFS 0.75 and 2.5 years respectively; p=0.006) or PCR negative pts after R-ASCT (median 3.3 years; p=0.0032; Figure 3A). PCR positivity rendered 5-year overall survival of 40% when compared to 76% for PCR negative pts after R (p=0.0186) and 86% PCR negative pts after R-ASCT (p=0.003). There was no difference in PFS or OS of PCR negative patients who received R or R-ASCT (Figure 3B). Presence of B symptoms at the time of initiation of therapy correlated with significantly shorter PFS (median 1.3 years) compared to symptom free patients (median 3.2 years; p=0.0058; Figure 4A). B symptoms were also connected with significantly shorter OS

compared to symptom free patients (5- year OS 10% AND 44% respectively; *p* =0.0412; Figure 4B). Patients that achieved CR had a longer PFS (3.1 years) when compared to patients with less than CR (0.6 years; $p<0.0001$). This translated into a better 5-year OS 78% and 38% for patients in CR and less then CR respectively (*p*= 0.0008). We have also noticed that more pretreated patients (2 or more lines of previous therapy) were more likely to experience disease relapse/progression than patients with less than 2 previous treatments with PFS 1.4 years and 3.2 years respectively (p= 0.0065). The 5-year OS was not different between patients with 2 or more and less than 2 previous lines of therapy; it was 78% and 58% for patients respectively.

The implications of PCR positive PBPC harvest

The total of 17 PBPC harvests were available for evaluation by PCR. The total of 8 (47%) of the harvests were PCR negative. All 9 (100%) patients who were transplanted with PCR positive PBPC autograft relapsed compared to only 2 (25%) relapses in patients who were transplanted with PCR negative PBPC (*p*=0.0023). Patients who received contaminated PBPC also had significantly shorter PFS (44.4 % at 3 years) compared to patients with PCR negative autografts (85.7% at 3 years, *p*= 0.0008; Figure 5A). However the OS was not statistically different between both groups of patients (75% vs 100% at 5 years; *p*=ns; Figure 5B).

In univariate analysis the PBPC contamination was not affected by any of the following clinical characteristics: sex, age at time of therapy, histological diagnosis, molecular marker, clinical stage, presence of B symptoms, IPI, number of previous therapies, and disease status at the time of rituximab initiation. These data suggest that patients transplanted with contaminated PBPC may still achieve not only clinical remission, but even MCR. They however always relapse. At the same time the OS of these patients remains excellent.

DISCUSSION:

In order to cure lymphoproliferative disorders, residual disease at the molecular level must be eradicated. This is demonstrated by PCR negativity. [22] Major advantages coming from the introduction of PCR monitoring include the rapid evaluation of the anti-tumor activity and early identification of patients with high-risk of disease recurrence. This is particularly important in the era of monoclonal antibodies, which may induce PCR negativity without adding significant toxicity. While it is known that cells with t(14;18) (specific for FL) may be detected in the peripheral blood of healthy individuals without causing lymphoma [23] and some patients with follicular lymphomas remain in long remission despite PCR positivity [24] there is a growing number of studies which have shown that patients who achieve molecular remission have better prognosis. [4,6,12,25,26] Coiffier et al. presented retrospective data from two lymphoma trials (GELF-86 and GELF-94) showing that treatment of relapsed FL represented by a combination of salvage chemotherapy and rituximab followed by HDT was superior to rituximab-containing chemotherapy alone. [27] These observations were recently expanded by publication of the first randomized trial using PCR results in FL patients (Italian GITMO ILL trial) The authors reported that achieving MCR is critical for effective disease control, regardless of which treatment is used, but they also stated that the high dose sequential therapy ensures superior disease and molecular outcome over standard chemoimmunotherapy. [25] While our study examined a small and relatively heterogeneous group of patients, we were able to confirm that attaining MCR after treatment is a strong prognostic factor. Our observations are in harmony with the studies of our French [27] and Italian colleagues.[25] Patients who achieved PCR negativity had better outcome regardless the therapy, and the combination of chemoimmunotherapy followed by HDT achieves MCR in a high number of patients.

We have observed longer OS in our patients with PCR negativity. Interestingly, their PFS is similar to PCR positive patients. This is an intriguing observation; we will discuss several factors that may have contributed this situation.

The addition of rituximab to current treatment regimens has already changed the management of CD20 positive B-LPDs. Rituximab based therapy has been used as an in vivo purging method, which by itself leads to high rates of MCR. [25,28,29] In our experience, combination of rituximab with chemotherapy induced PCR negativity in approximately two thirds of patients. We have noticed that OS and PFS of patients who were in MCR after R based treatment was not different from patients who completed R-ASCT. Based on these observations, one could perhaps consider harvesting PBPC at that moment and then to hold off with high dose therapy followed by ASCT until the time of progression or relapse. Our results also confirm previous reports [13,30] showing that patients with low levels of contamination can still achieve and maintain MCR in serial PB and BM samples post-transplant and that level of contamination (assessed by PCR) correlated with risk of relapse after transplantation. [30] In our experience, all patients that received PCR positive PBPC relapsed. These relapses contributed to a relatively high relapse rate in R-ASCT group (61.1%), which was similar to therapy with R group (61.1%). A similar observation was previously made from a sub-group analysis of a 100 low grade lymphomas from the European Bone Marrow Transplantation (EBMT) study when there was no difference in PFS for patients with purged autografts compared to unpurged autografts. However patients with purged autografts had improved OS compared with unpurged autograft recipients (p=0.0016). [31]

All patients in our study that were transplanted with positive PBPC achieved PCR negativity (in BM or PB), but eventually relapsed (median 2.24 years). It is very plausible that their relapses

occurred from the contaminated PBPC. In such case the contaminating cells may have homed initially to tissues other than BM and did not circulate through PB until relapse. Another possible explanation may be that these cells may remain dormant at levels below PCR detection for a long period of time prior to relapse.

The relapses in patients with positive PBPC also appeared to be relatively well controlled by salvage therapy, which was reflected by the fact that their OS remained excellent. Several studies have reported that rituximab may be used as consolidation after ASCT and showed great potential for eradicating MRD. [28] This approach seems to be therefore logical in patients transplanted with contaminated autografts and should be tested in a randomized trial.

We have also noticed observed that PCR negative CLL and MCL patients had a higher tendency to relapse (62.5% and 77.8% respectively) than PCR negative FL patients (50%). These differences may be explained by the different biology of the diseases. Patients with FL maintain a long term MCR and remain disease free when compared to other types of indolent B-LPD. [29] We have previously reported that CLL patients achieve PCR negativity at high rate after fludarabine, cyclophosphamide and rituximab followed by ASCT, but relapses continued to occur. [32] The sensitivity of PCR detection of CDRIII (mostly CLL patients) is lower than PCR detection of oncogene translocations (bcl-2/IgH for FL and bcl-1/IgH for MCL). Therefore PCR negative patients with CLL may have had actually higher levels of MRD than PCR negative patients with FL or MCL. The different PCR sensitivity however did not lead to a different PFS or OS in our study as CLL patients were less likely to achieve PCR negativity. It is also known that PCR negativity in BM and or PB after rituximab does not always confer CR. [33] Unfortunately in our relatively small group of patients we would not be able to evaluate the pattern of the disease recurrence. Although it is not clearly established which tissue should be

used for PCR monitoring in the follow up period, many researchers use monitoring of peripheral blood performed whenever available and BM is tested less frequently. Evidence is lacking to guide frequency of sampling and testing.

We can conclude that both therapeutic modalities; ASCT and rituximab, lead to high rates of PCR negativity, which correlates with significantly prolonged PFS and OS. Combination of R followed by ASCT has been successful in induction of molecular remission which in turn led to significant improvement of overall survival. The achievement of molecular remission by chemoimmunotherapy and autologous stem cell transplantation is a realistic goal in patients with lymphoproliferative disorders and should be the aim of treatment.

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TABLES and FIGURES:

Table 1:

Age at diagnosis	Median: 55 (23-76)
Female	28 (49%)
Dg.: CLL	18 (32%)
FL	27 (47%)
MCL	12 (21%)
CDRIII	30 (53%)
t(14;18)	20 (35 %)
t(11;14)	7 (12 %)
*CS: I	6 (11%)
II	1 (2%)
III	6 (11%)
IV	42 (76%)
*Symptoms: A	28 (58%)
B	20 (42%)
*IPI: 1	24 (53%)
2	16 (36%)
3	5 (11%)
Disease status: 1	22 (39%)
2	17 (30%)
3	10 (18%)
4	8 (14%)
Treatment lines: < 2	35 (61%)
>= 2	22 (39%)
R based therapy	39 (68%)
R – ASCT	18 (32%)
Best- CR	45 (79%)
PCR negative	43 (75%)
PCR positive	14 (25%)
Relapse/Progression	38 (67 %)
Death	16 (28 %)

Table 2.

	PCR negative		PCR positive	
Age at diagnosis	Median: 55 (23-76)			
	Median: 54 (23-76)		Median: 56.5 (44-68)	
	N	%	N	%
	43	74.4	14	24.6
Sex:	Females 28 (49.1%); Males 29 (50.9%)			
Sex: Female	22	51.2	6	42.9
Male	21	48.8	8	57.1
Dg.: CLL (p=0.00141)	9	20.9	9	64.3
FL (p=0.00141)	26	60.5	1	7.1
MCL (p=0.00141)	8	18.6	4	28.6
CDRIII (p=0.0361)	20	46.5	10	71.4
t(14;18) (p=0.0361)	19	44.2	1	7.1
t(11;14) (p=0.0361)	4	9.3	3	21.4
CS: I	5	11.6	1	8.3
II	1	2.3	0	0.0
III	5	11.6	1	8.3
IV	32	74.4	10	83.3
Symptoms: A (p=0.047)	25	65.8	3	30.0
B (p=0.047)	13	34.2	7	70.0
IPI: 1 (p=0.031)	19	51.4	5	71.4
2 (p=0.031)	16	43.2	0	0.0
3 (p=0.031)	3	5.4	2	28.6
Disease status: 1	19	44.2	3	21.4
2	12	27.9	5	35.7
3	7	16.3	3	21.4
4	5	11.6	3	21.40
Treatment lines: < 2	28	65.1	7	50.0
>= 2	15	34.9	7	50.0
R alone	15	34.9	5	35.7
R + Chemotherapy	28	65.1	9	64.3
Best- CR (p<0.00001)	43	100.0	2	14.3
Relapse/Progression (p=0.0216)	25	58.1	13	92.9
Death (p=0.0133)	8	18.6	8	57.1

Table 3.

	R		R-ASCT	
Age at diagnosis	Median: 55 (23-76)			
	Median: 57 (23-76)		Median: 53 (38-62)	
	N	%	N	%
	39	68.4	18	31.6
Sex:	Females 28 (49.1%); Males 29 (50.9%)			
Sex: Female	18	46.2	10	55.6
Male	21	53.8	8	44.4
Dg.: CLL	13	33.3	5	27.8
FL	17	43.6	10	55.6
MCL	9	23.1	3	16.7
CDRIII	21	53.8	9	50.0
t(14;18)	12	30.8	8	44.4
t(11;14)	6	15.4	1	5.6
CS: I	2	5.4	4	22.2
II	0	0.0	1	5.6
III	4	10.8	2	11.1
IV	31	83.8	11	61.1
Symptoms: A	18	54.5	10	66.6
B	15	45.5	5	33.3
IPI: 1	17	58.6	7	46.7
2	9	31.0	7	46.7
3	3	10.3	1	6.7
Disease status: 1	15	38.5	7	38.9
2	11	28.2	6	33.3
3	5	12.8	5	27.8
4	8	20.5	0	0.0
Treatment lines: < 2	22	56.4	13	72.2
>= 2	17	43.6	5	27.8
Best- CR (p<0.0214)	27	69.2	18	100.0
PCR neg (p=0.025)	25	64.1	18	100.0
Relapse/Progression	25	64.1	11	61.1
Death	14	35.9	2	11.1

Table 4. Cox regression analysis of risk factors.

Parameter	OS HR (95% CI)	*P*	PFS HR (95% CI)	*P*
Excluding CR*				
Lack of PCR negativity	4.6848 (1.6071-13.6567)	0.005	3.489 (1.7071-7.1309)	0.001
Pretreatment**	1.7017 (1.1756-2.4632)	0.005	1.6735 (1.2471-2.2457)	0.001
Excluding PCR*				
Lack of CR	5.3619 (1.7775-16.1812)	0.003	4.6041 (2.1213-9.99	<0.001
Pretreatment**	1.7419 (1.2061-2.5157)	0.003	1.6912 (1.2593-2.2713)	<0.001

*Only significant variables were included.
**Represents increase HR with each line of therapy received as pretreatment.

Figure 1:

A **B**

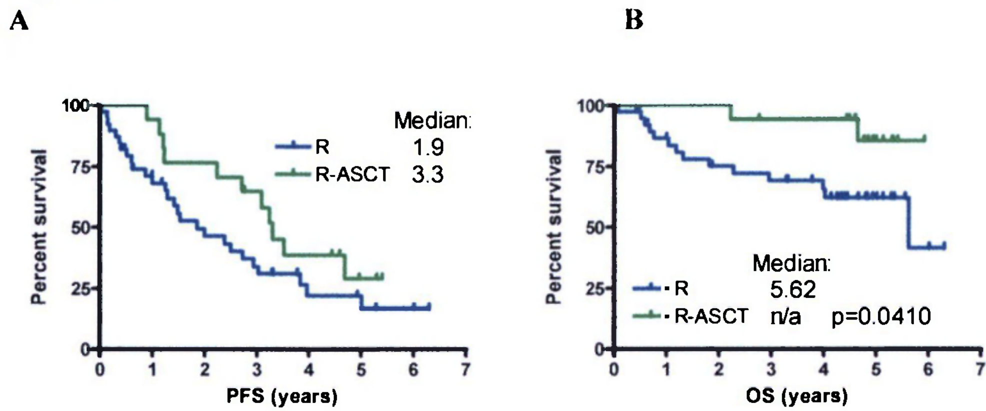
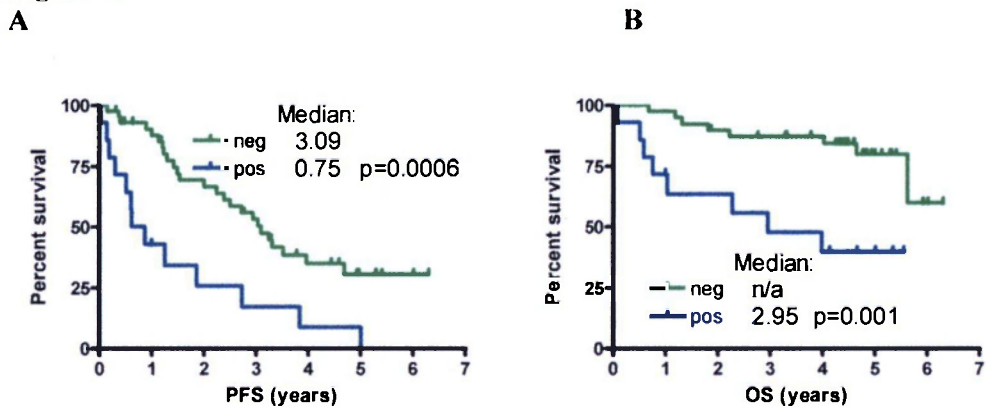
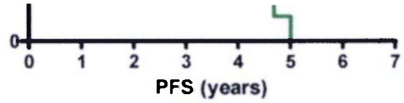


Figure 2:

A **B**





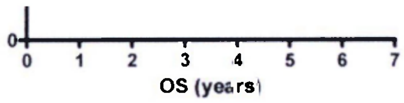
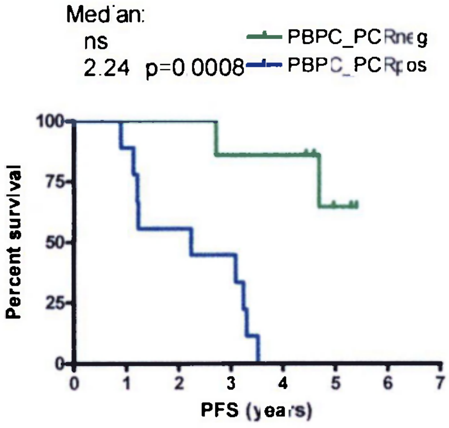


Figure 5:

A



B

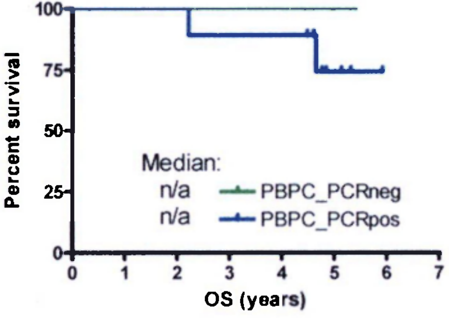


Figure 3:

A

B

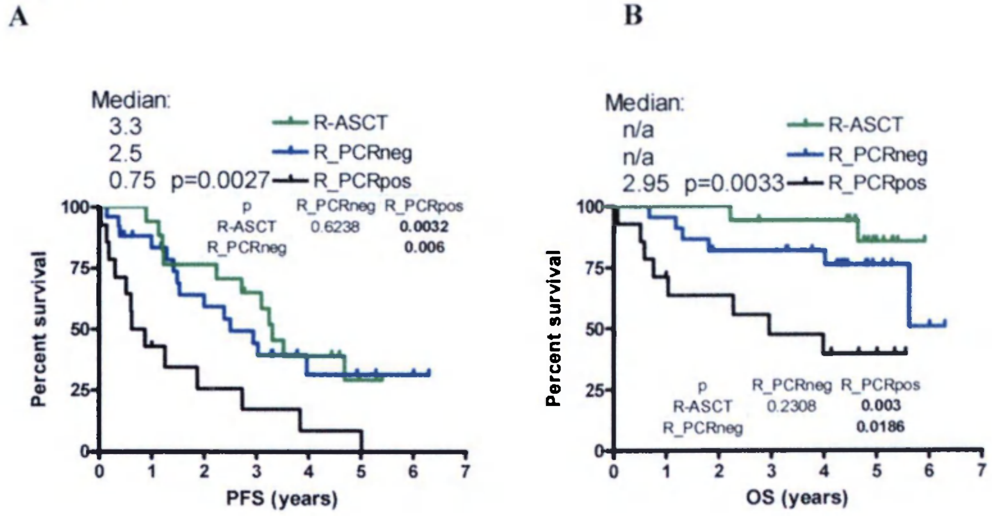
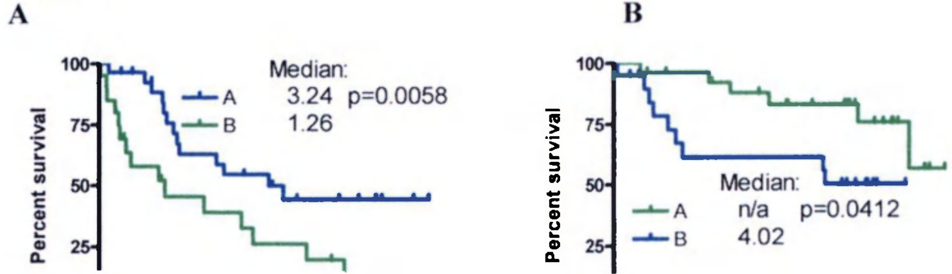


Figure 4:

A

B



LEGENDS:

Table 1: The clinical characteristics of all patients.

(Dg- diagnosis; CLL- chronic lymphocytic leukemia or small lymphocytic lymphoma, FL- follicular lymphoma, MCL- mantle cell lymphoma; CDRIII- complementarity determining region III; CS- clinical stage according to Ann Arbor (*patients with lymphomas) or according to Rai (*patients with CLL); Symptoms - general symptoms (*only pts with lymphomas evaluated); IPI- International Prognostic Index (*only for lymphomas); Disease status at start of therapy was as follows: 1- previously untreated or 1st partial remission; 2- 1st progression or 1st relapse; 3- 2nd partial remission or 2nd relapse; 4- 2nd and higher progression or resistant disease; R- rituximab +/- chemotherapy; ASCT- autologous stem cell transplantation).

Table 2: The clinical characteristics of patients based on PCR result after completed therapy.

(Dg- diagnosis; CLL- chronic lymphocytic leukemia or small lymphocytic lymphoma, FL- follicular lymphoma, MCL- mantle cell lymphoma; CDRIII- complementarity determining region III; CS- clinical stage according to Ann Arbor (lymphomas) or according to Rai (CLL); Symptoms - general symptoms (only pts with lymphomas evaluated); IPI- International Prognostic Index (only lymphomas); Disease status at start of therapy was as follows: 1- previously untreated or 1st partial remission; 2- 1st progression or 1st relapse; 3- 2nd partial remission or 2nd relapse; 4- 2nd and higher progression or resistant disease; R- rituximab +/- chemotherapy; ASCT- autologous stem cell transplantation).

Table 3: The clinical characteristics of patients treated with R or R- ASCT.

(Dg- diagnosis; CLL- chronic lymphocytic leukemia or small lymphocytic lymphoma, FL- follicular lymphoma, MCL- mantle cell lymphoma; CDRIII- complementarity determining region III; CS- clinical stage according to Ann Arbor (lymphomas) or according to Rai (CLL); Symptoms - general symptoms (only pts with lymphomas evaluated); IPI- International Prognostic Index (only lymphomas); Disease status at start of therapy was as follows: 1- previously untreated or 1st partial remission; 2- 1st progression or 1st relapse; 3- 2nd partial remission or 2nd relapse; 4- 2nd and higher progression or resistant disease; R- rituximab +/- chemotherapy; ASCT- autologous stem cell transplantation).

