Clonality Analysis in B-Cell Chronic Lymphocytic Leukemia (B-CLL) Associated with Richter's Syndrome

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Abstract

B-cell chronic lymphocytic leukemia (B-CLL) comprises 90% of chronic lymphoid leukemias in Western countries and patients with B-CLL have a heterogeneous clinical course. Approximately 3-5% of B-CLL patients encounter transformation to an aggressive lymphoma, mainly diffuse large B-cell lymphoma (DLBCL) or Hodgkin's lymphoma (HL) which has been defined as Richter's syndrome and is associated with a poor clinical outcome. The mutational status of the immunoglobulin heavy chain variable region (IgVH) gene not only implies the developmental stage at which the neoplastic transformation occurs in a given B-cell lymphoma, but also constitutes an important prognostic factor in B-CLL, since B-CLL patients with unmutated IgVH genes usually have a poor clinical outcome. Sparse molecular analyses performed in Richter's syndrome so far suggest that it can occur in B-CLL patients carrying mutated or unmutated IgVH genes, and tumor cells in DLBCL or HL can be clonally identical to the B-CLL clone or arise as an independent, secondary lymphoma.

To determine the clonal relationship between DLBCL or Hodgkin/Reed-Sternberg (HRS) cells and pre-existing B-CLL cells in a larger series, to identify the IgVH gene usage and the mutational status and to explore possible prognostic factors in B-CLL undergoing Richter's transformation, we utilized a PCR-based GeneScan approach with subsequent sequencing of the IgVH genes. In cases with HRS/HRS-like cells laser capture microdissection (LCM) was employed to isolate these cells. In addition, a thorough morphological and immunohistochemical analysis was performed.

In total, specimens from 48 patients were investigated including 40 cases of Richter's syndrome and additional 8 cases of B-CLL cases with the presence of CD30-positive HRS-like cells. Among 40 cases of Richter's syndrome, 34 B-CLL cases showed transformation to DLBCL and 6 cases transformed from B-CLL to HL. Sequencing was performed in 23 paired B-CLL and DLBCL cases. In 18 cases, B-CLL and DLBCL were clonally identical, whereas DLBCL developed as a clonally independent neoplasm in 5 patients. Among the clonally related pairs, 11 out of 15 cases carried unmutated IgVH genes in both the B-CLL and DLBCL component, whereas 5 of 6 B-CLL cases that

showed transformation to HL carried mutated IgVH genes. HRS cells in two samples and HRS-like cells in one sample were clonally distinct from the B-CLL clone and infected by EBV, whereas one sample of HRS-like cells was related to the clone from the surrounding B-CLL cells and did not express latent membrane protein-1 (LMP1).

The VH genes VH3-23, VH3-74, VH1-2 and VH3-9 were overused in B-CLL cases that transformed to DLBCL, whereas VH4-34 and VH3-48 were used in over half of the B-CLL cases with transformation to HL. Immunohistochemical staining of ZAP70 was significantly associated with unmutated IgVH genes in B-CLL cases undergoing Richter's transformation. Clinical follow-up data could be obtained from 24 patients. The median survival times of B-CLL patients with transformation to DLBCL or HL were 7 and 21 months, respectively. No significantly different survival times were found between clonally related or unrelated cases, or between IgVH-mutated or -unmutated cases.

We conclude that in Richter's transformation, DLBCL can evolve by clonal transformation of the pre-existing B-CLL clone or occur as an independent, clonally unrelated neoplasm. In the majority of cases (78% in our series), B-CLL and DLBCL are clonally identical. In a subset of patients, however, DLBCL develops as an independent secondary neoplasm that is not clonally related to the B-CLL. Clonal transformation into DLBCL predominantly occurs in B-CLL patients with unmutated IgVH genes, whereas most B-CLL patients that show transformation to HL or CD30-positive HRS-like cells carry mutated IgVH genes. The tendency that IgVH-unmutated B-CLL transforms to DLBCL and IgVH-mutated B-CLL transforms to HL implies different transformation pathways in the two subtypes of Richter's syndrome. In addition, important pathogenetic differences are likely to exist between DLBCL cases derived from a pre-existing B-CLL as compared to *de novo* DLBCL cases, since *de novo* DLBCL is usually characterized by mutated IgVH genes. The biased usage of IgVH genes in the two subtypes of Richter's syndrome suggests a possible role for antigen involvement in tumorigenesis also in B-CLL cases that undergo Richter's transformation. Finally, EBV-association in the HL variant of Richter's syndrome occurs more frequently in clonally unrelated secondary malignancies.

Zusammenfassung

Die chronische lymphatische Leukämie vom B-Zell-Typ (B-CLL) macht ca. 90% der chronischen lymphatischen Leukämien aus. Der klinische Verlauf der B-CLL ist heterogen, und bei 3-5% der Patienten kommt es im Verlauf der Erkrankung zu einer Transformation in ein aggressives Lymphom, meist in ein diffuses großzelliges B-Zell-Lymphom (DLBCL) oder in ein Hodgkin-Lymphom (HL). Eine solche Transformation wird als Richter-Syndrom bezeichnet und ist mit einer ungünstigen klinischen Prognose assoziiert. In B-Zell-Lymphomen (B-NHL) ermöglicht der Mutationsstatus der variablen Anteile der Immunglobulinschwerkettengene (*IgVH*) eine Aussage über das Entwicklungsstadium der B-Zelle, in dem sich die Transformation zu einem B-Zell-Lymphom ereignet hat. In der B-CLL stellt der Mutationsstatus auch einen bedeutenden prognostischen Faktor dar, wobei die Erkrankung bei Patienten mit unmutierten *IgVH* Genen meist einen ungünstigen klinischen Verlauf zeigt. Die wenigen bisher durchgeführten molekularen Analysen an Fällen von Richter-Syndromen deuten darauf hin, dass ein Transformationsereignis sowohl bei B-CLL-Patienten mit mutierten als auch mit unmutierten *IgVH* Genen vorkommen kann, und dass die Tumorzellen des DLBCL oder HL sowohl klonal identisch mit dem B-CLL-Klon sein können als auch unabhängig als sekundäres Lymphom entstehen können.

Um die klonale Beziehung zwischen DLBCL- bzw. Hodgkin/Reed-Sternberg (HRS)-Zellen und den Zellen der vorbestehenden B-CLL zu analysieren, den Mutationsstatus des *IgVH* Gens sowie mögliche prognostische Faktoren in B-CLL-Fällen mit Richter-Transformation zu identifizieren, wurde eine größere Fallserie mit Hilfe eines PCR-basierten GeneScan Ansatzes mit anschließender Sequenzierung der *IgVH*-Gene untersucht. In Fällen mit HRS bzw. HRS-ähnlichen Zellen wurden die CD30-positiven Tumorzellen mittels Laser-Capture Mikrodissektion (LCM) isoliert. Weiterhin erfolgte eine morphologische und immunhistochemische Analyse der Fälle.