Figure 1: Progression free survival (A) and overall survival (B) based on therapy. (R- rituximab, R-ASCT- rituximab followed by autologous stem cell transplantation, PFS- progression free survival, OS- overall survival).

Figure 2: Progression free survival (A) and overall survival (B) based on PCR status after therapy. (neg- PCR negative, pos- PCR positive, PFS- progression free survival, OS- overall survival).

Figure 3: Progression free survival (A) and overall survival (B) based on type of therapy and PCR result after completion of treatment. (R_PCRneg- PCR negative after rituximab, R_PCRpos- PCR positive after rituximab, R-ASCT- rituximab followed by autologous stem cell transplantation, PFS- progression free survival, OS- overall survival).

Figure 4: Progression free survival (A) and overall survival (B) based on presence of B symptoms. (A- absence of B symptoms; B- B symptoms: fever, weight loss, or night sweats; PFS- progression free survival, OS- overall survival).

Figure 5: Progression free survival (A) and overall survival (B) based on PCR status of peripheral blood progenitor cells (PBPC). (PBPC_PCRneg- PCR negative peripheral blood progenitor cells, PBPC_PCRpos- PCR positive peripheral blood progenitor cells, PFS- progression free survival, OS- overall survival).

Short Communication

The Use of Comparative Duplex PCR in Monitoring of Patients with Non-Hodgkin's Lymphoma and Chronic Lymphocytic Leukaemia

(duplex PCR / quantitative molecular monitoring / clinical significance)

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Abstract. Various quantitative PCR approaches have been utilized during the last years to provide information about the treatment efficacy and the risk of recurrent disease in haematological malignancies. Apart from the frequently used real-time PCR, cost-saving modified standard PCR methods may be applied as well. This report evaluates the utility of the end-point comparative duplex PCR. We have used this method for monitoring of 35 patients with either NHL or CLL and observed a good correlation between quantitative molecular results and clinical outcome. There was also an agreement between comparative duplex PCR and real-time PCR in patients who were monitored by both methods. We therefore believe that use of this technique should be strongly considered instead of simple qualitative detection in monitoring of therapeutic outcome in NHL or CLL patients.

The polymerase chain reaction (PCR) has become an essential tool for molecular biologists in haematological oncology. Currently, attention is namely drawn to the exploitation of quantitative estimation. Quantitative data can be obtained quite comfortably by real-time PCR. This, however, is also the most expensive method. Thus, several cost-saving standard PCR methods have been established. We have described standard comparative duplex PCR employing co-amplification of clone-specific markers and internal standards. We then evaluated relative quantitative changes of specific disease markers in consecutive DNA samples by gel densitometry. It is essential that duplex PCR is optimized so that amplicons of all samples are compared in sub-plateau phase of PCR. Using this approach, we have previously suggested co-amplification of the complementarity determining region (CDR3) of the immunoglobulin heavy chain gene (*IgH*) as the disease marker and a segment of the *Hras 1* gene containing codon 61 (ras) as the internal standard for monitoring B-lymphoproliferative disorders (Slavickova et al., 1999, 2000).

In the current study, we show other sets of disease-specific and normalizing markers, the agreement between results of comparative duplex and real-time PCR, and the applicability of the methodology in molecular monitoring of patients with non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukaemia (CLL).

Material and Methods

Patient samples

Thirty-five patients were followed by quantitative PCR methodology. These patients were treated with either conventional therapy, rituximab (MabThera^R, chimaeric anti-CD20 monoclonal antibody, F. Hoffmann-La Roche Ltd., Basel, Switzerland) in combination with fludarabine and cyclophosphamide, and several patients underwent high-dose therapy with autologous stem cell rescue. The group consisted of 23 chronic lymphocytic leukaemias, five mantle cell lymphomas, four follicular lymphomas and three diffuse large B-cell lymphomas. The diagnosis was established using clinical criteria, histology, histochemistry and immunophenotyping analysis according to the modified REAL classification. In all patients the clonal

Received July 21, 2005. Accepted October 14, 2005.

The work has been supported by the grants GA UK No. 203580, MSM No. 111100004 and presently by MSM No. 0021620808, Czech Republic.

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Abbreviations: CDR3 – the third complementarity determining region of the hypervariable domain of immunoglobulin heavy chain genes, CLL – chronic lymphocytic leukaemia, IgH – immunoglobulin heavy chain gene, mbr – the major breakpoint region of the bcl-2 gene, mtc – major translocation cluster region of the bcl-1 gene, NHL – non-Hodgkin lymphoma, PCR – polymerase chain reaction.

rearrangement of complementarity determining region 3 (CDR3) of the immunoglobulin heavy chain gene (*IgH*) was monitored. In addition, the interchromosomal translocation t(14;18-locus mbr) was used in three patients and the t(11;14) in two patients.

DNA was isolated from bone marrow aspirates and peripheral blood by the "salting out" procedure (Miller et al., 1988), dissolved in TE and stored at 4°C until use.

Principle of comparative duplex PCR, evaluation of results

Serial dilutions of consecutive DNA samples were submitted to the same duplex PCR. The primer sets 5' AGA CGT GCC TGT TGG ACA TC 3' with 5' CGC ATG TAC TGG TCC CGC AT 3' or 5' CAA CTT CAT CCA CGT TCA CC 3' with 5' GAA GAG CCA AGG ACA GGT AC 3' were used for ras and β-globin, respectively, as normalizing markers in co-amplification with the primers for disease-specific markers. The Jh consensus primer 5' ACC TGA GGA GAC GGT GAC CAG GGT 3' and 5' TAT GGT GGT TTG ACC TTT AG 3' were used for detection of the t(14;18-mbr) translocation. The Jh consensus primer and 5'ACA CGG CC/TG/C TGT ATT ACT GT 3' were used for the amplification of clonal CDR3. For detection of the t(11-mtc; 14) translocation the Jh consensus was then used with either 5' ATA TTC GGT TAG ACT GTG ATT AGC 3' or 5'CTA CTG AAG GAC TTG TGG GTT GCT 3'. All primers were products of Genosys Biotechnologies, Inc. (The Woodlands, TX). Amplification products were analysed on 10% polyacrylamide gels stained with ethidium bromide (Fig. 1). The amount of PCR products was determined by gel densitometry using gel documentation system (Ultra-Lum Inc., Claremont, CA) and gel analysis software (Media Cybernetics, Silver Spring, MD), as described earlier (Slavickova et al., 2000). The values obtained from sub-plateau phase of both amplifications were analysed further, that is, the amount of the specific product was related to the respective amount of the normalizer and the differences of specific markers in two or more successive samples (found either directly or by extrapolation at identical amounts of normalizing products) were used in the assessment of molecular regression (decrease of the specific marker in the later of two consecutive DNA samples) and molecular progression or relapse (increase of the specific marker). The molecular results obtained from stored DNA samples were then correlated with the corresponding clinical observations retrospectively.

Real-time PCR

The real-time PCR quantification was performed using the LightCycler and the t(14;18-mbr) Quantification Kit of Roche Molecular Biochemicals exactly as recommended by the manufacturer.

Results and Discussion

Correlation of clinical outcome and quantitative molecular monitoring

We have observed a good correlation between molecular follow-up and clinical outcome in a total of hundred intervals (ranging from 1 to 18 months) when a molecular regression/progression (quantitative decrease/increase of the clonospecific marker) was in accordance with the clinical state (regression/ progression). In a group of 17 patients with clinically stable disease, but increasing markers as detected by our quantitative molecular method, ten patients developed disease progression and the remaining seven relapse. In seven of 33 cases (21%) of clinical complete remissions the molecular residual disease was still detected. Besides, in available 14 cases of paired samples of bone marrow and peripheral blood no significant difference

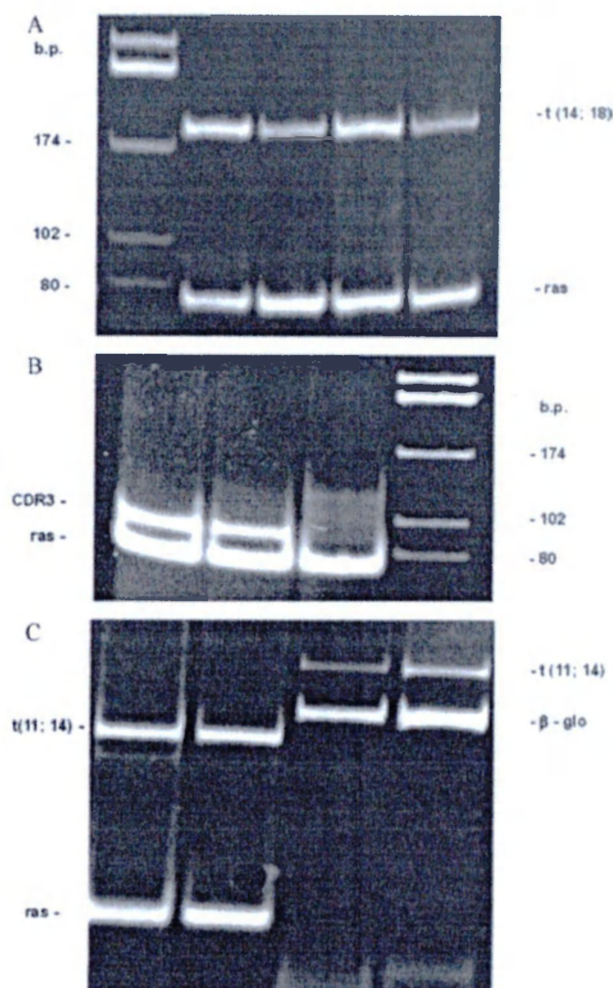

Fig. 1. 10% PAGE of duplex PCR. The products of duplex PCR for ras as a normalizing marker and the t(14;18-mbr) translocation as the disease-specific marker(A); the products of duplex PCR for ras and the clonal CDR3 (B); the products of duplex PCR for ras and t(11-mtc;14) translocation (left) and β-globin and the t(11-mtc;14) translocation (right).

was observed, indicating that the molecular evaluation of peripheral blood may also be informative, where it is applicable.

We have also shown other suitable combinations of disease-specific and internal standards (Fig. 1). Furthermore, the agreement between comparative duplex and real-time PCR was demonstrated (Fig. 2). Interestingly, while a good correlation of molecular results in general (increase or decrease) was observed, the methods display different sensitivities as shown in Fig. 2. That is, the lower sensitivity of IgH/ras PCR was sufficient for describing kinetics of the disease as well as the t(14;18)-real time or duplex PCR for mbr/ras. Similar results were already observed in monitoring NHL patients after stem cell transplantation (Mitterbauer et al., 2001).

Summarized, it seems that the comparative duplex PCR may be a method of choice for monitoring NHL and CLL patients as it may provide prognostic information. The predictive value of this method depends on the frequency of sample taking as well as on the assay sensitivity. The sensitivity should be checked in any single assay (Dölken, 2001). Despite the increasing pool of data on molecular monitoring and its prognostic role in lymphoproliferative diseases the issue of quantitative molecular monitoring has not been settled in a large clinical study and neither guidelines nor standards were established. Thus it is suggested that PCR amplification techniques and their correlation with clinical outcome need further observation and should remain investigational for the present (Gribben, 2002). This is in contrast with chronic myelogenous leukaemia, where it was already suggested that quantitative molecular monitoring of leukaemic cells might be helpful in individualizing therapeutic strategies (Lion, 1994).

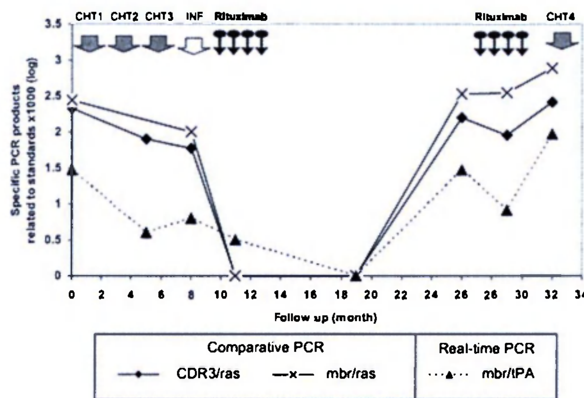

Fig. 2. Monitoring of treatment impact by comparative duplex and real-time PCR. The patient with follicular lymphoma treated with chemotherapy (CHT), interferon á (INF) and rituximab achieved PCR negativity at clinical complete remission. The negativity, however, was disproved by hybridization with a probing sequence from the mbr region labelled with digoxigenin. The disease progressed after several months, further therapeutic effort failed and the molecular quantification was in accordance with this.

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JOURNAL OF CELLULAR PHYSIOLOGY 199:217–226 (2004)

Biallelic IgH Rearrangements in Patients With Indolent Lymphoproliferative Disorders: Molecular and Practical Implications

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We report a group of patients (pts) with indolent lymphoproliferative disorder who had both alleles for the immunoglobulin heavy chain genes rearranged (biIgH). This group of 17 pts consisted of 9 small lymphocytic lymphomas (SLL) and 8 chronic lymphocytic leukemia (CLL). The polymerase chain reaction (PCR) amplification of clonal immunoglobulin heavy (IgH) rearrangement using the complementarity determining region III (CDRIII) constantly retrieved two distinct bands in all PCR informative samples of those pts. To rule out biclonality, we evaluated samples by fluorescein activated cell sorting (FACS) analysis and sequenced the PCR products. We were able to obtain both IgH sequences from 12 patients. FACS suggested biclonality in one case, which also correlated with sequencing results as both IgH rearrangements were in-frame. Recently, we reported a patient who sustained transformation into an aggressive disease after biIgH was detected in the setting of monoclonal disease (Cerny et al., 2003b, Haematologica 88(05):ECR15 B.). We decided to compare clinical characteristics and prognosis of 17 pts with biIgH and 37 pts with monoIgH rearrangements. Although we found some minor differences in disease characteristics between both groups, these did not translate into a significantly different overall survival. Our findings suggest that true biclonal cases of CLL are rare. J. Cell. Physiol. 199: 217–226, 2004. © 2003 Wiley-Liss, Inc.

B-cell lymphoproliferative disorders (B-LPDs) are caused by proliferation of a clone derived from a progenitor at specific stage of differentiation that correlates with the stage of B-cell receptor development. The crucial role in the B lymphocyte development is represented by the immunoglobulin heavy (*IgH*) and light chain (kappa or lambda, *IgK*, *IgL*) genes rearrangements. This process begins with assembly of variable-region (*V*), diversity (*D*), and joining (*J*) genes. The light chains are then assembled from V and J elements. This results from a random recombination of germ-line segments to produce an entirely new expressed *Ig* gene. On one hand, it is a purely stochastic process, not driven by any instructive or selective pressure and these recombination events are not inherited. On the other hand, the VDJ recombination is a highly orchestrated lymphoid-specific process (Rajewsky, 1996). There are many different V, D, and J segments in the germ line, and therefore each B-cell generates a particular set of genes for its heavy chain and light chain regions that differ from those of other B-cells and encode a distinct antibody. These distinct rearrangements also equip each B-cell with individual, molecular clonal markers—an essential feature for the molecular analysis of B-LPDs. It has been shown that the expression of antibody as an antigen receptor on the surface of B-cells is not critical only for the development, but also for the survival of B-cells (Lam et al., 1997). In case, the first IgH allele is rearranged out-of-frame the process moves to the second allele. If the first rearrangement is productive the process of allelic exclusion disables initiation of the recombination on the second allele through chromatin remodeling (Johnson et al., 2003). Similar actions continue on alleles for the light chains where four attempts are possible (two for kappa and two for lambda). The only surviving developing B-cells are those that acquired IgH

Contract grant sponsor: Ministry of Education; Contract grant number: 11110004; Contract grant sponsor: GAUK; Contract grant number: 15/2001/C/1. LF.

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Received 19 May 2003; Accepted 3 September 2003

DOI: 10.1002/jcp.10459

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and IgL rearrangement that can be translated into protein because they preserve the correct reading frame ("in-frame rearrangements" or "productive rearrangements"). Cells bearing out-of-frame VDJ rearrangements on both alleles are subjected to apoptosis. In addition the antibody specificity is tested in newly formed B-cells. Auto reactive or weakly reacting cells are either removed by apoptosis or "edit" their receptors by means of secondary VDJ rearrangements (Wilson et al., 2000). Expressing an "innocent" B-cell receptor the B-cell leaves the BM to become a mature, naive (i.e., not yet exposed to any antigen) B-cell (Nussenzweig, 1998). Further modification of the specificity of the B-cell antigen receptor by genetic means is resumed in a later phase of B-cell differentiation in germinal centers. This process is called somatic hypermutation and involves V regions of the IgH. Patients with unmutated *VH* genes have been previously reported to have poorer outcome than patients (pts) with mutated *VH* genes, e.g., in chronic lymphocytic leukemia (CLL) (Fais et al., 1998; Damle et al., 1999; Hamblin et al., 1999). While the Ig mutational status of B-cell lymphomas and leukemias has been confirmed to have prognostic impact other markers such as expression of CD38 in CLL patients is still a matter of a discussion. Some studies have shown a correlation of CD38 expression with the presence of unmutated *VH* genes and an unfavorable clinical outcome (Damle et al., 1999; Ibrahim et al., 2001; Lin et al., 2002) and others did not (Thunberg et al., 2001).

There are several situations when more than one IgH rearrangement can be detected in patients with B-LPD. (1) CLL patients may have a cell clone expressing two different Ig molecules on the cell surface. The lack of allelic exclusion has been shown to be higher in CLL than in normal cells (Rassenti and Kipps, 1997). (2) On the other hand, bimorphic lymphomas and biclonal leukemias represent situations when patients have two malignant clones each expressing different Ig molecule. Both common and separate clonal origins of the two tumor parts have been reported (Fend et al., 1999). (3) Patients have two IgH rearrangements detectable by polymerase chain reaction (PCR) (one in-frame and one out-of-frame; biallelic rearrangements), but only the productive is translated into an Ig protein.

It is important to identify patients with biclonal disease as they may have poorer outcome (Fakan et al., 1984). Furthermore, the Ig molecule expressed by a B lymphocyte is responsible for the actual function of a particular B-cell. There are some motifs found in IgH rearrangements that can be associated with some autoimmune complications (e.g., autoimmune hemolytic anemia; AIHA). Therefore, it must be emphasized that Ig mutations and other Ig sequence assessing studies should be performed on the productive (in-frame rearranged) Ig allele.

The situation (3) is described in this study. We present a group of 17 patients with CLL or SLL who had two IgH rearrangements, but only one of them had biclonal disease (according to fluorescein activated cell sorting analysis (FACS) and sequencing). We were interested whether the clinical characteristics and outcome is same in patients who have two IgH rearrangements and in patients with only one IgH rearrangement.

MATERIALS AND METHODS

Patients

The patients in this study are a subset (n = 50) of the cohort (n = 87) of patients diagnosed with chronic lymphocytic leukemia (CLL) or small lymphocytic lymphoma (SLL) who were positively tested by PCR for clonal IgH rearrangement at Molecular Biology Laboratory at 1st Department of Medicine Charles University, Prague, Czech Republic (between May 1994 and November 2001). Patients were selected for the present study based on the availability of detailed clinical histories and the availability of DNA. The clinical courses of the patients that were analyzed in this study were not significantly different from those that could not be studied because of lack of sample or follow-up. There were 36 males and 14 females in this group, with a median age of 59.5 years (range, 29–78). The median ages of the monoallelic (median, 60; range, 29–78) and biallelic (median, 59; range, 49–78) cases were similar. Further characteristics are in a Table 1.

Clinical monitoring

To assess all sites of disease involvement, baseline evaluation included clinical documentation, radiography of the chest, computed tomography (CT) of the chest, abdomen, and pelvis, and unilateral bone marrow biopsy. Laboratory testing included routine hematology, serum chemistries, serum immunoglobulin levels, lactate dehydrogenase, and beta-2-microglobulin assays in blood and urinalysis. Monitoring was represented by hematology and serum chemistry evaluations together with full tumor restaging. The clinical and molecular monitoring was performed before therapy and during the follow-up period till relapse or progression occurred.

Immunophenotypic studies

Diagnostic BM samples were collected in the solution of phosphate-buffered saline (PBS) with EDTA anticoagulant. In all cases, samples preparation a flow cytometric data acquisition was performed within the 4 h after collection. Marrow samples were processed by a whole marrow lysis technique and then by a direct immunofluorescence technique. Specimens were lysed with hypotonic NH_4Cl solution and washed twice in PBS containing 1% bovine serum albumin and 0.2% sodium azide (PBSA). The cell suspensions (5×10^6/ml) were incubated with flurochrome-conjugated monoclonal antibodies (MoAb) at room temperature in the dark for 15 min then washed once in PBS. After centrifugation, the cell pellet was resuspended in PBS with 1% paraformaldehyde. In all cases, antigen expression was analyzed using triple combination of the following MoAbs (Immunotech, a.s. Prague, Czech Republic) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) and PE–cyanine 5.1 (PC5) flurochrome tandem: CD23 (9P25)–FITC/CD5 (BL1a)–PE/CD19 (J4.119)–PC5, CD10 (ALB1)–FITC/CD38–PE/CD19 (J4.119)–PC5, FMC7–FITC/CD79b (CB3-1)–PE/CD19 (J4.119)–PC5, CD20 (B9E9)–FITC/CD11c (BU15)–PE/CD19 (J4.119)–PC5, anti kappa (polyclonal)–FITC/anti lambda (polyclonal)–PE/CD19 (J4.119)–PC5, and isotypic control IgG1–FITC/isotypic control IgG2a–PE/CD19 (J4.119)–PC5. Specimens were analyzed on a

TABLE 1. Clinical characteristics of patients

Age	Monoallelic Median: 60 (29–78) n	%	Biallelic Median: 59 (49–78) n	%
Sex				
F	10	30.3	4	23.5
M	23	69.7	13	76.5
Dg				
CLL	23	69.7	8	47.1
SLL	10	30.3	9	52.9
CS				
0	1	3.0	1	5.9
I	7	21.2	2	11.8
II	4	12.1	1	5.9
III	1	3.0	2	11.8
IV	20	60.6	11	64.7
Symptoms[a]				
A	6	60.0	4	44.4
B	4	30.0	5	55.6
IPI[b]				
0	1	10.0	0	0.0
1	5	50.0	5	55.6
2	4	40.0	3	33.3
3	0	0.0	1	11.1
No. of Rel/Pr				
0	14	42.4	6	41.2
1	8	24.2	6	41.2
2	4	12.1	2	11.8
≥3	7	21.2	1	5.9
Transformation	5	15.1	2	11.8
No. of Th				
0	2	6.1	2	11.8
1	7	21.2	4	23.5
2	9	27.3	6	35.3
>3	15	45.5	5	29.4
CD 38	14	42.4	4	23.5
CD 79b	2	6.1	5	29.4*
Light chain				
K	23	69.7	8	47.0
L	10	30.3	7	41.1
K & L	0	0.0	1	5.9
Death	8	24.2	2	11.8
ASCT	4	12.1	4	23.5
Rituximab	11	33.3	5	29.4
Comorbidities	6	18.2	7	41.1
Dg changes	0	0.0	7	41.1**

F, female; M, male; Dg, diagnosis; CLL, chronic lymphocytic leukemia; SLL, small lymphocytic lymphoma; CS, clinical stage according to Ann Arbor (SLL) or according to Rai (CLL).
[a]Symptoms, general symptoms (only patients (pts) with SLL evaluated).
[b]IPI, International Prognostic Index (only SLL); Rel/Pr, relapse or progression; No. of Th, at least three cycles of standard or two cycles of salvage chemotherapy, radiotherapy, or ASCT; K, kappa; L, lambda; ASCT, autologous stem cell transplantation.
*$P = 0.033$.
**$P < 0.0002$.

FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Calibration of the instrument was performed using Calibration beads (CALIBrite^{TM3}, Becton Dickinson). The CELLQuest$^{(tm)}$ software (BD) was used for the data acquisition and analyzing. Monoclonality was assessed based on light chain restriction as well as other surface molecule expression (Fig. 1; Table 2A,B).

Molecular detection and sequencing of IgH rearrangements using the complementarity determining region III (CDRIII)

The molecular studies were performed on bone marrow samples (BM), peripheral blood samples (PB), or lymph node samples (LN) at the time when disease was active (diagnosis, progression, or relapse) and detected by other diagnostic technique such as FACS or histology. All samples were collected and DNA was extracted using standard procedures and the usual precautions to avoid cross-contamination. Approximately 1 mg DNA was amplified using FR3A-(ACACGGCYSTGTATTACTGT) oligonucleotide, together with JH-(ACCTGAGGAGACGGTGACC) oligonucleotide and RedTaq polymerase (Sigma Prague, Czeck Republic). Each reaction included: 5 min at 94°C then 30 cycles, each comprising 3 steps at 94°C for 45 sec, at 58°C for 1 min 30 sec, at 72°C for 1 min 30 sec, followed by 7 min at 72°C.

For detection of t(14;18) or t(11;14), approximately 1 µg DNA was amplified using either MBRA-(TATGGTGGTTTGACCTTTAG) and/or MTC C-(CTACTGAAGGACTTGTGGGTTGCT) specific oligonucleotides, together with JH-(ACCTGAGGAGACGGTGACC) oligonucleotide and RedTaq polymerase (Sigma). We used a touchdown PCR technique that has been described previously (Cerny et al., 2003a). Each reaction contained positive (CDRIII, MBRA, or MTCC positive DNA) and negative control (normal DNA) for indication of contamination or false positivity due to the sub-optimal efficiency of amplification and was used for standardization of PCR efficiency variation. PCR products were visualized on agar gel stained with ethidium bromide. Under these conditions, the sensitivity of CDRIII detection was routinely 10 positive cells in 10^5 normal cells, the PCR detection of t(14;18) or t(11;14) had even higher sensitivity (Slavickova et al., 2000).

Amplified PCR products of the rearranged IgH CDRIII region were separated by electrophoresis on 3% Metaphor agarose (FMC Bioproducts, Rockland, ME) gels stained with ethidium bromide. The products of PCR amplification were extracted from gel slices using the crush and soak technique (Sambrook et al., 1989). The PCR products were then purified with Microcon-100 (Millipore, Foster City, CA, USA) purification columns sequenced using FR3A and JH primers with BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed on ABI Prism 310 Genetic Analyzer (PE BioSystems) according to the manufacturer's instructions. The sequence data were assigned on the basis of their similarity to BLAST sequences and confirmed by homology to each germ-line sequence (Buluwela et al., 1988; Ichihara et al., 1988; Matsuda et al., 1990, 1993). Assignment of D and JH segments was performed as defined by Ichihara et al. (1988) and Yamada et al. (1991).

Software analysis of digitalized pictures of a gel electrophoresis

We used two video documentation systems UltraLum PHD 2000 and GEL DOC to save the pictures of gels. The digitalized pictures were stored in TIFF format and analyzed by GEL PRO Analyzer 3.0 (Media Cybernetics, L.P., Silver Spring, MD). First, individual sample lanes as well as individual bands had to be defined. To minimize any artifacts and to adequately subtract the background intensity a proper width of sample lanes and individual bands must have been set (Goulding, 2000). Only the intensities of fluorescence from within the readability interval of the video documentation

system could be analyzed. The data were then exported to Microsoft Excel table and fluorescence intensities of both CDRIII products were compared from each sample. The ratio between intensities of the two CDRIII products was constant even when different samples were used for PCR (see Fig. 2).

Definitions

The complete remission (CR) and/or partial response (PR) in NHL patients were scored according to the criteria recently reported by Cheson et al. (1999). The National Cancer Institute (NCI)—sponsored Working Group guidelines were applied for the definition of response and time to progression (TTP) in patients with CLL (Cheson et al., 1999). TTP is defined as time from the diagnosis (mostly CLL) or the first application of therapy (mostly NHL) to detection of progression.

Statistical analysis

The significance of associations between clinical characteristics was determined using Fisher's exact test, non-parametric Wilcoxon and Mann–Whitney tests. The differences were tested at the level of significance 0.05 (P value). The survival curves were estimated by the method of Kaplan and Meier (1958) and compared by the log rank test (Mantel, 1960).

RESULTS

Clinical characteristics of patients with IgH rearrangement on one (monoIgH) and both alleles (biIgH)

There were 36 males and 14 females in the studied group, with a median age of 59.5 years (range, 29–78). The median ages of the monoIgH (median, 60; range, 29–78) and biIgH (median, 59; range, 49–78) cases were similar. We found no significant difference in distribution of the main clinical characteristics (e.g., age at diagnosis (Dg), sex, histology, clinical stage, presence of general symptoms, number of relapses, number of therapies, etc.) between mono- and bi-IgH pts (P = ns; further see Table 1). There were 5 (15.1%) and 2 (11.8%) transformations into aggressive disease recorded in mono- and bi-IgH pts, respectively (P = ns). Similarly the fraction of monoIgH pts treated with autologous stem cell transplantation (ASCT; 4; 12.1%) or rituximab (11; 33.3%) was not significantly different (P = ns) when compared to biIgH pts treated with ASCT (4; 23.5%) or rituximab (5; 29.4%).

A

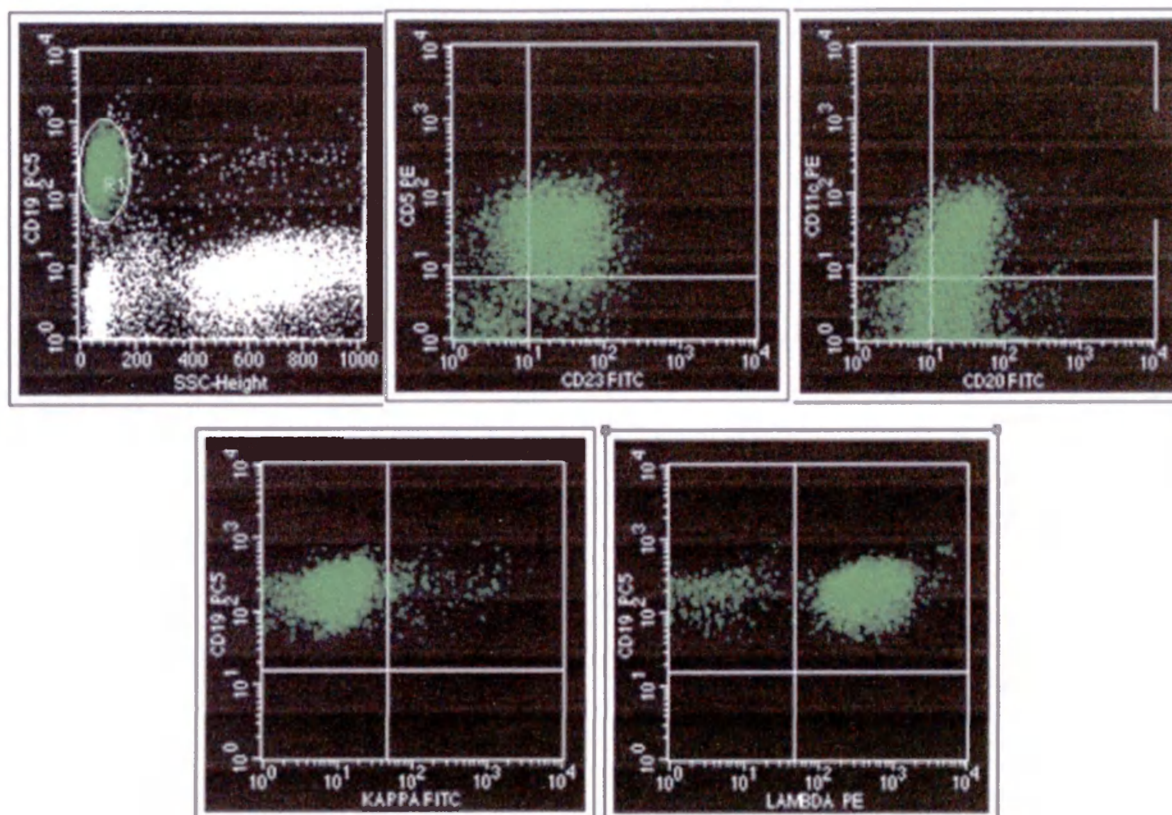

Fig. 1. A: Fluorescein activated cell sorting (FACS) analysis of chronic lymphocytic leukemia (CLL) patients. A patient with monoclonal disease. The malignant clone is positive for CD19, CD5, CD23, CD11b, and lambda light chain. B: FACS analysis of CLL patients. A patient with biclonal disease. The malignant clones are both positive for CD19, CD5, CD23, one has higher and second has lower expression of CD11b, one of the clones expresses lambda light chain while the second expresses kappa light chain. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

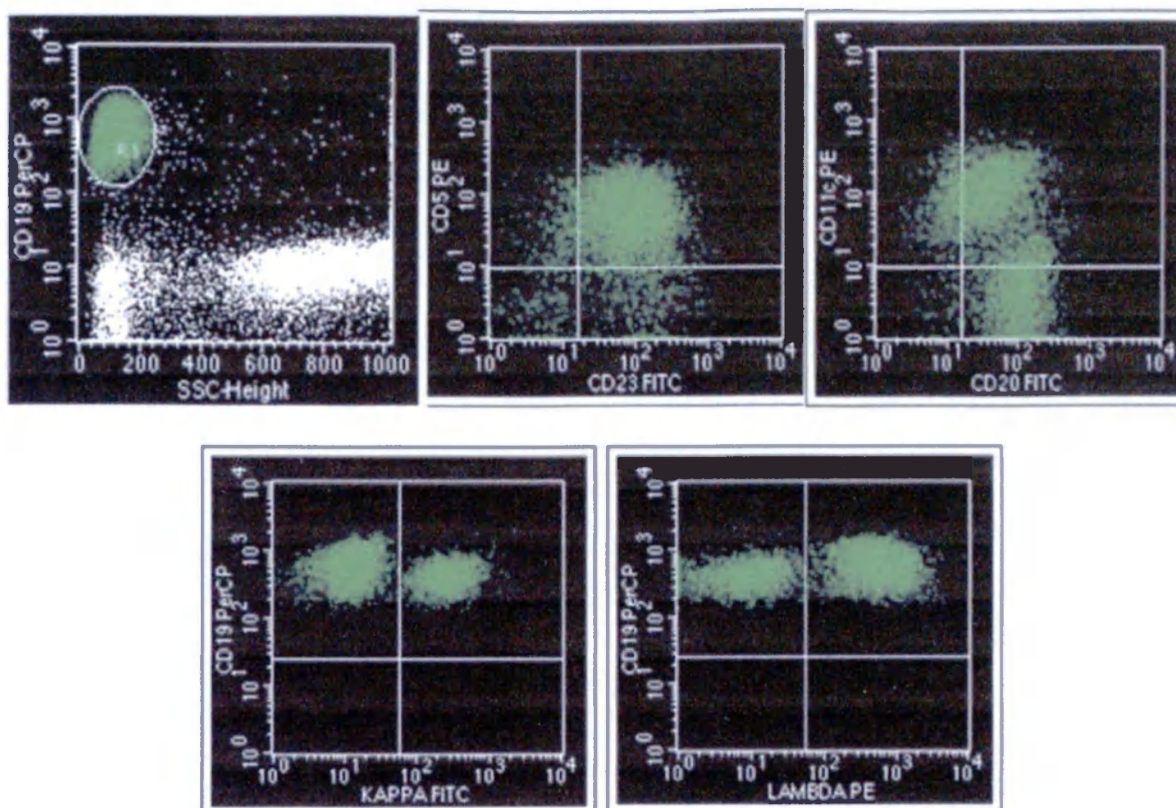

Fig. 1. *(Continued)*

We have noticed a higher number of comorbidities in pts with biIgH rearrangement. We divided them into oncological and immune system related disorders. The oncological ones included: two kidney carcinomas, melanoma, myelodysplastic syndrome, diffuse large cell lymphoma, glioblastoma multiforme, and urinary bladder carcinoma. The immune disorders were represented by three AIHA, four diabetes mellitus, four peptic ulcer disease, ANA positive polyarthritis, Wegener's granulomatosis, and Dupuytren's contracture. One patient had paraprotein (pp) IgM in urine and another one had triclonal pp IgG. We compared the frequency of oncological and autoimmune diseases between the mono- and bi-IgH pts. There were 6 (18.2%) monoIgH pts and 7

TABLE 2A. Clinical, immunophenotypic, and molecular characteristics of sequenced biIgH patients

Pt no.	Age	Sex	Dg	CS	Symptoms	IPI	Transf	No. of recur	ter_S	ter_L	L_S	L_L	D_S	D_L	JH_S	JH_L
39	59	M	CLL	0	na	na	—	0	TAG	—	24	78	x	x	6	5
40	56	F	CLL	1	na	na	—	0	TAG	—	72	87	3_22	2_15	4	6
41	66	M	SLL	4	B	3	—	0	—	TAG	84	87	5_5	2_2	4	6
42	78	M	CLL	3	na	na	—	0	TGA	—	57	81	x	x	3	6
43	64	F	SLL	4	A	1	—	0	—	TAG	63	89	3_10	x	5	6
44	52	M	CLL	4	na	na	—	1	TGA	—	66	89	2_2	3_3	4	6
45	58	M	SLL	4	B	1	RS	3	—	TAG	80	212	2_8	3_22	6	4
46	55	M	CLL	2	na	na	—	1	—	—	77	81	1_20	D4?	5	6
47	65	M	CLL	1	na	na	—	1	—	—	52	77	3_3	3_10	4	6[a]
48	52	F	CLL	4	na	na	RS	1	TGA	—	58	77	x	2_2	3	3
49	42	M	SLL	3	A	1	—	2	—	TAG	59	130	7_27	5_5	5	4
50	54	M	SLL	4	B	2	—	1	—	TGA	77	98	3_22	3_3	4	6

Pt, patient; F, female; M, male; Dg, diagnosis; CLL, chronic lymphocytic leukemia; SLL, small lymphocytic lymphoma; CS, clinical stage according to Ann Arbor (SLL) or according to Rai (CLL); symptoms, general symptoms (only pts with SLL evaluated); IPI, International Prognostic Index (pts with SLL evaluated); transf, transformation into aggressive disease; no. of recur, number of recurrences; ter_S or L, termination codon in short or long IgH; L_S or L, length of a sequenced polymerase chain reaction (PCR) product; D_S or L, D segment; JH_S or L, JH segment; LC, light chain; na, not applicable; RS, Richter syndrome; K, kappa; L, lambda; MCL, mantle cell lymphoma; FL, follicular lymphoma; MZL, marginal zone lymphoma; difCC-CB, diffuse centrocytic–centroblastic lymphoma.
[a]Patient with biclonality that was confirmed by fluorescein activated cell sorting (FACS).

TABLE 2B. Clinical, immunophenotypic, and molecular characteristics of sequenced biIgH patients (continued)

Pt no.	Dg	CD19	CD5	CD23	CD20	FMC7	CD11c	CD38	CD79b	LC	Initial Dg
39	CLL	+	+	+	+	+	+	−	−	L	
40	CLL	+	+	+	+	+	+	+	−	K	
41	SLL	+	+	+	+	−	+	−	+	K	MCL
42	CLL	+	+	+	+	+	+	−	−	L	
43	SLL	+	+	+	+	−	+	−	−	L	FL
44	CLL	+	+	+	+	−	+	−	−	K	MZL
45	SLL	+	+	+	+	+	+	−	+	L	difCC–CB
46	CLL	+	+	+	+	−	+	+	+	K	MCL
47	CLL	+	+	+	+	+	+	−	+	LK	[a]
48	CLL	+	+	+	+	−	+	−	−	L	
49	SLL	+	+	+	+	−	+	−	−	K	difCC–CB
50	SLL	+	+	+	+	−	+	+	+	L	MCL

Pt, patient; F, female; M, male; Dg, diagnosis; CLL, chronic lymphocytic leukemia; SLL, small lymphocytic lymphoma; CS, clinical stage according to Ann Arbor (SLL) or according to Rai (CLL); symptoms, general symptoms (only pts with SLL evaluated); IPI, International Prognostic Index (pts with SLL evaluated); transf, transformation into aggressive disease; no. of recur, number of recurrences; ter_S or L, termination codon in short or long IgH; L_S or L, length of a sequenced polymerase chain reaction (PCR) product; D_S or L, D segment; JH_S or L, JH segment; LC, light chain; na, not applicable; RS, Richter syndrome; K, kappa; L, lambda; MCL, mantle cell lymphoma; FL, follicular lymphoma; MZL, marginal zone lymphoma; difCC–CB, diffuse centrocytic–centroblastic lymphoma.
[a]Patient with biclonality that was confirmed by fluorescein activated cell sorting (FACS).

	Lane	1	2	3	4	5	6	7	8	9	10
	bp	marker	FI	FI	FI	FI	FI	FI	FI	FI	FI
IgH1	252	na	na	na	na	na	9742	15151	11828	na	13205
IgH2	117	na	na	na	na	na	12038	18362	14165	na	16641
	IgH1 / IgH2	na	na	na	na	na	0.81	0.83	0.84	na	0.79

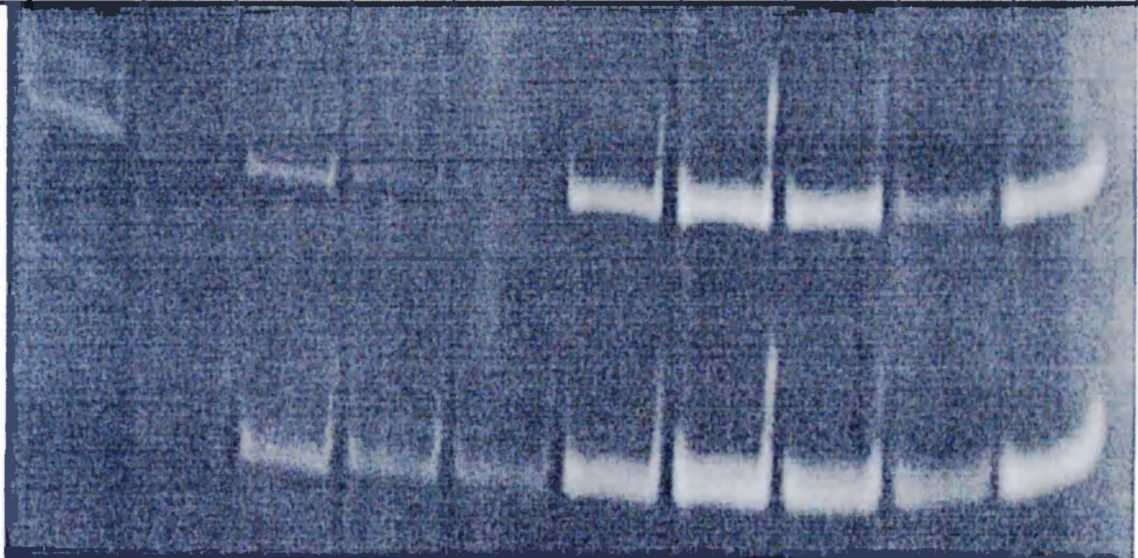

Fig. 2. Software analysis of gel electrophoresis of several different samples of one patient with biallelic immunoglobulin heavy (IgH) rearrangements. Ten percent polyacrylamide electrophoresis of polymerase chain reaction (PCR) for complementarity determining region III (CDRIII); each lane represents PCR products of samples collected from one patient at different times; lane 1 (marker), standard of molecular weight—pUC18 × *Hae* III; lanes 2–5 and lane 9, the fluorescence intensities (FI) of PCR products are outside of the sensitivity interval; therefore only lanes 6–8 and lane 10 can be compared; na, not applicable; the ratio between fluorescence intensities of both PCR products remains constant when samples from different times are compared; the length of the first IgH rearrangement (IgH1) is 252 bp, the length of the second IgH rearrangement (IgH2) is 117 bp.