Insgesamt wurden 48 Patientenproben untersucht, darunter 40 Fälle mit Richter-Syndrom sowie weitere 8 B-CLL-Fälle mit CD30-positiven HRS-ähnlichen Zellen. Unter den 40

Proben mit Richter-Syndrom zeigten 34 B-CLL-Fälle eine Transformation in ein DLBCL, in 6 Fällen erfolgte eine Transformation in ein HL. In 23 Fällen mit B-CLL und DLBCL wurde eine Sequenzierung der *IgVH* Gene durchgeführt. In 18 Fällen waren B-CLL und DLBCL klonal identisch, in 5 Fällen war das DLBCL als klonal unabhängige Neoplasie entstanden. Unter den klonal verwandten Paaren zeigten 11 von 15 Paaren unmutierte *IgVH*-Gene sowohl in der B-CLL als auch im DLBCL, wohingegen 5 von 6 Fälle mit Transformation einer B-CLL in ein HL mutierte *IgVH* Gene trugen. HRS-Zellen in 2 Fällen und HRS-ähnliche Zellen in einem Fall waren klonal verschieden vom B-CLL-Zellklon.

Die *VH*-Gene *VH3-23*, *VH3-74*, *VH1-2* und *VH3-9* waren überproportional häufig in B-CLL Fällen mit einer späteren Transformation in ein DLBCL vertreten, wohingegen *VH4-34* und *VH3-48* in mehr als der Hälfte der Fälle mit Transformation zu einem HL nachgewiesen werden konnten. Der immunhistochemische Nachweis von ZAP70 zeigte eine signifikante Assoziation mit unmutierten *IgVH*-Genen in B-CLL mit nachfolgender Richter-Transformation. Bei 24 Patienten konnte der klinische Verlauf eruiert werden. Die mediane Überlebenszeit von Patienten mit B-CLL mit stattgehabter Transformation in ein DLBCL oder ein HL betrug 7 beziehungsweise 21 Monate. Es fanden sich weder signifikante Unterschiede der Überlebenszeit zwischen klonal verwandten und nicht verwandten Fällen, noch zwischen *IgVH*-mutierten und -unmutierten Fällen.

Zusammenfassend kann festgestellt werden, dass bei der Richter-Transformation einer B-CLL in ein DLBCL dieses einerseits aus dem präexistenten B-CLL-Klon entstehen kann, andererseits aber auch als unabhängige, klonal nicht verwandte Neoplasie auftreten kann. In der Mehrzahl der Fälle (78% in dieser Serie) sind B-CLL und DLBCL jedoch klonal identisch und nur gelegentlich entsteht ein DLBCL ohne klonale Beziehung zur B-CLL als unabhängige, sekundäre Neoplasie. Eine klonale Transformation in ein DLBCL tritt vorwiegend bei B-CLL-Patienten mit unmutierten *IgVH*-Genen auf, wohingegen die Mehrzahl der B-CLL-Patienten mit einer Transformation in ein HL oder CD30-positive HRS-ähnliche Zellen mutierte *IgVH*-Gene aufweist. Dieser Befund lässt auf unterschiedliche Transformationswege der beiden Subtypen des Richter-Syndroms

schließen. Weiterhin existieren vermutlich wesentliche Unterschiede in der Pathogenese zwischen DLBCL-Fällen, die sich aus einer vorbestehenden B-CLL entwickelt haben, und *de novo* DLBCL-Fällen, da *de novo* DLBCL zumeist durch mutierte *IgVH*-Gene charakterisiert sind.

Chapter 1

Introduction

1.1 B-Cell Chronic Lymphocytic Leukemia (B-CLL) and Richter's Syndrome

Chronic Lymphocytic Leukemia (CLL) is the most common form of leukemia in adults and comprises 90% of chronic lymphoid leukemias in Western countries. The overall incidence is approximately 3/100,000 per year. Chronic lymphocytic leukemia of B-cell origin (B-CLL) represents more than 95% of all CLL cases and only approximately 2-5% of patients with CLL exhibit a T-cell phenotype (Perry and Rasool 2005). Cases with a T-cell phenotype include large granular lymphocytic leukemia, T-cell prolymphocytic leukemia and the leukemic phases of various T-cell lymphomas. The incidence among the black and white population is approximately the same, but CLL is rare in Asia comprising only 10% of all leukemias (Perry and Rasool 2005). The low incidence of CLL in Asian people is independent of the lifestyle and the geographic area. This suggests genetic differences in the susceptibility to CLL and this is further supported by the fact that 10% of CLL are familial cases(Caporaso, Marti et al. 2004).

CLL is a disease that primarily affects elderly individuals. The majority of patients are more than 50 years old and the median age is 65 to 68 years. The incidence is higher in males than in females, with a male to female ratio of 2:1 (Jaffe 2001).

B-cell chronic lymphocytic leukemia (B-CLL) is a monoclonal disorder characterized by a progressive accumulation of functionally incompetent lymphocytes (Perry and Rasool 2005). Morphologically, it is a neoplasm of monomorphic small, round B-lymphocytes in the peripheral blood, bone marrow and lymph nodes with the presence of prolymphocytes and paraimmunoblasts. Immunophenotypically, B-CLL cells usually express CD20, CD5 and CD23 (Jaffe 2001).

The onset of B-CLL is usually indolent and the diagnosis of B-CLL is frequently made by chance, e.g. in a routine blood examination. Alternatively, B-CLL patients may suffer

from generalized lymphadenopathy. The symptomatic B-CLL patient usually has non-specific complaints of fatigue, anorexia, weight loss, dyspnea, or a sense of abdominal fullness (from an enlarged spleen or palpable nodes). With progressive disease, there may be pallor due to anemia. Skin infiltration may be a feature of T-cell CLL patients. A predisposition to bacterial, viral, and fungal infection occurs in late disease because of hypogammaglobulinemia and granulocytopenia (Keating 2006).

The clinical course of B-CLL patients is heterogeneous and some patients survive for decades without treatment, while others die from drug-resistant disease shortly after presentation (Dighiero and Binet 2000). The median survival of patients with B-CLL is about 10 years (Keating 2006). Clinically, the staging systems by Rai (0- IV) and by Binet (A-C) are used to estimate the prognosis. Both staging systems are based on the extent of lymphadenopathy, splenomegaly and hepatomegaly on physical examination and on the degree of anemia and thrombocytopenia in peripheral blood cell counts. Anemia and thrombocytopenia are the major clinical adverse prognostic variables for B-CLL patients. For example, Rai stage 0 refers to the involvement of bone marrow and blood lymphocytosis only and patients may survive for more than ten years; Rai stage III or IV comprises patients that have lymphocytosis including anemia or thrombocytopenia and patients are more likely to die within 3-5 years (Rai, Sawitsky et al. 1975). Binet stage A or B is characterized by lack of anemia or thrombocytopenia and fewer or more than 3 areas of lymphoid involvement (Rai stages 0, I, and II); Binet stage C is characterized by anemia and/or thrombocytopenia regardless of the number of areas of lymphoid enlargement (Rai stages III and IV) (Binet, Auquier et al. 1981).

Therapies for B-CLL patients include a wide range of options. Chemotherapy has long been the standard therapy for B-CLL and usually alkylating agents like chlorambucil are used, either alone or in combination with corticosteroids. In recent years, it has been shown that fludarabine, a nucleoside analogue, causes more effective remissions than other treatments. However, survival advantage has not been demonstrated yet (Rai, Peterson et al. 2000). In patients with immunohemolytic anaemia and thrombocytopenia corticosteroid therapy is frequently applied. Local irradiation may be applied to areas of lymphadenopathy or liver and spleen involvement for transient symptomatic palliation

(Johannsson, Specht et al. 2002). Total body irradiation in small doses has occasionally been successful in the remission of CLL patients (Roncadin, Arcicasa et al. 1994). Finally, biological therapy, such as the application of the anti-CD52 monoclonal antibody (Campath), bone marrow or stem cell transplantation, and gene therapy are additional therapeutic options in B-CLL (Kalil and Cheson 2000).

Despite this wide range of therapeutic options, B-CLL is usually incurable, with the possible exception of younger patients who receive stem cell transplantation. It is not usual to treat patients who have early-stage disease or have no clinical symptoms, because there is no evidence that early treatment prolongs survival for these patients (Dighiero 1997).

Although most B-CLL patients survive many years with little or no treatment, there are approximately 15% of patients who will undergo rapid progression with poor clinical outcome. One unfavourable form is chronic lymphocytic leukemia/prolymphocytic leukemia (B-CLL/PLL) with an increasing number of prolymphocytes. Another unfavourable subset of B-CLL patients suffers from Richter's syndrome with rapidly progressing large cell lymphoma. These patients have a poor response to the treatment and usually die shortly after presentation (Jaffe 2001).

1.2 Definition of Richter's Syndrome

Richter's syndrome refers to the occurrence of an aggressive lymphoma in patients with B-cell chronic lymphocytic leukemia (B-CLL) /small lymphocytic lymphoma (SLL) (Jaffe 2001).

In 1928, Maurice N. Richter first described this phenomenon which was published in the American Journal of Pathology. He reported on a patient with rapidly fatal generalized lymphadenopathy and hepatosplenomegaly associated with chronic lymphocytic leukemia. Histological examination of the lymph nodes, liver, and spleen revealed two types of cells: 'leukemia' and 'tumor' cells. Leukemic cells referred to lymphocytes of

small size and tumor cells referred to numerous, polymorphous endothelial cells, several times as large as the lymphocytes, with abundant basophilic cytoplasm, well defined nuclei, and several prominent nucleoli (Richter 1928).

The term "Richter's syndrome" was coined by Lortholary in 1964, when he described 4 patients and reviewed the cases of 10 previously reported patients with presentations similar to that in Richter's first report (Lortholary, Ripault et al. 1964) .

Originally, Richter's syndrome referred to the development of aggressive Non-Hodgkin's Lymphoma (NHL) during the course of B-CLL. The main subtype describes B-CLL transformation to diffuse large B-cell lymphoma (DLBCL), also regarded as classical Richter's syndrome. It is characterised by confluent sheets of large cells that may resemble paraimmunoblasts, but more often are centroblast- or immunoblast-like (Jaffe 2001). Cases resembling Hodgkin's Lymphoma (HL) also occur in a small number of B-CLL patients, including scattered Reed Sternberg (RS)-like cells and their variants in the background of B-CLL or infiltrates of classical HL. The latter has been referred to as "HL variant of Richter's syndrome" (Brecher and Banks 1990) or Richter's syndrome with HL features (Rubin, Hudnall et al. 1994; Fayad, Robertson et al. 1996).

Subsequently, Richter's syndrome was expanded to include other lymphoid malignancies that develop in patients with B-CLL, such as prolymphocytic leukemia (PLL), small noncleaved cell lymphoma (Litz, Arthur et al. 1991), lymphoblastic lymphoma (Pistoia, Roncella et al. 1991) and hairy cell leukemia (Duchayne, Delsol et al. 1991). In rare cases, patients with B-CLL may develop high-grade T-cell NHL (Raziuddin, Assaf et al. 1989; Strickler, Amsden et al. 1992; Lee, Skelly et al. 1995; Novogrudsky, Amorosi et al. 2001).

1.2.1 Incidence of Richter's Syndrome

The reported incidence of transformation to Richter's syndrome in patients with B-CLL ranges from 3-10% (Robertson, Pugh et al. 1993; Mauro, Foa et al. 1999). Transformation of B-CLL to HL occurs in about 0.5% (Brecher and Banks 1990).

In the largest study reported to date 39 of 1,374 B-CLL patients developed Richter's syndrome (2.8%) during a 20-year period between 1972 and 1992 in the USA (Robertson, Pugh et al. 1993). During the following ten years (1992-2002), Richter's syndrome occurred in 105 of 2,147 patients with B-CLL (4.9%).

Another retrospective analysis of B-CLL patients observed at a single institution in Italy was performed in 1,011 patients with B-CLL, among which 22 patients developed Richter's syndrome (2.2%). These included 18 cases with DLBCL (1.8%) and four cases with the HL variant of Richter's syndrome (0.4%). The occurrence of Richter's syndrome was significantly higher in younger patients (</=55) (5.9%, 12 patients versus 1.2%, 10 patients; $P < .00001$), most likely, because tissue biopsies and post-mortem examinations were not performed in some of the elderly patients. A significantly higher male/female ratio in younger patients was found (2.85 v 1. 29; $P <.0001$)(Mauro, Foa et al. 1999). In the HL variant of Richter's syndrome, males predominated by a factor of 3 in a series of 88 cases reviewed by Adiga et al. (Adiga, Abebe et al. 2003).

Since B-CLL is less common in Asia compared to Western countries, the extremely low occurrence rate of Richter's syndrome is no surprise in China and Japan. Although four cases of Richter's syndrome have been reported in Japan (Nakamura, Kuze et al. 2000) and one case in Taiwan (Dunn, Kuo et al. 1995), the accurate frequency of Richter's syndrome outside Western countries is still unknown.

1.2.2 Morphology of Richter's Syndrome

The major subtype of Richter's syndrome is constituted by the transformation of B-CLL to DLBCL showing classic morphology including the centroblastic (CB) and immunoblastic (IB) variants. The immunoblastic variant of DLBCL reveals a diffuse proliferation of large-sized cells (immunoblastic cells >90%) with a single centrally located nucleolus and a moderate amount of cytoplasm. Richter's syndrome with the centroblastic variant of DLBCL shows a diffuse and monotonous proliferation of centroblasts with oval to round, vesicular nuclei with fine chromatin and 2-4 membrane

bound nucleoli. In contrast, paraimmunoblasts are medium to large-sized immunoblast-like cells that are frequently found to be scattered in pseudofollicles of B-CLL, but do not show a confluent growth pattern.