(41.1%) biIgH pts who had one or more comorbidities. Multiple cases of comorbidities were evenly distributed between both groups of pts (P = ns).

Another interesting issue was the fact that the initial diagnosis has been changed in 7 (41.1%) biIgH cases during the course of the disease while there were no changes in diagnosis in the monoIgH group ($P < 0.0002$). Further details are in a Table 1. We did not detect either t(14;18) or t(11;14) in any of the biIgH patients.

Presence of biallelic rearrangements does not influence the clinical prognosis

The estimated TTP of monoIgH patients was not significantly different from the biIgH patients (54.5% vs. 54.5% at 3 year) (P = ns; see Fig. 3A). We also did not observe significant difference in overall survival between both groups (79.4% vs. 100.0% at 3 year) (P = ns; see Fig. 3B). We did not see different probability of overall survival in patients who relapsed or progressed (66.4% vs. 67.5% at 2 year) (P = ns; see Fig. 3C).

Patients with biallelic rearrangements have similar immunophenotype as to patients with monoallelic rearrangements

The majority of patients in both groups had a typical immunophenotype for CLL or SLL when expressing CD5, CD23, CD20, and CD19 with similar frequencies of weak FMC7 positivity (P = ns). Expression of CD38 was also not significantly different between mono-(14; 42.4%) or bi-IgH (4; 23.5%) cases (P = ns). The expression of CD79b was significantly higher in biIgH (5; 29.4%) pts and low in monoIgH group (2; 6.1%) ($P = 0.033$; further see Table 1).

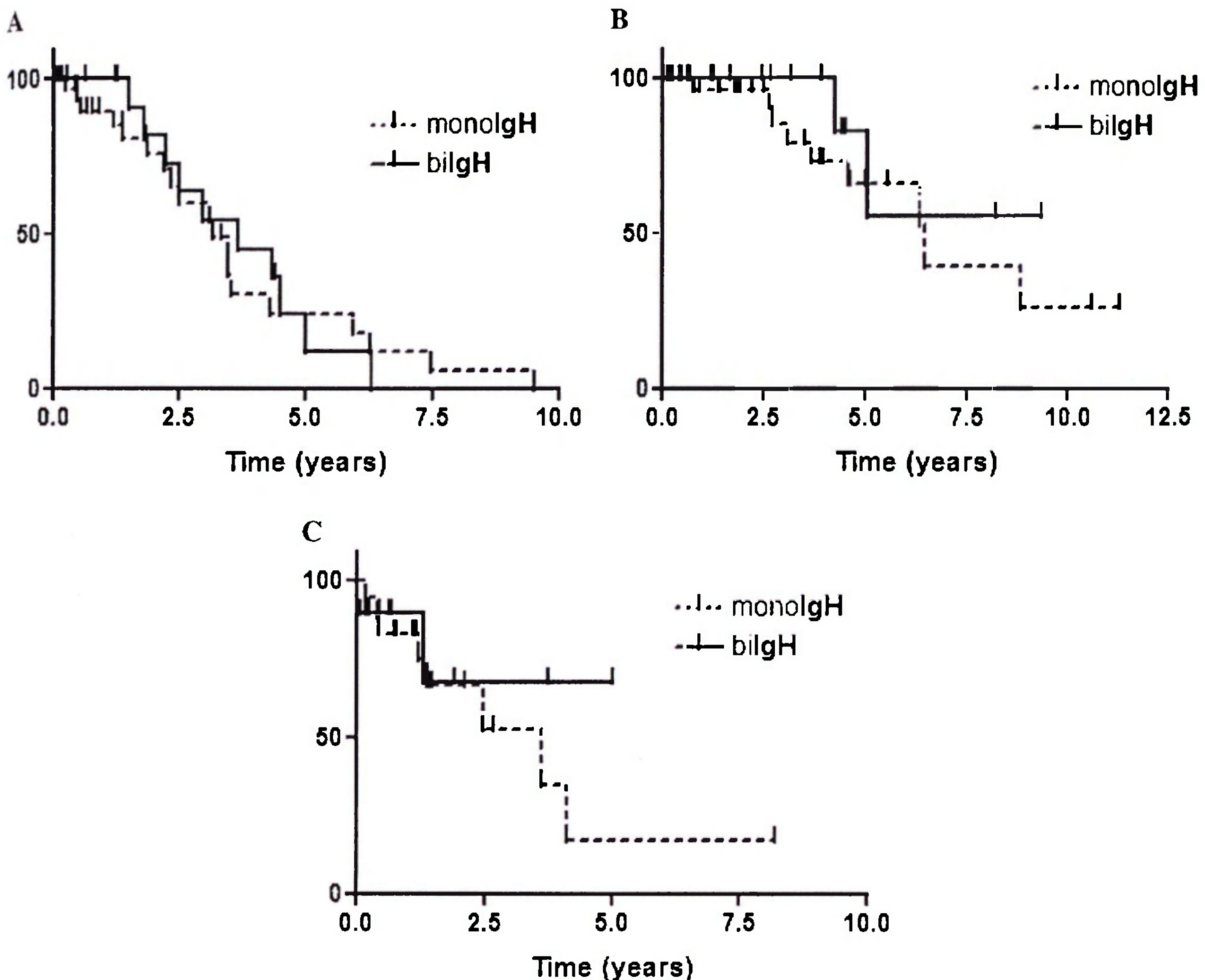

Fig. 3. A: Time to treatment progression. The time of follow-up of the patients is shown on the X axis; the percentage of patients without relapse or progression OR remaining in remission; monoIgH, ··I··; biIgH, –I–; P = ns. B: Overall survival. The time of follow-up of the patients is shown on the X axis; the percentage of patients surviving is shown on the Y axis; monoIgH, ··I··; biIgH, –I–; P = ns. C: Overall survival after 1st relapse or progression. The time of follow-up of the patients is shown on the X axis; the percentage surviving patients after 1st relapse or progression is shown on the Y axis; monoIgH, ··I··; biIgH, –I– ; P = ns).

Presence of biallelic IgH rearrangements is a frequent feature while true biclonalities are rare

We were able to obtain sequences of both CDRIII rearrangements in 12 out of 17 biIgH patients and we also sequenced CDRIII rearrangements of 7 monoIgH pts for comparison. The presence of both PCR bands correlated with the actual clinical situation. It was easy to detect in patients with active disease and more difficult to find in patients who were responding to their therapy. Furthermore, the biIgH result can be evaluated as a result of a duplex PCR (Fig. 2). Using software analysis we observed equal ratio between fluorescent intensities of both IgH rearrangements from different samples of one patient. Since the ratio remained constant over time this was another although indirect proof that these IgH rearrangements originated from one cell. One patient had biclonal disease according to FACS (see Fig. 1B). Both of his IgH rearrangements were in-frame. In ten cases, we found that only one of the two IgH rearrangements was in-frame. We did not detect a stop codon within both CDRIII sequences of one patient, however, FACS did not confirm biclonality. The stop codon might be in the sequence that we did not study (e.g., *VH* gene).

Overall, biclonal disease was detected in 1 (1.1 %) patient and we observed biIgH rearrangements in 17 (19.5 %) pts out of a total of 87 pts with CLL or SLL who were positively tested by PCR for clonal CDRIII rearrangement.

Frequency of *VH*, *D*, and *JH* genes used in productive rearrangements is same as in previously published reports

In our study, we focused on CDRIII sequences in order to determine clonality, therefore, we could not evaluate the usage or mutational status of *V* genes. In common with other reports the majority of the monoIgH tumors used JH4 (three of seven; 42.9%) or JH6 (three of seven; 42.9%), which is also similar to their use in normal CD5 + B-cells (52 and 27%, respectively) (Brezinschek et al., 1997). The short rearrangement of biIgH had mostly JH4 (5 of 12; 41.7%), then even distribution of JH3 (2 of 12; 16.7%), JH5 (3 of 12; 25%), and JH6 (2 of 12; 16.7%). The majority of long rearrangements of biIgH cases used JH6 (8 of 12; 66.7%), then JH4 (2 of 12; 16.7%), JH3 (1 of 12; 8.3%), and JH5 (1 of 12; 8.3%). There was no difference of statistical significance in the use of *JH* genes between any of the group of pts. The productive IgH rearrangements in biIgH cases consisted of JH4 (4 of 12; 33.3%), JH5 (4 of 12; 33.3%), JH6 (3 of 12; 25%), and JH3 (1 of 12; 8.3%). Because of extensive mutations or N additions or nucleotide loss, a *D* segment gene could not be assigned to the sequences in three short rearrangements and three long rearrangements of the biallelic cases. The most commonly detected *D* genes were D3-3 (2× in long rearrangements, 1× in short rearrangements) and D 3-22 (1× in long rearrangements, 2× in short rearrangements). There was no preference of D segments used in out-of-frame rearrangements. We also observed that the length of the rearrangement does not determine if the rearrangement is productive or not. The median length (including primer sequences) of the short rearrangements was 61 bp (range, 24–80 bp). The median length of the long rearrangements was 87 bp (77–212 bp). The median length of the productive rearrangements was 77 bp (51–89 bp).

DISCUSSION

Despite the fact that our study was partially retrospective and we had to use archival samples, we were able to obtain both IgH sequences in 12 pts. The present study was undertaken to rule out biclonal disease, to evaluate a clinical impact of presence of two CDRIII bands in samples from CLL/SLL pts. In B-LPD the detection of a clonal IgH rearrangement by PCR serves as a sensitive tool to "highlight" a predominantly present clone (predominant IgH rearrangement) among the background of normal B-cells (polyclonal IgH rearrangements) (Gleissner et al., 2000). Therefore, by definition, more than one discrete PCR product could be viewed as a failure to detect a malignant clone. Patients in our study were diagnosed with B-LPD and had active disease at the time of PCR analysis, which excluded the possibility that we were dealing with pseudoclonal IgH bands due to low B-cell numbers (Elenitoba-Johnson et al., 2000).

It is important to determine the presence of bimorphic lymphomas or biclonal leukemias as they are of prognostic significance (Fakan et al., 1984). Detection of biIgH one of which is due to a secondary VDVDJ recombination in a patient with SLL may precede a transformation into an aggressive disease (Cerny et al., 2003b). We did not identify a secondary rearrangement in any other pts with biIgH.

Extensive analyses of primary developing B-cells and of Abelson virus transformed cell lines have shown that pro-B-cells invariably contain D-to-JH rearrangements on both IgH alleles (Alt et al., 1984; Ehrlich et al., 1993; Rolink et al., 2000). Since the VDJ recombination is blind to translational reading frame, only one-third of rearrangements are productive, leading to an estimated maximal frequency of cells expressing two in-frame gene rearrangements at a given locus of no more than 0.3% (Casellas et al., 2001). This number agrees with recent estimates of dual light chain expression. The cell loss during the normal B-cell development in the setting of allelic exclusion approximates 90% and in the setting of dual receptor expression might approach 99%, making it likely that very few dual receptor-expressing B-cells would survive (Osmond, 1986). One explanation for the presence of two IgH rearrangements in one B-cell is a lack of allelic exclusion. Data from Rassenti and Kipps (1997) suggested that up to 5% of CLL cases lack allelic exclusion. Signaling through the B-cell receptor is important for survival of a particular cell. It is apparent that this signaling is defective in CLL cells. Aberrant expression of CD79b was previously suspected to explain the allelic exclusion, but no difference in CD79b expression was observed between CLL samples that expressed more than one IgH allele and those with normal IgH allelic exclusion (Rassenti and Kipps, 2000). Interestingly, we found significantly higher CD79b expression in biallelic (29.4%) pts and low in monoallelic group (6.1%) ($P = 0.033$). Based on the sequencing and FACS data we conclude that the allelic exclusion was unlikely to be responsible for the presence of two IgH rearrangements in our patients.

The combination of at least two methods must be employed in order to prove biclonality as suggested by Sklar et al. (1984). Sequencing and FACS analysis confirmed only one biclonal disease (1.1%; see Fig. 1B), which correlates with the expectations that true biclonal cases are rare. Based on the results of FACS analysis and molecular studies, we conclude that in the 16 remaining patients we detected rearrangements on both alleles for IgH (one in-frame and one out-of-frame) that were originating from one cell. Interestingly, Lossos et al. (2000) reported non-functional IgH rearrangements in 17% patients with diffuse large cell lymphoma. The authors did not mention the prognosis of those patients. Since we did not observe a significant difference in TTP and OS between both biIgH and monoIgH group it seems that the presence of complete biIgH rearrangements does not have an impact on the biology and therefore the prognosis of the disease. Nevertheless, some of the findings are worth discussing. We have observed a higher number of comorbidities in patients with biallelic (41.1%) than monoallelic (18.2%) rearrangement. The pathogenesis of AIHA and other autoimmune disorders or syndromes has been previously investigated (Martin et al., 1994; Crouzier et al., 1995; Efremov et al., 1996). Strikingly similar CDRIII regions that contained a single reading frame of the D 3-3 (*DXP4*) gene segment, an N-encoded proline at the DH/JH boundary, and a tyrosine-rich region encoded by the *JH6* gene segment were observed in CLL patients with AIHA (Efremov et al., 1996). We were able to evaluate sequences of two CLL pts with AIHA. We detected both D 3-3 segment and JH6 segment in one patient and JH6 segment in the second patient in the productive IgH rearrangements. Due to extensive mutations or N nucleotide additions we could not assign any D segment to one of the two CDRIII sequences of the second patient. The fact that certain CDRIII sequences are associated with AIHA in CLL patients may help to identify the patients at risk. While the current literature does not give a clear message about this issue, we would like to emphasize that the CDRIII from the productive allele must be used for these studies. This is also true for assessment of mutational status of *Ig* genes.

It is interesting that the histological diagnosis had to be reviewed and changed in 7 (41.1%) biallelic pts during the course of the disease while there were no changes in diagnosis in the monoallelic group ($P < 0.0002$). These changes were mostly down grading to CLL or SLL from 3× mantle cell lymphoma (MCL), 2× diffuse centrocytic–centroblastic lymphoma (difCC–CB_, 1× follicular lymphoma (FL) and 1× marginal zone lymphoma (MZL). B-cell lymphomas usually express a B-cell receptor with functional Ig, and the chromosomal translocations are predominantly located on the non-functional Ig alleles (Kuppers and Dalla-Favera, 2001). It has been also suggested previously that a prevalent stimulus for receptor editing might result in a high frequency of chromosomal translocation and consequent lymphoid malignancy (Schlissel, 2002). We did not detect any translocation t(14;18), which is a typical aberration of lymphoma cells arising from a germinal center and neither t(11;14) was detected. We can only speculate that biIgH rearrangement bearing cells perhaps represent an intermediate step in a malignant transformation process between a lymphoma bearing chromosomal translocation and lymphoma without translocation. The latter would be a case when the translocation was targeted by the recombination machinery and secondary VDJ rearrangement took place during the clonal evolution (Cerny et al., 2003b).

Finally, the fact that the sensitivity of PCR detection of minimal residual disease in patients with biIgH may be decreased may also negatively influence the patient's prognosis. Therefore allele specific PCR should be used for minimal residual disease monitoring of patients with B-LPD.

ACKNOWLEDGMENTS

The authors thank Monika Hofmanova and Helena Achylisova for technical support. This work was supported by grants from the Ministry of Education No. 11110004, GAUK No. 203580.

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Familial chronic lymphocytic leukemia

Familial aggregation of chronic lymphocytic leukemia (CLL) has been observed more frequently than familial aggregation of any other type of oncohematologic disorder. The presence of cells with a CLL-like immunophenotype (CLL-like celis) was recently documented in 13.5% healthy first-degree relatives of CLL patients.[1] We present a family with CLL in which 2 brothers, a sister and their mother were affected.

haematologica 2003; 88:1190-1191
(http://www.haematologica.org/2003_10/1190.htm)

We used polymerase chain reaction (PCR) and flow activated cell sorting (FACS) to investigate bone marrow samples (BM) and peripheral blood samples (PB) obtained from patients as described elsewhere.[2] PCR products were extracted from 3% Metaphor agarose gel using the crush and soak technique[3] and purified with Microcon-100 (Millipore) columns, sequenced with a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and analyzed on an ABI Prism 310 Genetic Analyzer (PE BioSystems).

Two patients (brothers) who were diagnosed (brother I at age 52; brother II at 60) and treated for CLL carried a PCR-detectable IgH rearrangement. Their mother and sister died (both had enlarged lymph nodes; Figure 1). In both cases the disease became refractory to chemotherapy. Transformation to a high-grade malignancy was suspected in brother I, but not confirmed. He died approximately 9 years after the initial diagnosis. In the second brother, the transformation to Richter's syndrome was confirmed and he died approximately 3 years after the diagnosis.

Both cases had a typical immunophenotype characterized by monotypic surface expression of CD19, CD5, CD23, CD20, CD11c, HLA-DR, and immunoglobulin light chain κ. Clonality (as mentioned above) was verified by PCR. Nucleotide sequence data analysis revealed the presence of two different CDRIII rearrangements (109 bp in patient I and 110 bp in patient II, Figure 2). The first patient's CDRIII sequence consisted of V(?) - D1-7 - JH6c gene rearrangement, was in- frame (see Figure 2 A). The second patient's CDRIII consisted of V(?) - D6-19 - JH3b segments. When we looked for the position of the reading frame, we found a TGA stop codon (unproductive rearrangement; Figure 2B). That means we have detected the unproductive allele for IgH. Since FACS confirmed monoclonality, the other allele was rearranged and productive, but not detected by

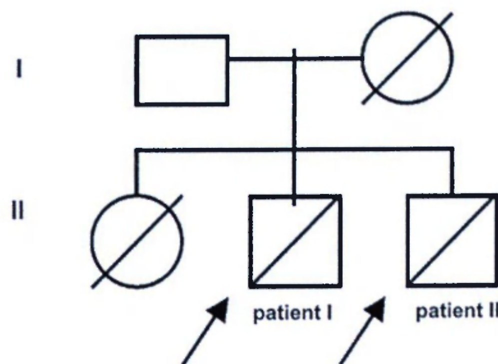

Figure 1. Pedigree of a family with CLL: Mother and daughter died from leukemia (both had enlarged lymph nodes). Two brothers (sons) were diagnosed as having CLL: one died 9 years after diagnosis, the other developed Richter's syndrome and died within 3 years of diagnosis.

Figure 2A. Sequence of CDRIII rearrangement obtained from PCR positive samples from patient I.

5'- AC-ACG-GCY-STG-TAT-TAC-TGT-GCG-AGA-
FR3A primer 3' end of VH gene

-GAG-G GA-
(N-nucleotides)

-ATA ACT-GGA-ACT-ACG-TAC-
D 1-7 segment

-ATT-ACT-ACT-ACT-ACT-ACA-TGG-ACG-TCT-GGG-GGA-AAG-

-GGA-CCA-C-
JH6c sequence

-GTC-ACC-GTC-TCC-TCA-G- 3'
JH primer

FR3A: framework region 3; D: diversity segment; JH: joining gene; N: nucleotides (small letters); sequence between rearranged VH, D and JH genes (capital letters); primer and D sequences underlined.

Figure 2B. Sequence of CDRIII rearrangement obtained from PCR positive samples from patient II.

5'- AC-ACG-GCY-STG-TAT-TAC-TGTGCG-AGA-
FR3A primer 3' end of VH gene

GGA-CTG-GAC-CCA-C-

-AT-AGC-AGT-GGC-TGG-CTG-GTT-GGG-
D 6-19 segment (N- nucleotides)

Stop codon
-TGA-TGC-TTT-TGA-TTC-TGG-GGC-CAA-GGG-ACA-ATG-
JH3b sequence

-TCA-CCG-TCT-CCT-CAG- 3'
JH primer

FR3A: framework region 3; D: diversity segment; JH: joining gene; N: nucleotides (small letters); sequence between rearranged VH, D and JH genes (capital letters); primer and D sequences underlined.

our PCR primers. The comparison of CDRIII sequences with the germ line sequences did not suggest mutation. However, since we used PCR to detect the CDRIII region we could not further evaluate the sequence and mutational status of the VH genes.

Our first patient (brother I) presented with an indolent disease. The second patient (brother II) was diagnosed approximately 5 years later, but his disease was more aggressive and he died after developing Richters syndrome. Richter's transformation was linked to a DNA mismatch-repair defect-initiated microsatellite instability.[4] This genetic alteration was initially identified in the hereditary non-polyposis colorectal cancer syndrome (HNPCC).[5] We tried to confirm the diagnosis of both mother and sister. Unfortunately, because of the long period between their death and diagnosis of the first brother, the old medical data were no longer available. Nevertheless our patients' history is suggestive of familiar CLL.

The mutational status of VH genes does not change during the course of the disease and has been documented to hold within a family with CLL.[6] In contrast, the behavior of CLL in both our patients was different (indolent versus aggressive) despite both having unmutated Ig status (poor prognosis). The second patient (brother II) was not tested for the presence of CLL-like cells prior to diagnosis, nor for VH gene mutations because he had been diagnosed and treated before studies of mutational status of VH genes[7] and data by Rawstron *et al.*[1] had been published. That is also why we had to use limited archive samples and PCR products for sequencing.