Because of the very low incidence of the Hodgkin (HL) variant of Richter's syndrome, the maximum number of reported cases in a single series was eight (Brecher and Banks 1990). Recently, Adiga and colleagues reviewed 88 cases from papers which have been published in the English language between 1975 and 2002. To analyze the clinical and histological features of patients with the HL variant of Richter's syndrome, four groups were created. In group 1, the biopsy showed B-CLL with scattered RS-like cells (n=27); in group 2, distinct sites of involvement by B-CLL and HL was observed simultaneously in the same anatomical location (n=24) or at different sites (n=15); group 3 consisted of HL with no evidence of present B-CLL, but with a documented past history of B-CLL (n=6); finally, group 4 included cases which could not be classified into any of the above groups due to inadequate or incomplete information (n=15). Information about the histological subtype of HL was available in 50 patients only and the most common histological type was mixed cellularity (n=30), followed by the nodular sclerosis (n=12), lymphocyte depletion (n=5), and lymphocyte predominant (n=3) subtypes. These subtypes were equally distributed within groups 2-4 (Adiga, Abebe et al. 2003).

1.2.3 Treatment and Clinical Outcome of Richter's Syndrome

Richter's syndrome usually presents with lymphadenopathy, systemic symptoms (fever, weight loss, night sweats) and hepatosplenomegaly. The major feature of Richter's syndrome is the presence of disseminated, enlarged lymph nodes, whereas the extranodal involvement including bone marrow, spleen (Nai, Cabello-Inchausti et al. 1998), liver, the gastrointestinal (GI) tract (Brousse, Solal-Celigny et al. 1985; Jasani, Gough et al. 1986; Wilson 1986; Desablens, Gineston et al. 1987; Faigel, Vaughn et al. 1995; Ott, Ott et al. 1995; Parrens, Sawan et al. 2001), central nervous system (CNS) (Narang and Bishop 1988; O'Neill, Habermann et al. 1989; Bayliss, Kueck et al. 1990; Mahe, Moreau et al. 1994; Robak, Gora-Tybor et al. 2004; Schmid, Diem et al. 2005), skin (Alluminio, Margarita et al. 1988; Novice, Mikhail et al. 1989; Zarco, Lahuerta-Palacios et al. 1993;

Fraitag, Bodemer et al. 1995; Cerroni, Zenahlik et al. 1996; Ratnavel, Dunn-Walters et al. 1999), eye (Hattenhauer and Pach 1996), testis (Houdelette, de La Taille et al. 1996) and lung or kidney (Foucar and Rydell 1980) is uncommon.

The interval between the diagnosis of B-CLL and subsequent development of Richter's syndrome ranged from 1 to 66 months, with a median of 21.9 months in the report of 544 B-CLL patients (Morrison and Rai et al. 1999). In the literature review of the HL variant of Richter's syndrome, the median interval between diagnosis of B-CLL and the evolution to HL was 61.8 months (n=52), with a range of 0 to 17 years (Adiga, Abebe et al. 2003).

Compared with the heterogeneous clinical course of B-CLL patients, the clinical outcome of patients with Richter's syndrome is very poor. The median survival of B-CLL with DLBCL is 3 to 5 months after presentation (Harousseau, Flandrin et al. 1981), whereas in B-CLL transformation to HL it is approximately 12 months (Brecher and Banks 1990; Adiga, Abebe et al. 2003).

It has been a great concern that therapy may further increase the risk of developing Richter's syndrome. However, until now, there is no clear evidence that alkylating agents or purine nucleoside analogues may be associated with an increased incidence of Richter's syndrome or secondary malignancies in patients with B-CLL (Cheson 1995; Cheson, Vena et al. 1999; Robak 2004).

No standard treatment has been established for patients with Richter's syndrome. Among other therapies, combined chemotherapy was used, but no significant results were obtained. Tsimberidou and colleagues reported that Yttrium-90 radioimmunotherapy had no significant antitumor activity and showed severe hematological toxicity in seven patients with Richter's syndrome (Tsimberidou, Murray et al. 2004). However, stem cell transplantation appears to be a promising therapeutic strategy for patients with Richter's syndrome. It has been reported that 3 patients (38%) achieved durable disease remissions and were free of disease at 14 months, 47 months, and 67 months after therapy. In the same study, 5 out of 8 patients who were treated with high-dose chemotherapy followed

by an allogeneic stem cell transplant, died of treatment-related toxicities (Rodriguez, Keating et al. 2000).

Data on the optimal treatment of the HL variant of Richter's transformation are limited (Alliot, Tabuteau et al. 2003; Robak, Szmigielska-Kaplon et al. 2003). Most patients respond to standard therapies for Hodgkin's disease (i.e., combination of doxorubicin, bleomycin, vinblastine, and dacarbazine [ABVD] or mechlorethamine, vincristine, procarbazine, and prednisone [MOPP]), with or without radiotherapy. Patients with the Hodgkin variant of Richter's syndrome are more resistant to therapy than patients with Hodgkin's Lymphoma alone, but are more sensitive than patients with classical Richter's syndrome (Alliot, Tabuteau et al. 2003). Currently, to our knowledge, it is unknown whether the two types of Richter's syndrome respond differently to therapy.

1.3 Pathogenetic Mechanisms in B-CLL

1.3.1 Chromosomal Abnormalities

With the development of new techniques such as fluorescence in situ hybridization (FISH) or comparative genomic hybridization (CGH), chromosome aberrations can be found in more than 80% of B-CLL cases. The most frequently observed abnormalities are deletions in band 13q14 (51%), followed by deletions in 11q22-q23 (20%), deletions in 17p13 (7%) and deletions in 6q21 (6%). The most common gains of chromosomal material are trisomies 12q, 8q and 3q. Chromosome translocations, which are frequently observed in other types of non-Hodgkin's lymphoma, are rare events in B-CLL (Stilgenbauer, Lichter et al. 2000).

One candidate tumor suppressor gene in 13q14 is the retinoblastoma gene 1 (RB1), although deletions and/or mutations of both alleles have rarely been detected in B-CLL (Liu, Szekely et al. 1993). However, two micro-RNA genes (miR15 and miR16) located at 13q14 are deleted or down-regulated in the majority (approximately 68%) of B-CLL

cases (Calin, Dumitru et al. 2002). Micro-RNA genes play a major role in gene regulation (He and Hannon 2004).

Other candidate genes which are affected by deletions in 17p and 11q22-23 aberrations appear to be p53 and ataxia telangiectasia mutated (ATM), respectively. In particular, 17p abnormalities and deletions in 11q22-q23 have already been shown to be among the most important independent prognostic factors identifying subgroups of patients with rapid disease progression and short survival (Dohner, Stilgenbauer et al. 2000). In addition, deletion of 17p has been associated with resistance to treatment with purine analogs (Dohner, Fischer et al. 1995).

Trisomy 12 is the fourth most common chromosome aberration and is associated with an increased proportion of prolymphocytes (B-CLL/PLL)(Matutes, Oscier et al. 1996). The frequencies of trisomy 12 in FISH studies range from 10 to 20% in most European series (Dohner, Stilgenbauer et al. 1997) and reach more than 30% in a study from the United States (Anastasi, Le Beau et al. 1992). The bands 12q13-q22 were identified as a minimally duplicated region, but the knowledge of potential candidate genes involved in B-CLL with trisomy 12 is still limited.