While recent data suggest that ZAP-70 expression should be included in the diagnostic work-up of patients with CLL as a more convenient prognostic marker than VH genes mutation,[10] the studies showing a high incidence of CLL-like cells in healthy first-degree relatives of CLL patients indicate that an early FACS analysis of these relatives should be considered as a screening for pre-CLL.[1] As we now have a variety of relatively non-toxic therapeutic modalities (e.g. monoclonal antibodies anti-CD20 or anti-CD52) available and early stages of CLL[8,9] respond better to such therapy, CLL with its later consequences, including Richter's transformation, could perhaps have been avoided and CLL, at least in its initial form, may become a preventable type of cancer.

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Key words: leukemia, familial, clonality, IgH rearrangement, polymerase chain reaction, clonal lymphocytosis of uncertain significance.

Funding: supported by grants from the Ministry of Education No. 11110004, GAUK No. 203580.

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Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received July 17, 2003; accepted August 18, 2003.

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A rare β-thalassemia mutation (C-T) at position -90 of the β-globin gene discovered in a Chinese family

We provide the first description of a Chinese family with three heterozygotes for a rare β-thalassemia mutation previously observed in a Portuguese carrier. The mutation (-90 C-T) changes the conserved promoter sequence within the proximal CACCC box of the β-globin gene; this can reduce β-globin transcription significantly.

haematologica 2003; 88:1191-1193
(http://www.haematologica.org/2003_10/1191.htm)

β-thalassemia is a varied group of disorders of hemoglobin (Hb) synthesis, most of which result from point mutations within the β-globin gene or the immediate flanking sequence. Over 200 different β-thalassemia mutations have now been characterized worldwide.[1] Within each population at risk for β-thalassemia a small number of common mutations are found. For example, in the Chinese population, five mutations, of the 30 known, account for more than 90% of all cases.[2-4] Here we describe a rare β-thalassemia mutation previously unreported in the Chinese population, the C-T substitution at position -90 in the proximal CACCC box of the β-globin gene.

The proband was a 27-year old woman from a Chinese family originating from Sihui county of Guangdong Province, southern China. We studied this family because the proband was found to have a typical hypochromic microcytosis during routine genetic screening for β-thalassemia but no known mutations reported in the Chinese population could be identified. Standard hematologic techniques were used to measure RBC counts and Hb concentration. Reverse dot blots (RDB)

Unusual sequence of vdj rearrangement revealed by molecular analysis in a patient with indolent lymphoma

We report a unique case of indolent lymphoma with an unusual VDJ rearrangement. Polymerase chain reaction (PCR) analysis of bone marrow at the time of diagnosis was positive for both BCL-2/JH and CDRIII rearrangements. After treatment, the patient achieved complete remission (CR) with slow disappearance of both rearrangements (CDRIII and then BCL-2/JH). Subsequently, two new CDRIII rearrangements were detected in bone marrow, peripheral blood, and lymph node tissue. After this conversion, fluorescent activated cell sorting (FACS) analysis demonstrated monoclonal disease, suggesting that both CDRIII rearrangements originated from one cell. Histological evidence of a B-cell small lymphocytic lymphoma (B-SLL) infiltrate in the bone marrow became evident approximately 1 year after the two CDRIII rearrangements appeared. Direct sequencing revealed that one of the CDRIII sequences consisted of a VDVDJ rearrangement. This is the first report of such a rearrangement in a case of indolent lymphoma. This type of rearrangement has been described to result from a secondary VDJ recombination in childhood acute lymphoblastic leukemia (ALL) leading towards oligoclonality and poorer prognosis. Our observations suggest that such a finding in an indolent lymphoma patient may precede transformation into an aggressive disease. Early detection by PCR could have substantial impact on the prognosis of such patients.

Haematologica 2007; 88:(5)e61-e63

Introduction. B-cell chronic lymphocytic leukemia/ small lymphocytic lymphoma (B-CLL/SLL) and Follicular lymphoma (FL) both typically represent distinct entities with different morphology, immunophenotype, histology and genetics. However, FL occasionally, can show morphological features of both FL and monocytoid B-cell lymphoma (e.g. marginal-zone lymphoma) making the diagnosis challenging. [1,2] These lymphomas have been called composite lymphomas. [3]The molecular pathogenesis of B-CLL/SLL remains unknown. There is no typical chromosomal translocation associated with this pathological entity. High level of *BCL-2* expression that is consistently seen in B-CLL/SLL results from other mechanism (e.g. oncogene hypomethylation).[4] B-CLL/SLL is characterized by an accumulation of neoplastic B-cells positive for CD5, CD23, and CD19 and negative for surface CD22 and FMC7 with a low proliferative rate. Based on studies of mutational status B-CLL/SLL can be divided into cases with unmutated and mutated VH genes. Cells with mutated VH genes have gone through the germinal center (GC) and are connected with more favorable course of disease.[5-7] Chromosomal translocation t(14;18)(q32;q21) that occurs between the BCL-2 protooncogene and the JH immunoglobulin gene region is a hallmark of follicular lymphoma (FL) and can be detected in 85% to 90% of FL.[8] The lymphoma cells originate from germinal center that means they have already encountered with antigen. On the cell surface they express CD19, CD20, CD22, CD24, CD10, immunoglobulins and are CD5 negative. Clinically FL and B-CLL/SLL belong to low grade lymphomas with median survival approximately 10 years. Up to 70% of low grade lymphomas tend to convert into an aggressive lymphoma with a diffuse large cell architecture over time.[8,9] Detection of BCL-2/JH translocation and/or the CDRIII of immunoglobulin heavy chain gene rearrangement by PCR is commonly used for diagnostic purposes and can be monitored for minimal residual disease (MRD) evaluation during the posttreatment follow-up.

Methods. The material tested by PCR was represented by bone marrow samples (BM), peripheral blood samples (PB) and lymph node (LN) obtained from patient. The DNA was extracted using standard procedures and the usual precautions to avoid cross-contamination. The presence of the BCL-2/JH translocation in the major breakpoint region (MBR) of the BCL-2 gene was examined using a touchdown PCR (TD-PCR)[10] modified for our conditions. The presence of the clonal immunoglobulin heavy chain gene rearrangement (CDRIII, complementary determining region) was examined by polymerase chain reaction (PCR), as described previously.[11] Each reaction contained positive and negative control. Amplified products were visualized on 2% polyacrylamide or 3% Metaphor agarose (FMC Bioproducts, Rockland, ME, USA) gels stained with ethidium bromide. Results were confirmed by repeat PCR at least once. The sensitivity was routinely better then 10 positive cells in 10^5 normal cells as determined by comparative PCR and also real time PCR.[12] PCR products were extracted from 3% Metaphor agarose gel slices using the crush and soak technique[13] and purified with Microcon-100 (Millipore) purification columns, sequenced with BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and analyzed on ABI Prism 310 Genetic Analyzer (PE BioSystems).Sequences obtained from each sample were compared with germ line sequences in the EMBL/GenBank and current databases (V-BASE sequence directory; I.M. Tomlinson, MRC Center for Protein Engineering, Cambridge, UK) and the closest sequence was assigned.[14] Attribution of the D segments was based on the identification of at least 6 consecutive bases without mismatches. The nomenclature proposed by Corbett *et al.* was adopted.[15]

Case history I. A 58-year-old man was diagnosed with a stage IV nonhodgkin lymphoma which was described as folicullar (FL) type in 1993. The PCR revealed positivity for BCL-2/JH translocation in BM. The BM was also positive for immunoglobulin heavy chain gene rearrangement (CDRIII). The patient received 6 cycles of ProMACE-MOPP and then 4 cycles of ProMACE-CYTABOM (Prednisone, Doxorubicin, Cyclophosphamide, Etoposide, ara-C, Bleomycine, Vincristine, Methotrexate, Leucovorin) and reached complete remission. He remained PCR positive for BCL-2/JH and CDRIII rearrangements. Then first CDRIII disappeared followed by BCL-2/JH clearance. This was due to more sensitive PCR technique (see sample from 1995 in table 1). The next PCR test (see sample from 1996 in table 1) revealed surprising result as the patient converted from positivity for BCL-2/JH and CDRIII into BCL-2/JH negativity and CDRIII double positivity in BM. These findings were consistent also in subsequent samples and other tissues (PB and LN). The two distinct CDRIII rearrangements were confirmed by direct sequencing. Later on also the histology and the FACS analysis was consistent with BM infiltration with a B-CLL/SLL infiltrate. Slow progression in BM occurred in XI/1999, approximately 3 1/2 years after the molecular conversion. Then the patient presented with generalized lymphadenopathy within the matter of weeks. Upon histological evaluation a large cell infiltrate in BM sugested a

Table 1: [illegible]

	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
date	LN	BM	PB	PBPC	BM	BM
[illegible]		[illegible]				
[illegible]			[illegible]			
[illegible]		[illegible]				
[illegible]			[illegible]			
[illegible]			[illegible]			
[illegible]				[illegible]		
[illegible]				[illegible]		
[illegible]				[illegible]		
[illegible]				[illegible]		
[illegible]				[illegible]		
[illegible]		[illegible]			[illegible]	[illegible]
[illegible]		[illegible]			[illegible]	[illegible]
[illegible]		[illegible]			[illegible]	[illegible]
[illegible]		[illegible]			[illegible]	[illegible]
[illegible]		[illegible]			[illegible]	[illegible]
[illegible]			[illegible]			
[illegible]		[illegible]			[illegible]	[illegible]
[illegible]		[illegible]			[illegible]	[illegible]
[illegible]			[illegible]		[illegible]	[illegible]
[illegible]			[illegible]		[illegible]	[illegible]
[illegible]			[illegible]		[illegible]	[illegible]
[illegible]	[illegible]				[illegible]	[illegible]
[illegible]			[illegible]		[illegible]	[illegible]
[illegible]		[illegible]			[illegible]	[illegible]
[illegible]			[illegible]		[illegible]	[illegible]

Table 2A: [illegible]

5'- AC ACG GCY GTG TAT TAC TGT [illegible]
FR3 primer — 3' end of VH gene

[illegible]
D 2-8 segment — (N nucleotides)

[illegible]
JH6b sequence

[illegible]
JH primer

Table 2B: [illegible]

5'- AC ACG GCY GTG TAT TAC TGT [illegible]
FR3 primer — 3' end of VH gene

[illegible]
(N nucleotides) — D 3-22 segment

[illegible]

[illegible]
Stop codon

[illegible]
VH 4-4 gene [illegible]

[illegible]
(N nucleotides) — D 3-21 segment

[illegible]
JH4b sequence — JH primer

disease transformation. The patient obtained 1 cycle of salvage chemotherapy (ESAP), but further progressed. The treatment was then changed to fludarabine and cyclophosphamide together with rituximab (anti-CD20). The disease remained resistant to therapy and the patient died due to disease progression.

Results and discussion. This is a first report of patient with indolent lymphoma bearing a secondary VDJ rearrangement. This report discusses also a rare case of indolent lymphoma with both alleles for IgH rearranged in the malignant clone. Sequencing revealed two distinct CDRIII rearrangements (117 bp and 252 bp, see table 2) in BM, PB as well as LN. The result of each PCR can be evaluated as a duplex PCR. Using software analysis we observed equal ratio between fluorescent intensities of short and long CDRIII from different samples. This result was another proof of monoclonality. We wanted to sequence the BCL2-JH translocation and CDRIII rearrangement from the diagnosis samples. However, we were not able to successfully amplify any of those archival samples due to lack of DNA. That the two CDRIII rearrangements originated from single malignant clone was confirmed by FACS analysis. The immunophenotype of tumor cells was characterized by monoclonal surface expression of CD 19, CD 5, CD 23, CD 20, CD 11c, and immunoglobulin light chain kappa, FMC 7 and small % CD 79b. The histology resembled B-CLL/SLL at that time so one explanation for presence of two CDRIII rearrangements in the patient's BM could be that those result from lack of allelic exclusion. Rassenti *et al.* presented that up to 5% of CLL cases lack allelic exclusion. [16] Aberrant expression of CD79b was previously suspected to lead to lack allelic exclusion, but no difference in CD79b expression was observed between CLL samples that expressed more than one IgH allele and those with normal IgH allelic exclusion. [17] We compared both CDRIII sequences with the corresponding germline sequences using the GenBank/EMBL database computer-based search. The shorter CDRIII resembled a usual V(¿) - D2-8 - JH6b gene rearrangement and was within reading frame and productive (see table 2A). The longer CDRIII consisted of V(¿) - D3-22 - VH 4-4 - D2-21 - JH4b segments and we found a stop codon TAG (unproductive rearrangement; see table 2B). The comparison of CDRIII sequences with the germ line sequences did not suggest mutation. However mutational status of VH genes was not evaluated in detail. Without the initial BCL-2/JH and CDRIII sequences from the diagnosis it is rather difficult or speculative to explain whether this molecular conversion resulted from a clonal evolution or if our patient had a composite lymphoma (the originally dominant FL clone was eradicated and the minor clone of B-SLL expanded). However, the presented data still suggest a presence of secondary VDVDJ recombination that is unusual for indolent lymphoma. The V(D)J recombinase possesses latent transponase activity. Errors of V(D)J recombination may generate chromosomal translocations consistent with those present in lymphomas and leukemias. Lymphomas bearing t(14;18) have been classified by phenotype and genotype as malignant disorders originating from germinal center (GC) B cell. The GC is a site of affinity maturation where B cells undergo V(D)J hypermutation, Ig class switch and may also support secondary V(D)J recombination. The BCL-2/JH translocation is typical for tumor cells of GC B origin and remains stable even after the transformation occurs. [18] In our patient this marker disappeared as the patient reached remission. The CDRIII is often used as molecular marker in B cell lymphoproliferative disorders. Our patient was originally positive for one CDRIII, then was shortly CDRIII negative. Later on we detected two CDRIII rearrangements. This was followed by the disease progression and transformation. It is known that in 30-50% of cases of childhood B precursor acute lymphoblastic leukemia the CDRIII may change along the course of the disease leading to oligoclonality. This is believed to result from a secondary V gene to DJ rearrangement. [19] There is also some evidence about receptor revision through secondary VH gene rearrangements in normal human B-lymphocytes. [20] We are not

aware of data about such phenomena in indolent lymphomas or leukemias. It is believed that secondary rearrangements are driven by somatic hypermutation, which takes place in GC. It is difficult to explain the VDVDJ rearrangement in presented case since we did not observe any mutations within both CDRIII sequences. Our observation suggests that occurrence of a secondary rearrangement is possible also in indolent lymphomas. Since it may be followed by transformation into aggressive disease, detection of such CDRIII sequence should be considered seriously and the patient managed carefully before a full-blown clinical relapse/progression of aggressive disease develops.

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Chemotherapy alone may lead to a PCR negative stem cell harvest in transformed lymphoma refractory to rituximab

We report a case of patient with transformed low-grade lymphoma who was treated with chemotherapy alone for her 4th relapse and achieved polymerase chain reaction (PCR) negative stem cell harvest. Based on our observations we would like to discuss the strengths and weaknesses of PCR monitoring in lymphoma patients. Chromosomal translocation t(14;18)(q32;q21) between the BCL-2 protooncogene and the JH immunoglobulin gene region can be detected in 85% to 90% of follicular lymphoma (FL). Up to 70% of low-grade lymphomas tend to convert into an aggressive lymphoma with diffuse large cell architecture over time.[3,4] The histological shift is generally accompanied by the accumulation of secondary genetic changes that lead to poor outcome (p53 mutations, mutations of BCL-6 etc.).[5] The BCL-2/JH translocation remains stable even after the transformation and thus is used as a clonal marker. Polymerase chain reaction (PCR) followed by sequencing is a rapid technique for diagnosis confirmation. PCR monitoring is of prognostic value when used for minimal residual disease (MRD) evaluation during the follow-up.[6]

Haematologica 2007; 88:(2)e19-e20

The DNA was extracted from bone marrow (BM) and peripheral blood stem cells (PBSC) using standard precautions to avoid cross-contamination.[7] The presence of the BCL-2/JH translocation in the major breakpoint region (MBR) of the BCL-2 gene was examined using a touchdown PCR (TD-PCR)[8] modified for our conditions (for details contact author).

Each reaction contained positive and negative control. The sensitivity was routinely better then 10 positive cells in 10^5 normal cells. PCR products were purified with Microcon-100 (Millipore) purification columns, sequenced with BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and analyzed on ABI Prism 310 Genetic Analyzer (PE BioSystems).

The patient was a 58-year-old female diagnosed with nonhodgkin's lymphoma (NHL) in 1992. The histology was concluded as clinical stage IVB of small lymphocytic lymphoma (cervical, axilary, inquinal nodes, spleen and BM involvement). She received 10 cycles of COP and 3 cycles of chlorambucil with prednisone and reached partial remission (PR; BM morphologically negative, spleen borderline). She was asymptomatic untill 1st progression (Pr) occurred in 1997 (in BM, paraaortic, mesenterial and all external lymphnodes). She received chemotherapy (6xCHOP then 6xM2) with radiotherapy of spleen and reached PR again. The 2nd Pr was treated by radiotherapy of involved lymph nodes (axillary) and spleen again (II/1999). In IX/1999 the patient was first time evaluated by PCR. BCL-2/JH translocation was confirmed in BM. The histology diagnosis has been changed into follicular lymphoma.

The patient was also positive for immunoglobulin heavy chain gene rearrangement (CDRIII) by PCR. PCR for BCL-2/JH translocation was used for further monitoring. The 3rd Pr (IV/2000; in BM, spleen, liver, paraaortic and mesenterial lymphnodes) was accompanied by transformation into aggressive diffuse large cell lymphoma (partially expressing also CD30). The patient received 4 doses of rituximab (monoclonal antibody anti-CD20) in combination with ESAP chemotherapy and achieved CR including PCR negativity in BM. In XII/2000 she presented with her 4th relapse in chest and abdominal lymphnodes, BM histology was negative, but PCR positive with a molecular confirmation of the malignant clone. The patient received 2 cycles of salvage chemotherapy (IVE) and was able to collect a stem cell harvest that was negative by PCR. However, being heavily pretreated she developed profound pancytopenia complicated by sepsis and renal failure. The patient succumbed to these complications.

Using TD-PCR and sequencing we obtained a BCL-2/JH translocation of approximately 200 bp (within published range) both prior and at the time of 4th relapse. (see Table 1)

Thus the initial diagnosis was changed into follicular lymphoma. It is obvious that a PCR technique should be attempted in order to establish a precise diagnosis especially in cases when the histology or other laboratory tests are not conclusive.

Median survival of patients with transformed low-grade lymphomas is less than 1 year despite further conventional therapy.[9] Therefore patients with chemosensitive transformed NHL should be seriously considered for high-dose therapy and autologous stem cell support.[10] Numbers of patients in reports showing the outcome after treatment with rituximab for transformed malignancies are low, especially those evaluating the molecular features of malignant cells.[11,12] We observed that it is possible to obtain a PCR negative autograft by using salvage chemotherapy even in patients with a 4th relapse of transformed NHL.

We would like to highlight some issues of PCR monitoring in NHL. Our patient's BM was PCR negative (VIII/2000) and CT studies were also negative shortly

Table 1A: MBRA-JH sequence obtained from PCR positive BM samples: MBRA (blue)- major breakpoint region A, NDN (black)- sequence between break of MBRA and JH genes (blue); D segment (green), primer sequences, D segment and break sequence are underlined.

5'- [illegible]
MBRA primer

[illegible]
break sequence

[illegible]

[illegible]

[illegible]
JH6 sequence

[illegible] -3'
JH primer

Table 1B: CDRIII rearrangement sequence obtained from PCR positive BM samples: FR3A (blue)- farmework region 3, NDN (black)- sequence between rearranged VH genes and JH genes; JH gene (blue), primer sequences underlined.

5'- [illegible]
FR3A primer

[illegible]
NDN sequence [illegible]

[illegible]
JH sequence JH primer

[illegible] -3'

before the 4th relapse.

That relapse occurred in lymph nodes allows us to reason that residual tumor cells in lymph nodes were responsible for the recurrence. More frequent PCR evaluation has a prognostic impact in patients with acute lymphocytic leukemia. [13] Our experience suggests that more frequent PCR testing especially in patients with aggressive lymphomas should be warranted during the follow up. Secondly, unlike in leukemia where the disease typically presents within easy-to-sample tissues (BM, PB), in NHL the most reliable tissue for PCR follow up has not yet been clearly established. We recently presented that rituximab alone or in a combination with chemotherapy may induce PCR negativity in the BM of high percentage of NHL patients and that is connected with a better prognosis. [6, 14] However, patient with PCR negativity in BM may still have active disease e.g. in lymph nodes. [15] These observations bring to our attention other methods for early disease detection such as positron emission tomography (PET). [16] We believe that PET imaging may have a prognostic impact in patients without molecular marker and may be also considered in PCR informative patients as the disease may recur in sites or organs that are difficult to sample regularly. Initiation of randomized trials is needed to validate this hypothesis.

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přehled

VÝZNAM MINIMÁLNÍ REZIDUÁLNÍ NEMOCI A METODY JEJÍHO STANOVENÍ U PACIENTŮ S NĚKTERÝMI HEMATOLOGICKÝM MALIGNITAMI

THE SIGNIFICANCE OF MINIMAL RESIDUAL DISEASE AND METHODS OF ITS DETECTION IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES

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Souhrn: Rychlý vývoj cytogenetiky, průtokové cytometrie a molekulární biologie rozšiřuje možnosti detekce minimální reziduální nemoci. V obecné části je uveden stručný přehled nejčastěji užívaných laboratorních technik. Ve specializované části jsou pak shrnuty literární údaje o klinickém významu detekce reziduální nemoci u některých hematologických malignit.

Klíčová slova: minimální reziduální nemoc, cytogenetika, průtoková cytometrie, molekulární biologie, polymerázová řetězová reakce, molekulární kompletní remise, transplantace krvetvorných kmenových buněk, monoklonální protilátka

Summary: The rapid development of cytogenetics, flow cytometry and molecular biology broadens the spectrum of options for minimal residual disease detection. A brief overview of the most frequently used laboratory techniques is given in the general part. The specialized part summarizes the data from literature concerning the clinical significance of the minimal residual disease detection in certain hematological malignancies.

Key words: minimal residual disease, cytogenetics, flow cytometry, molecular biology, polymerase chain reaction, molecular complete remission, transplantation of hematopoietic stem cells, monoclonal antibody

Úvod

Prakticky všechna onkologická onemocnění představují klonální proces, kdy se určitá část buněčné populace, klon, vymkla kontrolním mechanismům růstu. V hlavní roli se zde uplatňují specifické změny v genomu buňky (primární změny). Jejich přítomnost může vyvolat nádorovou transformaci normální buňky v nádorovou buňku určitého typu (např. translokace t(9;22) u chronické myeloidní leukémie, CML). V patologicky změněném genomu dochází v průběhu času nadále k mutacím (sekundární změny). Jejich výsledkem je vznik subklonů buněk, které jsou čím dál tím méně ovlivnitelné vnitřními fyziologickými mechanismy kontroly růstu buněk, stejně tak jako jsou i méně citlivé k protinádorové terapii. Sekundární změny jsou tedy zodpovědné za rezistenci, progresi onemocnění (např. mutace proteinu p53). Předpokladem vyléčení nemocného s onkologickým onemocněním je úspěšné zničení maligního klonu buněk, tj. navození „kvalitní" kompletní remise onemocnění.[1] Kvalitu remise můžeme měřit s různou citlivostí. Podle vybrané metodiky můžeme hovořit o kompletní remisi klinické (clinical complete remission, CCR), cytogenetické, imunologické, nebo molekulární (molecular complete remission, MCR).

CCR je možno definovat jako vymizení známek onemocnění pod úroveň detekovatelnou při použití běžných vyšetřovacích metod (radiodiagnostických i laboratorních). Obecně platná kriteria klinické kompletní či parciální remise (partial remission, PR) jsou pak ještě upravována jednotlivými pracovními skupinami jako je tomu např. pro nehodgkinské lymfomy (NHL).[2] U hemato-onkologických pacientů lze dosáhnout s vysokou pravděpodobností CCR, dochází však také ve vysokému procentu k návratu nemoci, relapsu. Relapsy jsou ve své konečné fázi nejčastější příčinou selhání protinádorové terapie. Předpokládá se, že k rekurenci onkologických onemocnění přispívají reziduální maligní buňky. Subklinickým zbytkům nádoru se říká minimální reziduální nemoc (minimal residual disease, MRD). Rozsah a vývoj MRD v průběhu onemocnění lze sledovat s různou citlivostí laboratorních technik.

Metody:

- *cytogenetické* (cytogenetika; popř. fluorescein in situ hibridization, FISH), mohou detekovat 1 nádorovou buňku mezi 10^2 buňkami normálními
- *imunologické* (imunofenotypizace; průtoková cytometrie, fluorescein activated cell sorting, FACS), vykazují vyšší citlivost tj. 10^{-2}-10^{-3}
- *molekulární* jsou nejcitlivější (zejména polymerase chain reaction, PCR nebo reverse transcriptase-polymerase chain reaction, RT-PCR), citlivost 10^{-3}-10^{-6}

Tak jako je možné označit stav pacienta bez detekovatelné MRD jako molekulární kompletní remise (molecular complete remission, MCR) lze podobně definovat i molekulární nebo také PCR relaps, což je označení užívané situace, kdy je zejména pomocí kvantitativní PCR zjištěna expanze patologického klonu o 1 log bez závislosti na intervalu od předchozího vyšetření bez známek klinického relapsu.[3]

Cytogenetika

Chromozomální analýza lidských buněk používá jednak klasických cytogenetických metod, kdy se na metafázických chromozómech hodnotí jejich počet, velikost, tvar a strukturu chromozómů.[4] DNA sond (prób) identifikujících určitý úsek DNA lze použít i pro buňky v interfázi. Zprvu radioaktivně značené sondy byly nahrazeny sondami fluorescenčními (*FISH*), což

umožnilo rozvoj cytogenetiky. Dnes jsou k dispozici tzv. barvicí sondy specifické pro každý chromozóm, kterými lze odhalit několik aberací najednou a mimo jiné také delece a amplifikace genetického materiálu.
Technika *komparativní genomické hybridizace (comparative genomic hybridization, CGH)* porovnává barvení chromozómů nádorových a normálních buněk. Pokud není ztráta nebo nadbytek DNA, daný úsek chromozómu je žlutý (rovnováha mezi červenou a zelenou barvou). V případě imbalance se daný úsek zbarví červeně nebo zeleně. Z této metodiky vychází i tzv. microarrays. Novější metodou je tzv. *multibarevná FISH (multicolor FISH, M-FISH)*,[5,6] kdy se jednou či více barvami označené chromozómy normálních buněk porovnávají s chromozómy buněk nádorových. Touto metodou lze tedy identifikovat změněné chromozómy, avšak bližší specifikace vyžaduje použití specifické DNA próby.
Cytogenetická odpověď např. u CML představuje procento jader negativních pro danou cytogenetickou změnu, tj. t(9;22) historicky Philadelfský chromozóm (Ph). Podle stupně se odpověď dělí následovně: žádná, minimální (1% až 32% Ph neg), minor (33% až 65% Ph neg), major (66% až 99% Ph neg) a kompletní (100% Ph neg).[7]

Imunofenotypizace
Průtoková cytometrie používá značení povrchových nádorových antigenů pomocí monoklonálních protilátek vážících se specificky na tyto povrchové znaky.[8] Většina analýz FACScm závisí tedy na detekci povrchových antigenů buněk, nejčastěji tzv. CD (cluster differentiation). V případě B buněčných lymfoproliferací se ještě k určení klonality využívá exprese lehkých imunoglobulinových řetězců.[9] Přestože jsou tyto techniky citlivější než morfologické metody mají svá omezení. Vzhledem k variabilitě povrchových znaků nádorových elementů mají některé metody informativní hodnotu jen u vybraných případů.[10] Významným pokrokem u metod monitorování MRD pomocí FACS je uplatnění vícebarevné fluorescence, které umožní analýzu značně velkého množství buněk a identifikaci i jen velmi malé buněčné subpopulace. Tuto metodiku lze použít v široké míře, je kvantitativní a zachovává si i dobrou sensitivitu (10-5).[11]

Polymerázová řetězová reakce (polymerase chain reaction, PCR)
Metody PCR mají mnoho podob a modifikací. Mohou být *kvalitativní* (plus, minus) nebo také *kvantitativní (Q-PCR)*. Mezi klasické postupy patří *jednostupňová PCR*, kdy je cyklicky (30x- 50x) množena vybraná sekvence ohraničená párem primerů. Tato metodika pracuje s citlivostí zhruba 10^{-3}-10^{-5}. *Dvoustupňová PCR (nested PCR)* využívá navíc tzv. vnitřních primerů, které rozpoznávají úseky genetické informace již v předem namnožených sekvencích z jednostupňové PCR. Tím se může zvýšit citlivost na přibližně 10^{-5}-10^{-6}.[12] Nevýhodou je nutnost znát dva páry primerů, které vymezují hledanou sekvenci, což sebou mimo jiné přináší také pracnější optimalizaci reakce. Mezi jednostupňovou a dvoustupňovou PCR se svou citlivostí může za určitých podmínek vmezeřit i tzv. *„touchdown PCR“*, u které je cyklační program upraven tak, aby ani při vysokém počtu cyklů nedocházelo ke vzniku nespecifit.[13]
Metoda *RT-PCR* detekuje přítomnost nádorové mRNA, která je v prvním kroku přepsána do cDNA a dále již je množení cílové sekvence stejné jako u klasické PCR.[14]
Skutečnost, že přítomnost některých markerů nádorových buněk byla detekována u jedinců bez onkologického onemocnění[15] a u pacientů v dlouhodobě kompletní remisi,[16,17] vedla k rozvoji kvantitativních metodik. Rozsah MRD lze hodnotit *semikvantitativně* (nárůst, pokles, stagnace) nebo *absolutně*, kdy je možno určit počet patologických buněk v daném vzorku. PCR metodika *limitujícího ředění* určuje koncentraci (ředění) cílové sekvence ve vzorku, při které ještě lze detekovat PCR pozitivitu při znalosti citlivosti reakce.[18] Tato metoda byla využita k *semikvantitativním* stanovením MRD a její výsledky byly v souhlase s klinickým obrazem.[19,20] Pro přesnější kvantifikace pak byla navržena *komparativní PCR* užívající koamplifikace CDRIII jako patologického markeru a H-ras onkogenu jakožto referenčního markeru (interní standard). Obě metodiky poskytují souhlasné výsledky a vývoj hladin buněk nesoucích sledovaný marker byl v souhlase s klinickým obrazem.[20-22] Nevýhodou koamplifikačních reakcí (*multiplex PCR*) je však snížení citlivosti detekce na 10^{-2}-10^{-3}.
Absolutní kvantitativní PCR metody jsou představovány *kompetitivními (většinou nested) PCR* nebo *RT-PCR*,[23] které s citlivostí 10^{-5}-10^{-6} patří mezi k nejužívanějším. *Kompetitivní PCR* je založena na porovnávání množství produktů PCR při známé koncentraci templátu (externí standard) v pozitivní kontrole.[24] Při provedení reakce v ředící řadě, lze pak získat množství templátu ve vzorku, tj. počet nádorových buněk v měřeném vzorku. Uvedená metodika je dobře propracována a doporučována pro monitorování MRD u leukémií zejména CML.[3,25]
Možnost, jak odlišit PCR pozitivní nemocné, kteří po léčbě setrvají v dlouhodobé CCR od nemocných ohrožených relapsem naznačují studie s nejnovější metodikou tzv. *„realtime PCR“* u akutních lymfoblastových leukémií (ALL),[26] mnohočetného myelomu (MM)[27] anebo folikulárních lymfomů (FL).[28] Tato technika využívá 5'-3' nukleázové aktivity Taq polymerázy a fluorescenčně značenou cílovou DNA próbu.[29] Během reakce je próba odštěpována, což vyvolá fluorescenci, která je přímo úměrná množství cílových sekvencí.
Jak již bylo naznačeno dříve PCR je představována amplifikací unikátní genové sekvence, která se nachází v genomu patologické buňky. Taková sekvence může být jednak specifická pro dané onemocnění (t(9;22) u CML; t(14;18) u FL; t(11;14) u lymfomů z buněk plášťové zóny, MCL apod.). Nebo může být dokonce typická pro daný buněčný klon (CDRIII klonální restrukturace genu pro těžký imunoglobulinový řetězec u B lymfocytů nebo klonální restrukturace genu pro receptor T lymfocytů u lymfoproliferativních onemocnění).[30] Postupy založené na PCR detekci klonálního IgH jsou považovány za metody s dobrou citlivostí, avšak i ty lze aplikovat např. jen u asi 70-80% pacientů, protože bývá mutacemi IgH genů často změněno místo pro navázání tzv. konsensus primerů. Citlivost PCR detekce IgH rearanžmá je snižována přítomností normálních lymfocytů, pozadí (max. 10^{-4}). PCR techniky, které používají primery specifické pro pacienta (allele-specific oligonucleotide ASO–PCR) mají větší sensitivitu (až 10^{-6}).[31] Jedná se ovšem o postupy náročné na pracnost a finance a výsledky nejsou ihned dostupné. Proto není použití ASO-PCR ideální pro standardní monitoring léčebné odpovědi. Kombinací ASO-PCR spolu s „real-time“ PCR lze získat kvantitativní výsledky s citlivostí 10^{-5}.[32]

Materiál vhodný k vyšetření a monitorování MRD
Sledování MRD zvláště pak u leukémií je prováděno z kostní dřeně (bone marrow, BM) nebo periferní krve (periferal blood, PB) již déle než deset let. U NHL je však situace složitější, neboť není zcela jasné z jakého histologického materiálu vyšetření provádět. Lymfom vzniká nejčastěji v lymfatické uzlině (LN), avšak existuje i mnoho extranodálních lymfomů. Otázkou, ve které tkáni monitorovat přítomnost MRD u NHL se zabývalo několik autorů s rozdílnými závěry. Gribben, ale i další autoři popisují významnější vliv pozitivity v BM nad pozitivitou v PB na prognózu nemocných s NHL,[33,34] zatímco jiní upřednostňovali monitorování v PB.[35,36] Lopez-Guilermo koreloval výsledky PCR vyšetření BM a PB po léčbě s klinickou odpovědí u folikulárních lymfomů (FL). Jeho pozorování jsou uvedena v tabulce 1 a vyplývá z nich, že PCR nálezy v PB se cca v 70% shodují s nálezy v BM a naopak.[36] To, že je celá tato problematika komplexnější a že právě u NHL se více uplatňuje vliv rozdílnosti kompartmentů dokreslil

Tabulka. 1: Shoda mezi terapeutickou odpovědí na molekulární úrovni v PB a v BM v různých časových intervalech od léčby (čísla uvedená v procentech). (BM - kostní dřeň; PB - periferní krev; n - počet pacientů)

	3-5 měsíců (n = 57)	6-8 měsíců (n = 41)	9-14 měsíců (n = 35)
PB(+)/BM(+) nebo PB(-)/BM(-)	70	66	77
PB(+)/BM(-)	13	5	9
PB(-)/BM(+)	17	24	14

i Gupta svým pozorováním, kdy došlo po terapii monoklonální protilátkou anti-CD20 k vymizení lymfomových elementů z periferní krve, avšak objevil se relaps v uzlině.37 Množství t(14;18) pozitivních buněk v PB před zahájením standardní terapie nekoreluje s výsledkem léčby.38 U nemocných s bulky postižením cirkuluje v periferní krvi méně t(14;18) pozitivních buněk než u ostatních nemocných, což je vysvětlováno zvýšenou expresí adhesivních receptorů (např. L-selectin) na povrchu lymfomových buněk v případě bulky NHL. Takové buňky také preferují tzv. „homing" spíše do uzliny než do kostní dřeně či cirkulaci v periferní krvi.[39] Skutečnost, že nádorové buňky nemusí být vyplavovány z tkáňových depot do oběhu je však vyrovnána tzv. „sampling errorem" (odběrová jehla mine patologické ložisko) při vyšetřování BM nebo jiných solidních tkání.

U NHL existuje obecná shoda v tom, že uzlina by měla být vyšetřena při stanovení diagnózy a MRD bývá často monitorována během onemocnění v BM spolu s PB.[35,36] Ostatní tkáně (LN, periferní kmenové buňky, slezina, sliznice, kůže, mozkomíšní mok, atd.) mají jistě významnou úlohu v individuálních případech či spíše konkrétních situacích (stanovené diagnózy či relapsu apod.), ale nelze jejich vyšetřování praktikovat rutinně.

Praktický význam stanovení MRD

Stanovení MRD má mimořádný význam též při transplantační terapii. Nejpravděpodobnější příčinou rekurence je persistence nádorových buněk v organismu, nelze však vyloučit ani podíl infiltrace autologního štěpu.[40,41] Tento fakt vedl jednak k intenzivnímu výzkumu klinického významu různých metod čištění (purgingu)[42] a také ke snaze použít AlloSCT u většího počtu pacientů. Fakt, že infiltrace BM je nejvýznamnější faktor ovlivňující čistotu (puritu) štěpu, který je pacientovi v rámci autologní transplantace krvetvorných buněk podán (autologous stem cell transplantation, ASCT), byl popsán u mnoha onkologických diagnóz. Existují práce popisující, že čistota štěpu může být ovlivněna jednak použitým mobilizačním režimem,[43] intenzitou předléčení (užitou indukční terapií) a riziko znečištění roste s vyšším počtem separací.[40] Studie zabývající se kontaminací štěpů krvetvorných kmenových buněk (peripheral blood stem cells, PBPC) nádorovými elementy vedly ke zjištění, že doba výskytu maximálního počtu krvetvorných kmenových a progenitorových buněk vyplavených z kostní dřeně do periferní krve o několik hodin předchází nebo se prakticky shoduje s maximem vyplavování buněk nádorově transformovaných.[40,44,45] Další studie ukázaly, že přítomnost nádorových elementů v podávaném štěpu má na prognózu pacienta negativní dopad.[45,46]

Metodiky molekulární biologie umožňují sledovat vývoj onemocnění a tím i předvídat prognózu nemocného a zároveň pomáhají při studiu patogeneze maligních onemocnění, která bývá představována genetickou lézí vedoucí k nádorové transformaci.

Poznatky vysvětlující biologické chování jednotlivých hematologických malignit se pozvolna začínají odrážet i v zavádění nových léčebných způsobů do klinické praxe. Např. u CML se v poslední době k léčbě používá inhibitor specifické nádorové tyrosinkinázy STI 571 (imatinib mesylat).[47]

ALL (akutní lymfoblastová leukémie)

CCR je definována jako přítomnost méně než 5% blastů v KD, což může představovat 0 až 10^{10} leukemických buněk. Pro sledování MRD se využívá detekce translokací t(12;21) (u dětí), případně t(14;18). Asi u 3-5% dětí a 25% dospělých s ALL lze detekovat t(9;22). Uvádí se, že zmíněné metodiky pracují s citlivostí až 10^{-6}. V některých případech se používá PCR detekce klonální restrukturace IgH nebo T receptoru (dohromady až u 95% případů).[30,48] Zhruba u 20-30% nemocných však dochází během onemocnění ke klonálnímu vývoji, který představuje riziko falešné PCR negativity, proto se doporučuje kombinace s další technikou (FACS).[49] PCR detekce MRD pomáhá při hodnocení odpovědi na terapii a tím nabývá důležitou prognostickou hodnotu. Pacienti, u kterých nedojde k negativizaci PCR během prvního roku mají vysoké riziko relapsu.[50] Vymizení fúzních transkriptů BCR-ABL po transplantaci kostní dřeně (TKD) a jejich znovuobjevení před relapsem bylo pozorováno u nemocných s *bcr-abl* pozitivní ALL.[51] Zdá se, že kvantifikace MRD i z pouhého jednoho vzorku může mít určitou prediktivní hodnotu u nemocných s ALL (viz Tab.č. 2).[50] Stejně tak semikvantitativní metodiky monitorování MRD dokáží předpovědět klinický relaps.[52] Persistence MRD u nemocných s akutní lymfoblastovou leukémií (ALL) po terapii znamenala vysoké riziko relapsu nemoci.[53] Prospektivní analýza ukázala, že u nemocných bez detekovatelné leukémie docházelo v 8% k relapsu, zatímco u ostatních nemocných se nemoc vracela ve 40% případů. Avšak ani absence MRD po indukční terapii není uznána jako dostatečná podmínka pro přerušení nebo ukončení léčby.

Tabulka 2: Výše MRD v jednom vzorku a riziko relapsu u pacientů s ALL.

Úroveň MRD	Relapsů
> 10-3	100%
10-3 až 2x10-5	40%
< 10-5	0% (setrvali v remisi)

CLL (chronická lymfatická leukémie)

CLL je onemocnění s indolentním průběhem, které však má variabilní prognózu v závislosti na rizikových faktorech. Medián přežití je asi 6 let a 20% pacientů přežije 10 let. Zatímco se věkový medián v čase diagnózy pohybuje asi kolem 65 let, 30% nemocných CLL je méně než 60 let. CLL není vyléčitelná konvenčními postupy a při přítomnosti nepříznivých prognostických faktorů je přežití kratší než 3 roky. Taková prognóza není akceptovatelná zejména pro mladší nemocné, proto se neustále hledají účinnější léčebné postupy, které by byly schopné eradikovat onemocnění či navodit dlouhodobou remisi.

Dvě nejúčinnější používané metody pro sledování MRD u CLL jsou založeny buď na FACS nebo PCR. PCR detekce CDRIII patří mezi nejcitlivější metody sledování MRD u CLL a při použití tzv. konsensus primerů ji aplikovat u asi 70-80% pacientů. Robertson pozoroval, že nemocní s CLL léčení fludarabinem mohou dosáhnout MCR (stanovené duálním FACS vyšetřením a analýzou Ig genů pomocí Southern-blotu). Kvalita remise měla prediktivní hodnotu ve smyslu trvání léčebné odpovědi.[8] Vuillier jako jeden z prvních prezentoval, že nemocní s CLL mohou dosáhnout PCR negativity.[54] Později Provan demonstroval, že persistující PCR pozitivita po ASCT znamená vyšší riziko relapsu. Naopak se ukázalo, že většina pacientů snegativní PCR je i bez známek onemocnění. ASCT tedy představuje jednu z terapeutických možností, která se může stát u určité skupiny nemocných s pokročilou CLL kurabilní.[55] Magnac popsal výsledky monitorování MRD u 12 nemocných po dosažení CR (doba sledování 17- 60 měsíců; viz Tabulka 3). Vzhledem k malému počtu sledovaných nemocných je třeba brát výsledky rezervovaně, avšak potvrdilo se, že HDT má větší

Tabulka 3: Výsledky monitorování MRD u 12 nemocných s CLL po dosažení CR. Standard- 8 x fludarabin (4-12 cyklů) a 1x CHOP, 3 x salvage (refrakterní na standardní chemoterapii)- ESHAP (3-6 cyklů).