1.3.2. Immunoglobulin Heavy Chain Variable Region (IgVH) Genes and their Mutational Status

The immunoglobulin heavy chain variable region (IgVH) is encoded by three separated germline gene segments, the variable (V), diversity (D) and joining (J) regions. Initially, 1 of the 27 D gene segments in the germ line links up with 1 of 6 J genes in pro-B-cells. Subsequently, 1 of the 51 V segments forms the VDJ unit in pre-B-cells. Additionally, several nucleotides are randomly inserted at the V-D and D-J junctions. In the later antigen-dependent phase, B-cells enter the germinal centre in lymphoid follicles and their IgVH genes undergo somatic hypermutation. The majority of mutations are point mutations, but rarely insertions and deletions are also found (Wilson, de Bouteiller et al. 1998). Importantly, the maturation of B-cells is reflected by the mutation status of

immunoglobulin genes (Ig genes). Pre-germinal centre B-cells (naïve B-cells) usually exhibit unmutated Ig genes in germline configuration, germinal centre B-cells have a high rate of mutations and evidence of ongoing mutations and post-germinal centre memory B-cells show mutated but stable receptors.

Originally, B-CLL was considered to be a homogeneous leukemia consisting of transformed naïve B-cells. This assumption was based on morphological aspects and surface marker analyses (Dameshek 1967). However, several studies have found that approximately 50% of B-CLL clones exhibit somatic mutation of their immunoglobulin genes, which suggests that some B-CLL clones arise from post-germinal centre, "memory" B-cells (Schroeder and Dighiero 1994; Fais, Ghiotto et al. 1998). Furthermore, two publications reported simultaneously on the prognostic significance of the IgVH mutational status in B-CLL (Damle, Wasil et al. 1999; Hamblin, Davis et al. 1999). This finding has been confirmed in subsequent studies (Jelinek, Tschumper et al. 2001; Thunberg, Johnson et al. 2001; Krober, Seiler et al. 2002). Hamblin and colleagues reported that the median survival was 8 to 9 years for patients with unmutated IgVH genes, but beyond 24 years for patients with somatically mutated IgVH genes. In these studies, a 2% cut-off was used to define mutated and unmutated IgVH genes (Hamblin, Davis et al. 1999) meaning that an IgVH gene that differs from the germ line sequence by at least 2%, is defined as being mutated.

Although the biologic basis of the IgVH gene mutation status for the varying clinical outcome is not clear, recent studies show that unmutated IgVH genes are associated with trisomy 12 and a chromosomal deletion in 11q23, which constitute two prognostically unfavourable genetic alterations. In contrast, B-CLL clones with mutated IgVH genes are associated with the more favourable genetic defect in 13q14. Dysfunctional p53, as a highly significant predictor of poor outcome, belonged to the unmutated IgVH subgroup (Krober, Seiler et al. 2002).

1.3.3 DNA Mismatch-repair and Microsatellite Instability

Microsatellite instability (MSI) represents one specific pattern of genomic instability and is one of the genetic lesions most frequently detected in human neoplasia, especially in solid cancer. There are several investigations about MSI in B-CLL, but most of them observed no microsatellite alterations in B-CLL/SLL (Perez-Chacon, Contreras-Martin et al. 2005). In contrast, Niv and colleagues recently reported that 4 out of 27 B-CLL patients had a replication error positive phenotype (RER+) which was determined by more than 30% MSI in examined loci. A larger proportion of patients with stage C B-CLL exhibited RER+ than those with stage A or B ($P < 0.05$). A higher prevalence of RER+ was demonstrated in a subgroup of patients with additional malignancies (3 out of 8 patients) in comparison to patients with B-CLL alone (1/19) ($P = 0.031$)(Niv, Bomstein et al. 2005). However, the low incidence of microsatellite instability (MSI) in malignant lymphoid neoplasms indicates that MSI does not seem to be involved in the pathogenesis of B-CLL.

1.3.4 Epstein-Barr Virus

The Epstein-Barr virus (EBV), a human herpes virus with oncogenic potential, was found to be widespread in all human populations. It is controlled by virus-specific cytotoxic T-lymphocyte (CTL) surveillance and can persist in B-cells of individuals without symptoms (Young and Rickinson 2004).

EBV has the unique ability to transform resting B-cells into permanent, latently infected lymphoblastoid cell lines (LCLs) in vitro. EBV expresses small non-coding RNAs (EBERs 1 and 2) and eight virus latent proteins, known as the EBV nuclear antigens (EBNAs) 1, 2, 3A, 3B, 3C and LP, and the latent membrane proteins LMP1 and LMP2 (Young and Rickinson 2004). LMP1 is the key EBV-protein, which is necessary for EBV-induced immortalization (Kaye, Izumi et al. 1993). LMP1 can upregulate anti-apoptotic proteins, it resembles CD40 and causes B-cell proliferation (Dawson,

Tramountanis et al. 2003). However, the mechanisms of EBV contribution to lymphomagenesis are still not clear.

Approximately 40% of Hodgkin's Lymphoma (HL) cases carry EBV in the malignant Hodgkin/Reed Sternberg (HRS) cells, with expression of the viral latent membrane proteins (LMPs) 1 and 2 (Young and Rickinson 2004). B-CLL cells do not regularly become activated or immortalised like normal B-cells after exposure to EBV (Maeda, Bandobashi et al. 2001).

1.4 Recent Studies on Richter's Syndrome: A Short Review

Since 1928, when Maurice N. Richter first described Richter's syndrome, an increasing number of investigations has been performed on this topic. The number of publications about Richter's Syndrome has been increasing from 4 articles in the 60s to more than 70 articles in the 90s. The research field evolved from single case reports to immunohistochemical studies, cytogenetic alterations and to PCR- and sequencing analysis of IgVH genes of B-CLL in Richter's syndrome. Molecular methods have been widely applied, but the mechanisms involved in the transformation of B-CLL to Richter's syndrome are still poorly understood. Current studies of Richter's syndrome focus on two aspects:

1. The clonal relationship between B-CLL and transformed components (DLBCL or HRS/HRS-like cells) and
2. Possible risk factors in B-CLL that are likely to develop to Richter's syndrome.

1.4.1 Study of B-CLL and DLBCL in Richter's Syndrome

To determine the clonal relation between the two lymphoma components, i.e. B-CLL and DLBCL, in the 70s, immunoglobulin isotypes (such as γ, α or μ heavy chains as well as κ or λ light chains) were detected and compared by using antibodies and

immunostaining methods. DLBCL cells carrying the same isotype as B-CLL cells may indicate a clonal evolution of the aggressive lymphoma from the pre-existing B-CLL (Harousseau, Flandrin et al. 1981). The detection of different immunoglobulin isotypes was interpreted as evidence that DLBCL and B-CLL are not clonally related (Splinter, Noorloos et al. 1978). However, this approach has clear limitations. First, two genetically unrelated lymphoma components have a 50% chance of having the same immunoglobulin light-chain isotype (kappa or lambda). On the other hand, immunoglobulin heavy- or light-chain isotypes can be discordant in clonally related components as a result of isotype switching (Hieter, Korsmeyer et al. 1981). Therefore, a definite proof of clonal relationship can not be obtained by this approach.

In the 80s, southern blotting was applied to investigate the clonal relationship between B-CLL and DLBCL in Richter's syndrome. By this method, a rearrangement of the same-sized band in both B-CLL and DLBCL was taken as an evidence for clonal relationship (van Dongen, Hooijkaas et al. 1984; McDonnell, Beschorner et al. 1986; Sun, Susin et al. 1990; Matolcsy, Casali et al. 1995). However, this method also suffers from limitations. First, the bands are detected only when a clonal immunoglobulin gene rearrangement is present. Second, the pattern of bands may change because of somatic mutations that cause single base-pair substitutions in the variable region at a restriction-enzyme cleavage site (Teillaud, Desaymard et al. 1983; Nakamine, Masih et al. 1992). Thus, a discordant southern blot analysis does not necessarily exclude clonal relationship between B-CLL and DLBCL (Matolcsy, Inghirami et al. 1994).

The development of PCR- and sequencing methods allows for the detailed analysis of the immunoglobulin heavy chain variable genes (IgVH) and thus offers the best way to prove clonal origin. Immunoglobulin heavy and light chains consist of constant regions and variable regions. Within the variable region, three complementarity-determining regions (CDRs) belong to hypervariable regions, in particular the CDR3 region. As described above, immunoglobulin genes undergo V-D-J rearrangements during B-cell development. Moreover, several nucleotides are randomly added by the terminal deoxynucleotidyltransferase during D-J rearrangement and V-D-J recombination thus

making this genetic region unique for a given B-cell clone. If the length and nucleotide sequence of the CDR3 regions are identical between B-CLL and DLBCL in Richter’s syndrome, this can be taken as an evidence of clonal relationship. In 1993, the first study was performed establishing the clonal relationship between B-CLL and aggressive lymphoma in Richter’s syndrome through primary structural analyses of the expressed IgVH genes (Cherepakhin, Baird et al. 1993). Since then, a good number of cases were investigated, and the DLBCL component in Richter's syndrome was found to be derived from the original B-CLL clone or arose as an independent, secondary lymphoma (Matolcsy, Inghirami et al. 1994; Nakamura, Kuze et al. 2000; Timar, Fulop et al. 2004). Upon review of the literature, approximately two-thirds of Richter’s syndrome cases (mainly B-CLL and DLBCL) were of identical clones (Nakamura and Abe 2003). However, the largest series reported to date comprises eight cases (Timar, Fulop et al. 2004).

Genetic abnormalities in Richter’s transformation have been documented in a small number of cases only. Alterations of chromosomes 12 and 13 as well as other abnormalities such as gains and losses at chromosomes 17p, 8p, 11q, and 14q were detected in Richter’s syndrome (Bea, Lopez-Guillermo et al. 2002). There were several reports about the occurrence of trisomy 12 in Richter’s syndrome. In one study, trisomy 12 was found in a proportion of cells from all five investigated specimens with high grade transformation, but only one had trisomy 12 before transformation. This suggests that trisomy 12 may be an acquired cytogenetic abnormality during the course of B-CLL and has a high frequency in Richter's syndrome (Brynes, McCourty et al. 1995). Another study demonstrated that seven out of eight patients who subsequently developed Richter's syndrome, initially had complex karyotypic changes with or without trisomy 12 (Han, Henderson et al. 1987). Moreover, Zhu and colleagues detected a deletion at 11q23 in three cases of Richter’s syndrome (Zhu, Monni et al. 2000). Taken together, no final conclusion can be drawn regarding an association of Richter’s syndrome with any specific chromosomal abnormality, possibly due to the low number of analysed cases.

It was reported that high levels of MSI were associated with Richter's transformation in four cases of B-CLL, and, in five cases of Richter's transformation the hMLH1 promoter was hypermethylated in both B-CLL and DLBCL samples. In this study, hypermethylation of the hMLH1 promoter was associated with a high-level of MSI in four cases and a low-level of MSI in one case. This may suggest that in some cases of Richter's transformation, a DNA mismatch-repair defect could initiate genetic instability which in turn may play a role in tumor progression (Fulop, Csernus et al. 2003). However, in a contradictory report on four Richter's syndrome cases no microsatellite alterations were detected (Sanz-Vaque, Colomer et al. 2001).

Regarding the role of EBV infection in Richter's syndrome, one study found that in 4 out of 25 (16%) Richter's syndrome patients EBV could be detected in DLBCL cells. In that series, EBV-positive patients had a medium survival of three months, whereas EBV-negative patients had a medium survival of nine months. However, this difference was not statistically different (Ansell, Li et al. 1999). Since 40% of Hodgkin's Lymphoma cases are associated with EBV infection, more data on the EBV association exists in the HL variant of Richter's syndrome than in Richter's transformation with DLBCL.

1.4.2 IgVH Gene Analysis in the Hodgkin's Lymphoma (HL) Variant of Richter's Syndrome

The reported incidence of the HL variant of Richter's syndrome is approximately 0.5%, occurring with an average interval of 61.8 months after the diagnosis of B-CLL. The medium survival after transformation is approximately 12 months. Patients with the HL variant of Richter's syndrome are more resistant to therapy than patients with Hodgkin's Lymphoma; however, they are more sensitive to therapy than patients with classical Richter's syndrome.

Similar to the investigation in classical Richter's syndrome, an interesting question is whether the HRS / HRS-like cells represent a clonal transformation of underlying B-CLL

cells, or whether they represent a secondary malignancy. Using immuno-laser capture microdissection and single cell PCR analysis, the rearranged immunoglobulin heavy chain variable region (IgVH) gene of HRS/HRS-like cells was amplified in several studies. After sequencing and comparison with the IgVH sequences of the B-CLL, either clonally identical cases or clonally distinct cases have been found. However, the number of cases investigated was very limited (Ohno, Smir et al. 1998; Kanzler, Kuppers et al. 2000; de Leval, Vivario et al. 2004; Fong, Kaiser et al. 2005). A summary of published literature is provided in Table 1.