Počet nemocných	Terapie	PCR- vs. PCR+	Relaps	Úmrtí
9	Standard	1/8	1/2	0/2
3	Salvage+ ASCT	3/0	0/0	0/0

potenciál k indukci MCR a také, že nemocní bez reziduální nemoci měli lepší prognózu.[56] Dokonce i při použití nečištěného štěpu PBPC lze indukovat MCR. Ve studii italských autorů bylo celkem 20 nemocných v CR po terapii fludarabinem transplantováno nečištěnými PBPC. 75% pacientů dosáhlo MCR (15/20) a tito pacienti měli lepší prognózu onemocnění.[57] Nedávno byla publikována práce, ve které byla MRD sledována pomocí citlivé metodiky FACS po terapii fludarabin + ASCT nebo monoklonální protilátky anti-CD52 + ASCT. Jako MRD- byli označeni nemocní s méně než 0.05% CLL buněk v BM. Terapií bylo dosaženo celkem u 76% (19/25) nemocných MRD-, zatímco zbývajících 24% (6/25) bylo MRD+. Event free survival (EFS) byl statisticky významně horší u MRD+ pacientů než u MRD- (*p*= 0.0001). Podobně i celkové přežití (overall survival, OS) bylo statisticky významně zkráceno u MRD+ skupiny oproti nemocným MRD- (*p*= 0.007). U všech MRD+ nemocných i pacientů s nízkými úvodními hladinami reziduální nemoci docházelo k postupnému nárůstu CLL buněk.[11]

AML (akutní myeloidní leukémie)

Pravděpodobnost navození CCR se u dospělých nemocných pohybuje mezi 70-80%. Podobně je však také vysoká také pravděpodobnost relapsu (60-70%).[58] Persistence MRD je studována pomocí vysoce sensitivních PCR detekcí specifických translokací [t(6;9), t(15;17), t(8;21) apod.]. K monitorování MRD se také využívá i RT-PCR detekcí transkriptů *AML1/ETO* u akutní myeloblastové leukémie [AML M2); t(8;21)][59] a *PML/RAR-alfa* u akutní promyelocytární leukémie (APL, M3).5[9-61] Protože však pouze malá část nemocných s AML má PCR detekovatelný marker je MRD často sledována pomocí FACS.[49] Práce publikované v první polovině devadesátých let nepřinesly zcela jasnou odpověď na význam MRD u AML. Při stabilním množství MRD zachycené v průběhu CCR nemusí u nemocných dojít k relapsu ani při opakované PCR pozitivitě. To platí zejména pro AML M2.[62] Naproti tomu u AML M3, akutní promyelocytární leukémie [APL; t(15;17); *PML/RAR-alfa*] je pozitivita zjištěná pomocí RT-PCR opět spojena s vysokým rizikem relapsu.[60] Stejně tak varující je trvalý nárůst molekulárního markeru, který může být spojen s pozdějším návratem onemocnění.[63] Z prací novějších je možno zmínit např. multicentrickou studii "AIDA" Trial (GIMEMA-AIEOP), v níž byla MRD monitorována pomocí RT-PCR pro *PML/RAR-alfa* s citlivostí 10^{-4}. Z 21 pacientů, u nichž došlo během CR ke konverzi z PCR- na PCR+ a PCR+ se opakovala ve dvou po sobě následujících vzorcích, dospělo 20 nemocných k relapsu (95%).[64] Výsledky randomizovaných studií EORTC/GIMENA AML-10 a EORTC/GIMENA AML-13 přinášející shrnutí a pro AML subjednotky obecně platné výsledky monitorování úrovně MRD u pacientů po indukční a konsolidační terapii byly publikovány nedávno (viz Tabulka 4). Vyšší hladina MRD korelovala s kratším obdobím bez relapsu (relaps free survival; RFS) a horším OS.

Tabulka 4: Vliv hladiny MRD stanovené po ukončení indukce nebo konsolidace na počet relapsů. (MRDInd+/- přítomnost či nepřítomnost MRD po indukční terapii, MRDCons+/- přítomnost či nepřítomnost MRD po konsolidační terapii; * nesignifikantní, ** p < 0,001)

Po ukončení	Indukce	Indukce	Konsolidace	Konsolidace
MRD (buněk)	(MRDInd+) >= 4,5 x 10^{-4}	(MRDInd-) < 4,5 x 10^{-4}	(MRDCons+) >= 3,5 x 10^{-4}	(MRDCons-) < 3,5 x 10^{-4}
Celkem pacientů	(n= 26)	(n= 30)	(n= 22)	(n= 29)
Počet relapsů (%)	15 (58)*	12 (40)	17 (77)**	5 (17)

Sledování vývoje MRD u pacientů po ukončení konsolidace ukázalo podobné výsledky (viz. Tabulka 5).[65] Použití PCR pozitivního štěpu k transplantaci u AML je spojeno s rizikem rekurence onemocnění po TKD.40,66

Tabulka 5: Vliv hladiny MRD stanovené po ukončení indukce a v následném sledování po konsolidaci na počet relapsů. (MRDInd+/- přítomnost či nepřítomnost MRD po indukční terapii, MRDCons+/- přítomnost či nepřítomnost MRD po konsolidační terapii; * p < 0,001 **, p < 0,007)

Skupina pacientů	MRD Ind+		MRD Ind-	
Celkem pacientů	n = 21		n = 30	
Po konsolidaci pacientů	MRDCons+/ 14	MRDCons-/ 7	MRDCons+/ 8	MRDCons-/ 22
Počet relapsů (%)	10 (71)*	0(0)	7 (87)**	5 (23)

CML (chronická myeloidní leukémie)

CML je z hlediska molekulární biologie asi nejlépe prostudované onemocnění. Objevení tzv. Philadelfského chromozómu (Ph) v roce 1960 jako první chromozómální aberace spojené s určitým typem leukémie doplněné o pozdější upřesnění, že se jedná o translokaci t(9;22), která dá vzniknout fúznímu genu *bcr-abl*, znamenalo významný přelom ve studiu biologie nádorů.

Skutečnost, že pomocí interferonu-alpha (IFN-alpha) lze navodit u CML cytogenetickou remisi byla dále ověřena v prospektivní studii Italské kooperativní skupiny pro CML, jejíž výsledky byly průběžně publikovány.[7] Také se ukázalo, že pacienti v cytogenetické remisi po IFN-aplha mají delší přežití v porovnání s chemoterapií. Studie Italské kooperativní skupiny pro CML neměla design plánovaný pro určení kinetiky a trvání cytogenetických odpovědí, proto byla provedena retrospektivní analýza dat. Také bylo pozorováno, že schopnost cytogenetické odpovědi na terapii IFN bylo možné předpovědět na základě Sokalova indexu,[67] hematologické odpovědi a že většina cytogenetických odpovědí nastala během prvního roku. Při dosažení pouze minoritní cytogenetické odpovědi (Ph neg, 33% až 65%), byla taková odpověď nestabilní a trvala krátce, zatímco medián trvání kompletní a majoritní odpovědi (Ph neg, 66% až 100%) byl asi 60 měsíců.[7] Tato data jsou v souhlase se pozorováním z klinických studií na M.D. Anderson.[68]

Pro detekci transkriptu fúzního genu *bcr-abl*, který se vyskytuje až u 95% CML lze využít nested RT-PCR.[69] První studie ukázaly, že pozitivní PCR detekce aspoň ve dvou po sobě následujících vzorcích může předpovědět relaps,[69] zatímco jedna pozitivní PCR se může ještě konvertovat na negativitu během 6-9 měsíců a pacient dále setrvá v CCR.70 PCR negativita doprovází silnou reakci štěpu proti hostiteli (graft versus host disease, GVHD) v prvních 3-4 měsících po allogenní transplantaci. Na druhou stranu někteří autoři pozorovali PCR pozitivitu po téměř 10 let u některých nemocných bez návratu onemocnění,[17] což může být vysvětlováno samotným přirozeným průběhem onemocnění, kdy chronická fáze CML může trvat roky. Tato fakta přiměla vědce ke zpřesnění kvantitativních metodik pro sledování MRD po transplantaci u nemocných s CML a vedla k vývoji kompetitivní nested RT-PCR.[71] Na základě studií sledujících klinický význam molekulárního monitorování navrhla EICML Group (European Investigators on CML) doporučení pro použití PCR vyšetření u CML. Vzestup MRD o 1 log představuje PCR relaps a klinický relaps se může objevit během 1-2 měsíců, proto se doporučuje zkrátit

následující intervaly PCR vyšetření.[3] Podle sdělení některých autorů je PCR relaps událostí relativně dobře zvládnutelnou pomocí infuze dárcovských lymfocytů. Studie používající genetického značení ukázaly, že by se na relapsech po autologních transplantacích krvetvorných buněk mohla podílet právě MRD v reinfundovaných štěpech PBPC, proto se zhruba do konce 90. let u CML doporučovalo hledat dárce a provést transplantaci allogenní (AlloSCT).[72] V současné době se předpokládá, že do léčebných protokolů zasáhne i nový lék STI 571, neboť se očekávají slibné výsledky studií u pacientů s CML.

MM (Mnohočetný myelom)

Klonální restrukturace genu pro těžký imunoglobulinový řetězec (IgH, tj. CDRIII, complementarity detemining region) bývá používána jako nádorový marker u nemocných MM k detekci residuálních myelomových buněk zejména pak po ASCT.[73-76] V současné době se standardní součástí léčby pokročilých stádií MM stala vysokodávkovaná chemoradioterapie s následnou ASCT[77-79] a v rámci klinických studií pak AlloSCT.[80-81] Ve většině dosud publikovaných souborech pacientů navodila AlloSCT kompletní remisi u 30-50% případů,[78] ta však neznamenala vyléčení.[77,80] Významnou úlohu sehrává přítomnost tzv. graft-versus-myeloma efektu,[82] což přispívá k prodloužení období bez relapsu (RFS, relapse-free survival) v porovnání s autotransplantacemi.[83] Nízká pravděpodobnost relapsu s sebou však nese vysoké riziko peritransplantační mortality (40-50%).[81] I přes existenci prací zabývajících se aplikací AlloSCT a jejím vlivu na MRD, není zatím úloha MCR jednoduše hodnotitelná.[74,76] Molekulární remise jsou prakticky výjimečné po autologních transplantacích,[74] a proto je v současnosti velmi obtížné stanovit význam MCR u nemocných s MM.

NHL (Nehodgkinské lymfomy)

U NHL je molekulární monitorování subklinické choroby rovněž možné díky přítomnosti specifických interchromozómálních translokací, na nichž se podílí některé onkogeny. K nejprostudovanějším patří *bcl-2* onkogen vstupující do t(14;18) u folikulárních (follicular lymphoma, FL) a některých difusních velkobuněčných lymfomů (diffuse large cell lymphoma, DLCL), dále onkogen *bcl-1* podílející se na t(11;14) u lymfomů z plášťové zóny (mantle cell lymphoma, MCL). Nadto se uvádí, že u zhruba 90% malignit z B-lymfocytů lze pomocí PCR detekovat klonální rearanžmá genu pro těžký imunoglobulinový řetězec (IgH).[34] U lymfomů z T buněk se používá PCR detekce klonálního rearanžmá genu pro TCR.[30]
Po konvenční terapii je MRD detekovatelná prakticky u všech nemocných s NHL.[85,86] Nicméně při použití intenzivního standardního chemoterapeutického režimu CHOD-Bleo/ESHAP/ /NOPP u pacientů s nízkomalignim lymfomem došlo u 68% pacientů s PCR pozitivní na *bcl-2* ke konverzi na PCR negativitu ve (13/19).[87] Cabanillas později prezentoval novější data z dlouhodobého sledování této skupiny nemocných, kde bylo dosaženo u 88% CCR a 72% MCR. Pravděpodobnost 5-letého přežití bez příznaků onemocnění (disease free survival, DFS) byla 73% pro PCR- a 28% pro PCR+ skupinu pacientů, avšak žádná z křivek přežití nedosáhla plateau.88 Použití chemoterapeutického režimu FMD (Fludarabin, Mitoxantron, Dexamethasone) přineslo 68% odpovědí na molekulární úrovni (17/25), přičemž PCR- bylo dosaženo ve 32% (8/25).[89] Vysokodávkovanou terapií s následnou ASCT lze navodit PCR negativitu u značného procenta nemocných s nízkomalignimi NHL, v rozpětí 42-70%.[34,46,90,92,93] Gribben se svými kolegy jako jeden z prvních publikoval vliv MRD po vysokodávkované terapii s následným podáním imunologicky čištěného štěpu na prognózu pacientů s NHL nízkého a středního stupně malignity (viz Tabulka 6).[92] V nedávném novém hodnocení dat této studie byl ve 12 letech OS 69% a DFS 42%.[91] Ve skupině PCR negativních pacientů po ASCT byl DFS 85% ve 12 letech pozorování, zatímco ve skupině PCR pozitivních pacientů jen 20%. Tato data spolu s dalšími pozorováními jiných

Tabulka 6: Analýza MRD u pacientů s nízkym (LG) a středním stupněm (IG) NHL léčených pomocí ASCT. (kont. PCR- pacienti s kontinuální PCR negativitou, kont. PCR+ pacienti s kontinuální PCR pozitivitou, PCR+ ǵ PCR- pacienti, u kterých došlo ke konverzi na PCR negativitu, PCR-ǵ PCR+ pacienti, u kterých byla opět detekována PCR pozitivita, PCR+/PCR- pacienti, u kterých byla střídavě zachycena PCR negativita či pozitivita)

Po ASCT	Kont. PCR-	kont. PCR+	PCR+ → PCR-	PCR- → PCR+	PCR+ / PCR-
Pacientů	43% (58)	26% (35)	14% (19)	10% (14)	6% (8)
Relapsů	0%	71% (25)	0% (19)	47% (6)	25% (2)

Tabulka 7: Navození MCR u nemocných s NHL pomocí monoklonální protilátky anti-CD20 s nebo bez chemoterapie.
(LG- low grade; IG- intermediate grade; HG- high grade; FL- folikulární lymfom; MCL- mantle cell lymphoma (lymfom z buněk plášťové zóny); R- rituximab (anti-CD20); CHOP- Cyklofosfamid, Vinkristin, Adriamycin, Prednison; Mit- Mitoxantron; Cy- Cyklofosfamid; DFS- disease free survival.)

Autor	NHL	Terapie	MCR	Počet relapsů	Poznámka
Czuczman[96]	LG	R-CHOP	88% (7/8)	na	
Solal-Celigni[97]	FL	R	57% (17/30)	6% (1/17)	za 1. rok
Emmanouilides[98]	LG	Mit+ Cy+ R	71% (5/7)	na	
Vose[99]	IG + HG	R-CHOP	85% (11/13)	na	předpoklad >DFS a OS
Foran[100]	MCL	R-CHOP	48% (11/23)	na	medián DFS 16 měs.

autorů ukazují, že eradikace molekulárního markeru z organismu pacienta snižuje riziko relapsů po ASCT a nemocní, kteří jsou PCR- mají prodloužený DFS.[34,46,91,93] PCR detekce *bcl-2* je uznávána jako potenciální marker dlouhodobého sledování u transplantovaných nemocných s FL. Pracovní skupina z MD Anderson považuje PCR monitoring v periferní krvi spolu s hodnotami beta-2-mikroglobulinu za významný prognostický faktor u pacientů s FL po léčbě. Hladina t(14;18) pozitivních buněk v PB před zahájením standardní terapie nemá vliv na její výsledek.[38]
V poslední době lze MCR u CD20 pozitivních NHL lze navodit i pomocí nové terapeutické modality, kterou je monoklonální protilátka anti-CD20 (rituximab).[86,94,95] Použití anti-CD20 protilátky spolu s chemoterapií nebo i jen v monoterapii vedlo k dosažení MCR u pacientů s NHL ve vysokém procentu u indolentních i agresivních lymfomů, s čímž je spojena lepší prognóza onemocnění (viz Tabulka 7).[96-100] Podávání anti-CD20 protilátky vede k vysokému procentu PCR negativit v BM.[86,94,95] Tohoto faktu lze využít při mobilizaci periferních progenitorových buněk v rámci tzv. in vivo purgingu.[101] Důležitý je jistě i fakt, že anti-CD20 protilátka navodí PCR negativitu v BM bez ovlivnění doby přihojení štěpu při následné ASCT.[102] Kromě prvních zpráv zveřejnil také první slibná data Magni ve studii porovnávající in vivo purging s použitím chemoterapie s anti-CD20 protilátkou a chemoterapie samotné. Chemoimunoterapie byla úspěšná v 93% CD20+ MCL a FL. Chemoterapií samotnou bylo možno získat 40% PCR negativních PBPC produktů (p< 0.007).[43] výše zmíněná práce navázala na předešlý výzkum Corradiniho, který použitím samotné tzv. vysokodávkované sekvenční chemoterapie (HDS) pro čištění in vivo dosáhl PCR negativních sběrů PBPC u 12% (1/9) pacientů s MCL a 42% (8/19) nemocných se FL.[46]
Nedávno byly prezentovány výsledky našeho pracoviště, které se týkaly srovnání pacientů v PCR negativních a PCR pozitivních pacientů po léčbě pomocí ASCT nebo anti-CD20 pro-

tilátkou pro B- buněčné lymfoproliferativní onemocnění (B-LPD). Nemocní v MCR měli statisticky významně lepší prognózu.[103,104] U skupin nemocných PCR negativních versus pozitivních po léčbě nemusí být při porovnání základních klinických charakteristik nalezeny statisticky signifikantní rozdíly.[103,104] Zdá se tedy, že pravděpodobnost konverze na PCR negativitu či případné vyléčení může být dána biologickými charakteristikami onemocnění. Tento pohled je v současné době také ještě podporován i novými studiemi, které srovnávají profil exprese genů pomocí tzv. microarrays a tento profil je nezávislým prognostickým faktorem.[105,106]

Závěr

Předpokladem vyléčení nemocného je nejenom dosažení kompletní remise, ale i eradikace zbytkové nemoci. V posledních letech se nakumulovaly doklady, že nemocní, kteří dosáhnou eradikace tumoru na úrovni PCR, mají lepší prognózu než pacienti s PCR positivitou. PCR status nemocných s NHL po ASCT je v současnosti vnímán jako statisticky významný prognostický faktor. Podobnou váhu má i stanovení MRD po indukční léčbě u akutních leukémií, u CML při terapii interferonem či AlloSCT. Na druhou stranu existují práce, které ukazující přítomnost některých markerů např. t(14;18), t (9;22) u jedinců bez onkologického onemocnění nebo u nemocných v dlouhodobé kompletní remisi. Pro tyto situace existují zatím většinou víceméně jen teoretická vysvětlení. Díky moderním metodám kvantifikace je však možno mapovat dynamiku MRD a tím významně přispět k vyhledávání rizikových pacientů, u kterých bude vhodné nasadit léčbu i bez klinických projevů nemoci a naopak bude možno určit pacienty, u kterých lze léčbu ukončit. Jakýkoliv marker onkologického onemocnění je nutno brát jako surogátní prognostický faktor, který znamená přítomnost určitého buněčného klonu, avšak nevypovídá nic o jeho biologickém chování, proto musí být hodnocen ve vztahu k určité konkrétní situaci (stav před léčbou, po léčbě, remise apod.). Molekulárně biologické sledování MRD je třeba chápat jako velmi užitečný doplněk běžných prognostických faktorů a indexů zejména v situaci, kdy tyto ztrácí svou rozlišovací schopnost (např. při dosažení klinické kompletní remise).

Poměrně naléhavá je nutnost vytvoření standardizovaných protokolů pro jednotlivé metody a patologické jednotky, což nebude jednoduché, neboť v oboru molekulární biologie dochází k velmi rychlému vývoji. Nicméně cesta ke změnám léčebných postupů povede nejspíše přes standardizaci molekulárně biologických metod monitorování MRD.

Pro určení prognózy pacienta bude důležité nejen kde, v jakém množství a v jaké fázi onemocnění můžeme detekovat nádorové buňky, ale důležité bude i poznat jejich biologický potenciál.

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PŮVODNÍ PRÁCE

KLINICKÁ RELEVANCE SEMIKVANTITATIVNÍHO MONITOROVÁNÍ LYMFOMŮ UŽITÍM KOMPARATIVNÍ POLYMERÁZOVÉ ŘETĚZOVÉ REAKCE

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ABSTRAKT

Východisko. Z vysoce citlivých molekulárně biologických metodik využívaných při monitorování léčby hemato-onkologických pacientů mohou být proto pro klinická využití přínosnější a jsou žádanější metodiky kvantifikující umožňující predikci i odhad odpovědi na léčbu. Mnohdy, spíše než absolutní kvantifikace, může být informativní i relativní výsledek metodik využívajících stanovení konečného množství amplifikačních produktů, např. komparativní polymerázové řetězové reakce (PCR).

Metody a výsledky. Naše provedení komparativní PCR využívá optimalizované duplexní koamplifikace specifické sekvence, kterou je buď klonální restrukturace genu pro těžký imunoglobulinový řetězec CDR3 nebo sekvence úseku interchromozomální translokace t(14;18) – bcl2/Jh a vnitřního standardu, úseku genu Hras 1 (ras). Tyto koamplifikace jsou prováděny s dlouhodobě uchovávanými vzorky DNA z kostních dření a periferní krve paralelně v jediném běhu PCR a poté jsou gelovou denzitometrií kvantifikovány poměry produktů této duplexní reakce v její exponenciální fázi. Metodika byla využita v průběhu několika měsíců až několika let při monitorování nemocných nehodgkinským maligním lymfomem (NHL) léčených konvenční terapií, vysokodávkovou terapií s následnou transplantací kmenovými buňkami nebo terapií monoklonální protilátkou anti CD20 (Rituximab). Při 50 kvantitativních molekulárních analýzách, které byly doplněny klinickými údaji až dodatečně, byl zjištěn souhlas s konstatováními klinickými ve všech případech klesajícího nebo stoupajícího množství markerů, u některých pacientů, u nichž bylo dosaženo PCR negativity, došlo později opět k PCR pozitivitě. Výsledky zjištěné v kostní dřeni se shodovaly s výsledky z periferní krve. Výsledky dosažené pomocí komparativní PCR se shodovaly s výsledky zjištěnými užitím PCR v reálném čase.