Table 1: Summary of the molecular analysis of the HL variant of Richter's syndrome

Authors	Year	Patients (n)	Diagnosis	Methodology	Molecular Results	EBV	Treatment Preceding RS
Rubin et al[29]	1994	2	CLL + HL	Southern blot	One single	EBV+	Fludarabine
			CLL + HL		rearrangement	EBV+	Fludarabine
Ohno et al[24]	1998	3	CLL + HRS cells	Single cell PCR	Same clone*	NA	NA
			CLL + HL		Same clone*	NA	NA
			HL		Inconclusive	NA	NA
Kanzler et al[17]	2000	3	CLL + HRS cells	Single cell PCR	Same clone*	EBV−	No treatment
			CLL + HRS cells		Different clones*	EBV+	COP
			CLL + HRS cells		Different clones*	EBV+	CHOP + RxT
Pescarmona et al[25]	2000	4	CLL + HL	Whole section PCR	Biclonal	EBV+	NA
			CLL + HL		Monoclonal	EBV+	NA
			CLL + HL		Monoclonal	EBV+	NA
			HL		Polyclonal	EBV+	NA
Küppers et al[20]	2001	1	CLL + HL	Single cell PCR	Same clonal precursor*	EBV−	NA
Van den Berg et al[34]	2002	1	CLL + HL + ALCL	Microdissection PCR	Same clone*	EBV−	NA
de Leval et al	Current	2	HL	Microdissection PCR	Different clones*	EBV+	Fludarabine
			CLL + HRS cells + HL		Different clones*	EBV+	Fludarabine

*Cases for which the clonal relationship was established with certainty.

EBV, Epstein-Barr virus; PCR, polymerase chain reaction; NA, not applicable; CLL, chronic lymphocytic leukemia; HL, Hodgkin lymphoma; HRS, Hodgkin Reed-Sternberg; ALCL, anaplastic large cell lymphoma; COP, cyclophosphamide, vincristine, prednisone; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; RxT, radiation therapy.

(From de Leval et al.: Am J Surg Pathol, 2004)

This table, provided by de Leval and colleagues (de Leval, Vivario et al. 2004), summarizes the molecular analysis of the HL variant of Richter's syndrome from 1994 to 2004. In a total 16 of cases, the clonal relationship between B-CLL and HRS/HRS-like cells in Richter's syndrome was analysed in nine cases by molecular analysis of the rearrangement of the IgVH gene. In five cases, HRS cells developed from the B-CLL clone, whereas four cases were clonally different. Furthermore, all four clonally distinct

cases were infected by EBV; in contrast, among the five clonally identical cases three were EBV-negative, and in the remaining two cases there was no information on the EBV status. Thus, these findings may support the hypothesis that the EBV+ HL variant of Richter's syndrome develops as an independent and clonally unrelated secondary malignancy, possibly as a consequence of the underlying immunodeficiency which often occurs in B-CLL patients (Rubin, Hudnall et al. 1994).

1.5 Aims of the Present Study

1.5.1 Richter's Syndrome : Lymphoma Progression or Composite Lymphoma?

In 1954, Foulds characterized "tumor progression" as the development of permanent, irreversible and qualitative changes of the tumor in one or more of its characters (Foulds 1954). In the same year, Custer introduced the term "composite lymphoma" as the manifestation of more than one histological pattern of malignant lymphoma occurring in different organs or within the same organ (Kim, Hendrickson et al. 1977; Kim 1993). Muller-Hermelink et al. proposed to limit the use of the term "lymphoma progression" to those cases that show a clonal evolution or transformation. In contrast, the term "composite lymphoma" was suggested to be used as a merely descriptive morphological designation for different lymphoma entities in one individual, irrespective of their clonal relationship (Muller-Hermelink, Zettl et al. 2001).

Therefore, the important question arises as to whether Richter's syndrome, which shows completely different histopathological and clinical characteristics between DLBCL/HL on one hand and B-CLL on the other hand, falls into the category of lymphoma progression or composite lymphoma. The answer to this question is the central topic of this study.

With the development of various techniques, several investigations confirmed that DLBCL in classical Richter's syndrome can develop from the original B-CLL clone, or

arise as a clonally unrelated lymphoma (Matolcsy, Inghirami et al. 1994; Nakamura, Kuze et al. 2000; Timar, Fulop et al. 2004). Hodgkin's Lymphoma or HRS-like cells in the HL variant of Richter's syndrome were also shown to be either clonally identical to the B-CLL clone or develop as secondary B-cell malignancy in the susceptible hosts (Ohno, Smir et al. 1998; Kanzler, Kuppers et al. 2000; de Leval, Vivario et al. 2004).

Thus, according to the literature, Richter's syndrome can be divided into two subtypes, one belonging to the "lymphoma progression" group which is clonally identical to the preceding B-CLL; the second subtype belongs to the "composite lymphoma" group in which the two B-cell malignancies are clonally unrelated. However, all published studies suffer from the inclusion of only a small number of cases with Richter's syndrome; therefore, a definite answer about the clonal relationship in Richter's syndrome is not available to date.

1.5.2 IgVH-Mutational Status of B-CLL Cells in Richter's Syndrome: Mutated or Unmutated?

It is known that B-CLL is a heterogeneous disease which can be divided into two subsets according to the mutational status of the IgVH gene. B-CLL with unmutated IgVH genes may arise from the naïve B-cells and is associated with progressive disease, whereas B-CLL with mutated IgVH genes may originate from antigen experienced memory B-cells and has a relatively favourable clinical outcome.

As mentioned previously, DLBCL and HL in Richter's syndrome can be clonally related to B-CLL or represent secondary neoplasms. However, the risk factors in B-CLL that are prone to develop DLBCL or HL are still not known. In particular, there is only little information on the IgVH mutational status of B-CLL and DLBCL/HL in Richter's syndrome. The first study that included sequencing analysis of IgVH gene to establish the clonal relationship between B-CLL and DLBCL, indicates that the clonally related DLBCL carried mutated IgVH genes showing the phenomenon of ongoing mutations

(Cherepakhin, Baird et al. 1993). Another study found unmutated IgVH genes in four cases with clonal progression from B-CLL to DLBCL (Matolcsy, Inghirami et al. 1994). In another report investigating three cases of Richter's transformation with presence of DLBCL, all showed mutated IgVH genes and one case was clonally identical to the original B-CLL, whereas the others were unrelated to the B-CLL clone (Nakamura, Kuze et al. 2000). Recently, mutational analyses have been performed in eight cases of Richter's syndrome with six cases of B-CLL expressing unmutated and two cases expressing mutated IgVH genes. In 5 of 6 unmutated cases, DLBCL expressed the same unmutated IgVH genes. In one of the unmutated and two of the mutated B-CLL cases in Richter's syndrome, the IgVH sequences of the DLBCL were distinct and expressed mutated IgVH genes (Timar, Fulop et al. 2004). In conclusion, it appears that the clonal progression to DLBCL may predominantly occur in B-CLL with unmutated IgVH genes, whereas secondary, clonally unrelated DLBCL may originate from a mutated subset of B-cells. In the HL variant of Richter's syndrome, only one report demonstrates a case of HRS-like cells progressing from a B-CLL carrying mutated IgVH genes. In contrast, HRS-like cells were clonally unrelated to the B-CLL clone in two cases with unmutated IgVH genes (Kanzler, Kuppers et al. 2000). Because of the limited number of analyzed cases, however, no definite conclusions can be drawn so far.