Závěry. Komparativní PCR je využitelná při monitorování účinnosti léčby. Možnost prediktivních závěrů na základě těchto výsledků závisí na frekvenci odběrů vzorků a na stupni citlivosti detekce, který by měl být nezbytně stanoven u negativních případů.

Klíčová slova: komparativní PCR, molekulární monitorování, nehodgkinský maligní lymfom.

ABSTRACT

Slavíčková A., Ivánek R., Černý J.: Clinical Relevance of Semi-Quantitative Monitoring by Comparative PCR in Lymphomas

Background. PCR techniques detecting interchromosomal translocation and clonal immunoglobulin gene rearrangement (IgH) as disease markers in non-Hodgkin's lymphomas (NHL) has been utilised past ten years. However, qualitative PCR detection of persisted minimal residual disease cannot provide clinically useful prognostic information and presently, quantitative approaches are required to predict patient outcome and assess response to the treatment. In some cases, „end-point" quantifying techniques, such as comparative PCR, are applicable and the relative estimation of differences in target quantity may serve in disease monitoring rather than absolute number of target copies.

Methods and Results. Our method of comparative PCR employs co-amplification of sequences of interest (clonal CDR3, bcl2/Jh) and the segment of Hras 1 gene(ras) as an internal standard. Serial dilutions of stored diagnostic DNAs from blood and bone marrow are examined in the same PCR and, after gel densitometry, the amount of initial target is assessed by comparing exponential products of co-amplification. The comparative PCR assay was utilized in monitoring of NHL patients cured either with conventional therapy, or with high-dose regimens and transplantation with stem cells, or with chimaeric anti-CD20 monoclonal antibody (Rituximab). Results from 50 monitored intervals obtained during several months up to several years were supplemented with clinical statements retrospectively. Some of patients became PCR-negative, reappearance of PCR-positivity was observed as well. The decrease or increase of disease marker corresponded to clinical observations. Results obtained from bone marrow were in agreement with those obtained from blood.

Conclusions. End-point quantifying PCR comparative assay may provide an information on the increased risk of relapse and impact of the therapy. The predictive value of these methods depends on the frequency of sample taking and on the sensitivity of the method, which should be monitored in negative cases.

Key words: comparative PCR, molecular monitoring, non-Hodgkin's Lymphoma.

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Čas. Lek. čes., 141, 2002, No. 23, p. 735–738.

V laboratořích sledujících hematologické malignity je polymerázová řetězová reakce (PCR) pro svou citlivost užívána k detekcím minimální reziduální choroby už po desetiletí. Nicméně, pouhé kvalitativní detekce přetrvávající reziduální choroby nemohou poskytnout informace prognostické a pro tento případ je pak významnější **znalost hladiny minimální reziduální choroby**, tedy výsledek **kvantitativní PCR**. Jednou z možností kvantifikace PCR (3) je relativně jednoduchá

Předneseno na 5. celostátní konferenci DNA diagnostiky s mezinárodní účastí konané 14.-15. prosince 2001 v Lékařském domě v Praze.

a dobře dostupná **komparativní PCR**, při které porovnáním množství produktů amplifikace specifických markerů maligních buněk a srovnávacího markeru všech buněk lze dojít k závěrům o podílu specifických markerů v celkové DNA sledovaného diagnostického vzorku. Výsledkem porovnání dvou či více vzorků při stejných reakčních podmínkách pak může být **informace o poklesu či nárůstu maligních buněk**.

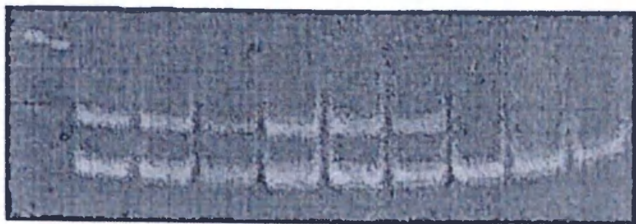
Z DNA abnormalit, které jsou využitelné při molekulárním monitorování nehodgkinských lymfomů (NHL), jsou nejčastěji sledovány klonální přestavba genu pro těžký řetězec imunoglobulinu (IgH) a interchromozomální translokace t(14;18) a t(11;14) se specifickými propojeními segmentu Jh genu IgH se segmenty onkogenů Bcl2, resp. Bcl1, k jejichž detekcím i kvantifikacím může být využita DNA PCR (1, 2). Pokud je tato využívána k relativní kvantifikaci, zejména při provádění v jedné zkumavce a množství PCR produktů má být mírou množství výchozí sekvence (specifického templátu), je k dosažení srovnatelných kvantitativních výsledků nezbytné, aby tato **duplexní PCR** byla dobře optimalizována. Při sledování a kvantifikaci translokace t(14;18) (8) a klonální přestavby IgH – oblasti CDR3 (4) byla zjištěna možnosti optimalizace koamplifikace se segmentem 2. exonu protoonkogenu Harvey *Ras* a dobrý **souhlas kvantitativních stanovení se zjištěními klinickými** (11, 12).

SOUBOR NEMOCNÝCH A POUŽITÉ METODY

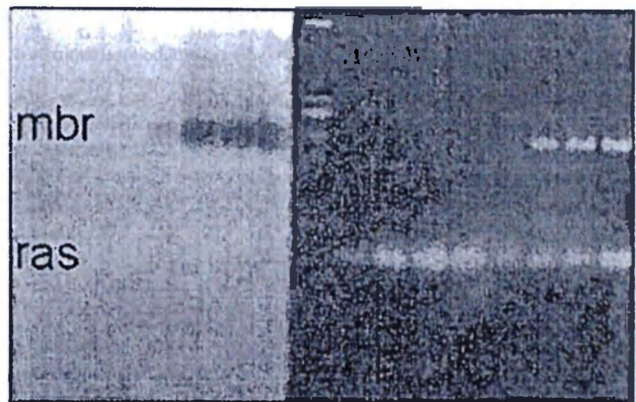
K monitorování byl užíván diagnostický materiál nemocných **nehodgkinským maligním lymfomem** (klasifikace REAL-WHO) léčených buď konvenční terapií, nebo terapií vysokodávkovou s následnou autologní transplantací periferními progenitorovými buňkami, nebo chimérickou monoklonální protilátkou anti-CD20 (rituximab). Vzorky diagnostických DNA byly připravovány z **periferní krve** (PK) a **kostní dřeně** (KD) standardní „vysolovací" technikou (9) a skladovány při 4 °C. Komparativní duplexní **IgH/ras PCR** byla prováděna, jak bylo již dříve popsáno (13), při t(14;18)/ras byl prováděn obdobně 45cyklový program PCR. Diagnostické vzorky DNA z různých období léčby pacienta byly porovnávány v jedné PCR, množství produktů reakce v její exponenciální fázi byla pak kvantifikována softwarovou analýzou (GelPro™ Analyzer, Media Cybernetics) digitalizovaných záznamů elektroforetických gelů. Takto získané relativní výsledky popisující vzestup nebo pokles markeru maligních buněk v porovnávaných vzorcích byly porovnávány s dalšími laboratorními (FACS – průtoková cytometrie) a klinickými šetřeními retrospektivně. Kontrolní kvantifikace t(14;18) užitím metody PCR v reálním čase (5, 10) byla provedena na přístroji LightCycler, detekční soupravou a dle návodu firmy Roche Molecular Biochemicals.

VÝSLEDKY

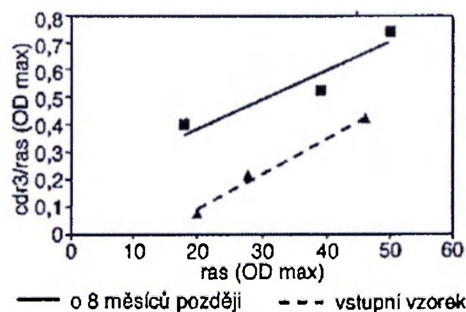
1. Optimalizace duplexních PCR (obr. 1 a 2) se vzorky s odlišným počátečním množství sekvencí, které mají být amplifikovány, se daří při neekvimolárních poměrech nukleotidových oček (primerů), např. 1:1 pro specifický marker a 0,5:0,5 pro marker referenční. Vzhledem ke skutečnosti, že z principielních důvodů mohou být analyzovány pouze výsledky z exponenciální oblasti PCR a pro srovnání množství specifického templátu ve dvou vzorcích mohou být užity pouze hodnoty při stejných amplifikacích referenčního markeru, je žádoucí provádět amplifikace každého vzorku DNA v několika ředěních (obr. 3 a 4).
2. Pokud byla možnost v průběhu léčby sledovat vývoj hladiny markeru maligních buněk v kostní dřeni i periferní krvi, byl pozorován shodný trend (obr. 5).
3. Při dlouhodobém sledování translokace t(14;18) a klonální restrukturace CDR3 u pacienta s folikulárním lymfomem



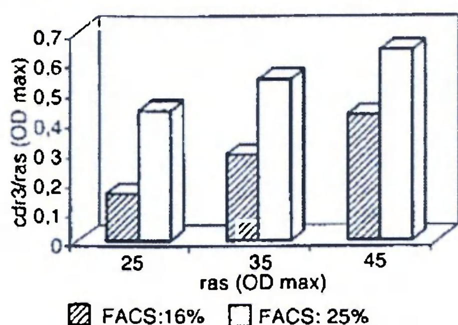
Obr. 1. IgH/ras PCR: koamplifikace markeru klonální přestavby CDR3 a segmentu ras
Detekce PCR produktů 10% polyakrylamidovou elektroforézou (CDR3 cca 100 párů bází, ras 73 p.b.)
Dráha 1: marker velikostí: shora 174, 102 a 80 p.b., dráhy 2–7: obdobné výsledky zjištěné ve dvou vzorcích (každý ve třech ředěních) DNA periferní krve odebrané v krátkém časovém úseku při diagnóze pacienta nemocného MCL, dráhy 8–10: ve vzorku PK odebraném po třech (ze čtyř) dávkách Mabthery (rituximab, chimérická monoklonální protilátka anti CD20) v kombinaci s chemoterapií byl zjištěn podstatný pokles CDR3



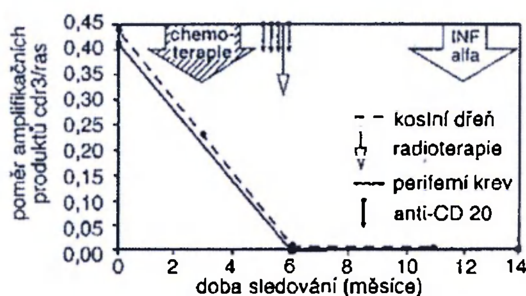
Obr. 2. Mbr/ras PCR: koamplifikace translokace t(14;18) a segmentu ras
Detekce PCR produktů (mbr cca 220 p.b., ras. 73 p.b.) 2% agarózovou elektroforézou (vpravo) a ověření specifického produktu hybridizací (vlevo). Zprava: výsledky reakce provedené ve čtyřech ředěních DNA z kostní dřeně pacientky s DLCL v relapsu a o 6 měsíců později po chemoterapii a vysokodávkové terapii s následnou transplantací kmenovými buňkami. Specifita a negativita specifické amplifikace v případě druhého vzorku byla ověřena hybridizací se sekvencí z oblasti Bcl2 označenou digoxigeninem. Molekulární remise byla pozorována při klinické kompletní remisi.



Obr. 3. Způsob kvantitativní analýzy produktů komparativní PCR
PCR je prováděna s několika koncentracemi DNA při shodných reakčních podmínkách. Denzitometrií digitalizovaného záznamu gelu (měřeno intenzitou proužku na gelu, např. OD_{max}), to jest užitím videodokumentačního systému a softwarovou analýzou (GelPro™, Media Cybernetics), jsou pro různé hodnoty amplifikace vnitřního standardu ras stanoveny poměry produktů specifických (zde CDR3) a komparačních (ras). Využity mohou být pouze hodnoty z exponenciální fáze PCR. U tohoto případu B-CLL kvantitativní analýza PCR odhalila nárůst markeru maligních buněk v pozdějším diagnostickém vzorku.



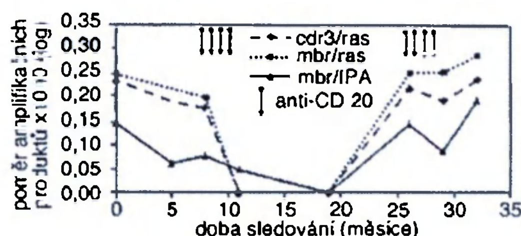
Obr. 4. Srovnávací analýza více diagnostických vzorků
Pokud není výsledek grafického výnosu v experimentu zjištěných hodnot (viz. obr. 3) pro příliš rozdíly v amplifikačních produktech zcela zřejmý, mohou být při zvolených hodnotách ras pro každý vzorek hodnoty kvantifikujících poměrů extrapolovány. Vyšší hodnoty jsou považovány za známku proliferace maligního klonu. Zde zjištěný nárůst molekulárního markeru v průběhu osmi měsíců v kostní dřeni pacienta s B-CLL byl v souhlase se stanovením maligních buněk dle FACS, půl roku poté došlo u k relapsu onemocnění.



Obr. 5. Molekulární monitorování účinku léčby v kostní dřeni a periferní krvi
U pacienta nemocného MCL, který léčbou Mabtherou (monoklonální protilátka anti CD20, rituximab) dosáhl klinické kompletní remise, byl monitorován současný rychlý nástup molekulární remise v kostní dřeni i v periferní krvi. Molekulární remise po této léčbě a během následné udržovací terapie interferonem alfa byla konstatována při funkční citlivosti metody přibližně 3x10⁻ buněk linie RAJI nesoucí klonální přeskupení CDR3 v 10^4 i více „normálních" buněk. PCR negativita byla potvrzena jednoduchou kvalitativní IgH PCR s citlivostí téměř o řád vyšší.

byl v průběhu choroby a léčby monitorován stejný trend změn hladin obou markerů (obr. 6).

4. Výsledky kvantifikace t(14;18) stanovené zde popsanou komparativní metodikou se shodovaly s výsledky zjištěnými užitím PCR v reálném čase (ob. 6).
5. Kvantitativní molekulárně biologické sledování vývoje hladiny markerů maligních buněk jsou v souhlase s pozorováními klinickými (tab. 1).



Obr. 6. Porovnání komparativní PCR a PCR v reálném čase
Shodnost výsledků zjištěných oběma metodami je velmi dobrá. PCR v reálném čase při užití přístroje LightCycler a reakční soupravy firmy Roche může vykazovat vyšší citlivost, jak je na grafu zachyceno v případě vzorku z 11tého měsíce sledování, deklarovaná analytická senzitivita je 20 kopií fragmentu mbr translokalace t(14;18), zatímco analytická senzitivita komparativní mbr/ras PCR se pohybuje zhruba kolem 60 kopií genomické DNA linie WL2 nesoucí tuto translokaci. U pacientky s folikulárním lymfomem nastal výrazný pokles obou markerů po čtyřech dávkách Mabthery (rituximab), pacientka však dosáhla pouze klinické parciální remise. Při negativitě v komparativní PCR byla zjištěna slabá pozitivita t(14;18) ve vzorku z 11tého měsíce rovněž PCR s následnou hybridizací se sekvencí z oblasti Bcl2 označenou digoxigeninem. Při následující progresi onemocnění neměly terapeutické snahy tak výrazný účinek, molekulární kvantifikace markerů malignity jsou v souhlase s touto skutečností.

DISKUZE A ZÁVĚRY

U mnoha translokací charakteristických pro NHL dochází ke chromozomovým zlomům nepravidelně, nebo je značná heterogenita chromozomových partnerů translokovaných k některému z onkogenů a jejich molekulární monitorování není proto využíváno v takové míře jako v případě leukémií (6, 7). PCR analýzy reziduálního onemocnění jsou zatím omezeny na FL a DLCL nesoucí translokaci Bcl2 nebo MCL nesoucí Bcl1 a na detekce klonální přestavby genu pro těžký imunoglobulinový řetězec, což je dáno relativní stálostí oblasti zlomů a možnosti využití univerzální primer Jh a tyto detekce mohou být i kvantifikovány (1, 8).

Komparativní PCR patří ke snadněji dostupným kvantifikujícím metodám. K dosažení srovnatelných kvantitativních výsledků je její optimalizace nezbytná z několika důvodů: Při vyhledávání minoritní klonální populace jsou v PCR užívány vysoké koncentrace celkové DNA a univerzální primery, tedy stanovení vhodného počtu cyklů by mělo být prvním optimalizačním krokem pro každý vzorek. Při duplexní PCR je rovněž nutná optimalizace množství polymerázy a vzájemného poměru primerů, který nemusí být vždy ekvimolární. Koamplifikace úseku 2. exonu protoonkogenu Harvey *Ras* a IgH, resp. t(14;18) PCR se zdá být při těchto optimalizacích výhodná.

Tab. 1. Relevance monitorování NHL užitím komparativní PCR a klinických nálezů

Klinický stav	výsledky molekulární kvantifikace			
	vzestup markeru	pokles markeru	marker beze změn	mol. remise (PCR negat.)
kompletní remise		5	2	10
regrese onemocnění		9		
stabilita onemocnění	2	3	8	
progrese	6			
relaps	5			

Při porovnání 50 monitorovaných intervalů v rozmezí 1 až 18 měsíců bylo mezi 17 případy klinické kompletní remise odhaleno 7 případů minimální reziduální choroby; mezi 13 případy klinicky stabilního onemocnění byl zjištěn třikrát pokles a dvakrát vzestup, čemuž odpovídal další vývoj onemocnění; 6 klinických progresí a 5 relapsů bylo asociováno se vzestupem molekulárního markeru a všech 9 klinicky konstatovaných regresí s molekulárním poklesem.

Vedle komparativních analýz umožňuje použitá řada koncentrací diagnostické DNA při amplifikacích se shodnou účinností také stanovení limitních koncentrací pro specifickou amplifikaci, a tedy i další možnost semikvantitativního odhadu markerů maligních buněk v porovnávaných vzorcích za předpokladu, že vyšší kritická koncentrace znamená nižší podíl specifického templátu. Nicméně, i při optimalizaci bývá citlivost specifické detekce výsledku duplexních amplifikací snížena a je proto nezbytné případnou negativitu ověřit jednoduchou specifickou PCR, jejíž citlivost je ověřena např. amplifikací řady koncentrací DNA buněčné linie nesoucí sledovaný marker.

Prezentovaná práce ověřuje využitelnost komparativní PCR při kontrole účinnosti léčby i monitorování hladiny reziduálního onemocnění NHL a částečně i možnost uplatnění při terapeutických rozhodováních. Prediktivní významnost těchto výsledků závisí na frekvenci odběrů vzorků a na stupni citlivosti detekce, který by měl být nezbytně stanoven u negativních případů.

Zkratky

CDR – complementarity determining region (oblast genu pro těžký imunoglobulinový řetězec)
CLL – chronická lymfatická leukémie
DLCL – difuzní velkobuněčný lymfom
FACS – přístroj pro průtokovou cytometrii
FL – folikulární lymfom
KD – kostní dřeň
mbr – major breakping region (oblast zlomů na genu pro protein Bcl2)
MCL – lymfom z plášťové zóny
PK – periferní krev
p.b. – páry bází
PCR – polymerázová řetězová reakce
tPa – tissue plasminogen activator (segment z oblasti tohoto genu pro je srovnávacím markerem při PCR v reálném čase)

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Práce je podporována grantem č. 203580, 15/01 GA UK.

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Jaké mohou být příčiny dětské erytrodermie?

Novorozenecké erytrodermie jsou poměrně vzácné příhody, jejichž přesný důvod lze jen stěží stanovit. Nejčastěji je choroba zařazována do kategorie *defektní imunitní odpovědi*. V dalším vývoji následuje obraz hrubé poruchy rohovění – ichtyóza, psoriáza nebo atopický ekzém nebo také vzácnější a komplikovanější Nethertonův syndrom. Dobrých 10 % zůstává neidentifikováno docela. V histologickém obrazu jsou hrubě poškozeny kožní rohovějící buňky – keratinocyty a kolem kapilár ve škáře jsou bohatě nakupené bílé krvinky – lymfocyty.

Důležité je, že imunitní porucha oslabuje obranu dítěte proti veškeré infekci, ta se může změnit i v sepsi, a proto nesmí být nic zanedbáno v péči o dítě během celé doby trvání erytrodermie, většinou během celého prvního roku života. Ovšem toto všechno dětský organizmus vyčerpává natolik, že je zpomalen růst a také ostatní zrání organizmu je poškozeno energetickou ztrátou a ztrácením elektrolytů. Je proto vždy naprosto nezbytné, aby se dítě s neonatální nebo infantilní erytrodermií dostalo do rukou skutečných ošetřujících expertů.

Prognóza takto postižených dětí je špatná a téměř 3/4 případů se nedaří zvládnout, přímá úmrtnost je až 15 % a dlouhodobé odolávání veškeré terapii s generalizovaným postižením kůže je až 35 %.

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