1.5.3 ZAP70, a Surrogate Marker for the IgVH Mutation Status of B-CLL in Richter's Syndrome?

The mutational status of the IgVH genes in B-CLL is an important prognostic factor. However, sequencing of IgVH genes is not suitable to be applied in routine laboratories, since it is costly and time consuming. DNA microarray analyses were used to define subsets of genes that may help to discriminate between the unmutated and mutated B-CLL subclones (Rosenwald 2003; Ferrer, Ollila et al. 2004). Zeta-associated protein 70 (ZAP70), a member of the tyrosine kinase family with a critical role in the initiation of T-cell receptor signalling, is normally expressed in T-cells and natural killer (NK) cells, but not in normal B-cells. Various studies demonstrated that ZAP70 is a good discriminator between IgVH-mutated and unmutated B-CLL that correctly predicts the mutation status

in 93% of patients (Wiestner, Rosenwald et al. 2003). Furthermore, ZAP70 expression does not change in its expression level in sequential analysis of B-CLL cases (Crespo, Bosch et al. 2003). Recently, a number of investigations in patients with B-CLL confirmed that ZAP70 can be applied as a surrogate marker for IgVH mutations in B-CLL (Orchard, Ibbotson et al. 2004; Carreras, Villamor et al. 2005). The ZAP70 protein expression can be evaluated by flow cytometry which allows for the discrimination of ZAP70-positive B-CLL cells from non-malignant T cells and NK cells (Orchard, Ibbotson et al. 2004). Alternatively, immunohistochemical assessment of ZAP-70 expression is also robust method and allows for the comparison of ZAP 70 expression in lymphoma cells with that in reactive T-cells. ZAP70 is expressed by many lymphoma subtypes but is rarely present in DLBCL and HRS-cells using immunohistochemical methods (Admirand, Rassidakis et al. 2004). In mantle cell lymphoma, ZAP70 expression was detected without difference between IgVH-mutated and -unmutated tumor cells (Carreras, Villamor et al. 2005). Interestingly, Chen and colleagues found that the expression of ZAP70 was associated with enhanced signal transduction via the B cell receptor (Chen, Widhopf et al. 2002). However, the reason why the ZAP 70 expression is associated with the IgVH gene mutational status and how the ZAP70 protein functions in the pathogenesis of B-CLL is still unclear. In conclusion, the third aim of our study is the investigation of ZAP70 as a surrogate marker for the IgVH mutational status in B-CLL cases that develop a Richter's syndrome.

Chapter 2

Materials and Methods

2.1 Materials

Paraffin embedded tumor specimens of 48 patients were identified in the archives of the Institute of Pathology at the Universities of Wuerzburg, Munich (both Germany) and Innsbruck (Austria) as well as in the files of the Laboratory of Pathology, National Cancer Institute, NIH (USA). Cases were diagnosed based on histopathologic and immunophenotypic criteria as detailed in the current WHO classification (Jaffe 2001). 34 cases were diagnosed as classical Richter's syndrome (B-CLL and DLBCL), 6 cases were classified as the HL variant of Richter's syndrome (B-CLL and classical HL) and additional 8 cases showed B-CLL with scattered CD30-positive HRS-like cells. Molecular analyses could be performed in 26 cases of classical Richter's syndrome, in 6 cases of the HL variant of Richter's syndrome and in 6 cases of B-CLL with CD30-positive HRS-like cells. Basic clinical data is summarized in Table 4 and Table 8 (see Results section on page 54 and page 66).

2.2 Reagents

2.2.1 Reagents for Immunohistochemical Staining

2.2.1.1 Immunohistological Staining Kit

The Histostain®-Plus bulk kit detection system (Zymed, USA) was used for immunohistochemical staining.

2.2.1.2 Primary Antibodies for Immunohistochemical Staining

Primary antibodies were purchased from the manufactures DAKO, NOVOCASTRA, UPSTATE and SANTA CRUZ. The optimal concentrations of antibodies are displayed in Table 2.

2.2.1.3 HistoGripTM and Peroxidase Blocking Solution

HistoGripTM (Zymed, USA) was used for pre-treatment of slides.

3% hydrogen peroxide (H_2O_2) (Zymed, USA) was prepared by adding 1 part of 30% H_2O_2 to 9 parts of Methanol (Merck, Germany).

2.2.2 Reagents for DNA Extraction

2.2.2.1 Digestion Buffer and Enzyme

Proteinase K (20mg/ml, PCR grade, Roche, Germany) was used for digestion.

The proteinase K digestion buffer was prepared as follows:

100mM NaCl

10mM TrisCL, pH8

25mM EDTA, pH8

0.5% SDS

Table 2: Primary antibodies used for immunohistochemical staining

Antibody	Source	Dilution	Method	Predominant Reactivity
CD5	NOVOCASTRA	1:20	LAB-SA method	T cells, B cells in B-CLL
CD20	DAKO	1:1000	LAB-SA method	B cells, except pre-pre-B cells and plasma cells
CD23	NOVOCASTRA	1:40	LAB-SA method	Coexpression in B-CLL
CD30	DAKO	1:10	LAB-SA method	Activated T or B cells, Reed-Sternberg (RS) cells
CD15	DAKO	1:30	LAB-SA method	Granulocytes; monocytes/histocytes; RS cells
LMP1	DAKO	1:100	LAB-SA method	EBV infected cells
Ki67	NOVOCASTRA	1:100	LAB-SA method	Proliferating cells
ZAP70	UPSTATE	1:200	LAB-SA method	T cells, NK cells
IRF-4	SANTA CRUZ	1:200	LAB-SA method	Plasmacellular differentiated B-cells, T cells
p53	DAKO	1:20	LAB-SA method	B cells with p53 mutations
CD10	NOVOCASTRA	1:40	LAB-SA method	Germinal center B cells
CD3	NOVOCASTRA	1:20	LAB-SA method	T cells
Bcl-6	DAKO	1:5	LAB-SA method	B-cells (centrocytes, centroblasts) Extrofollicular blasts

2.2.2.2 Phenol-Chloroform Reagent

Phenol-chloroform (Phenol/Chloroform/Isoamylalkohol 25:24:1, Roth, Germany) and Chloroform (P.A, Merck, Germany) were used to isolate DNA.

2.2.3 Reagents for PCR Reactions

2.2.3.1 Primers for PCR-GeneScan Analysis and Sequencing

The primers for PCR-GeneScan analysis and sequencing are given in the following table.

Table 3: Primers for PCR-GeneScan analysis and sequencing

Primer		Working Concentration (pM)
IgVH gene		
FR3A-for	5'-ACA CGG C(C/T)(G/C)TGT ATT ACT GT-3'	50pmol/ µl
FR2A-for	5'-TGG (AG)TC CG(AC) CAG (GC)C(CT) (CT)C(AGCT) GG-3'	50pmol/ µl
LJH/(HEX)	5'-TGA GGA GAC GGT GAC C-3'	50pmol/ µl
VLJH/(FAM)	5'-GTG ACC AGG GTN CCT TGG CCC CAG-3'	50pmol/ µl
M13-for	5'-TGT AAA ACG ACG GCC AGT-3'	10pmol/ µl
M13-rev	5'-GAG CGG ATA ACA ATT TCA CAC AGG-3'	10pmol/ µl

All primers were obtained from MWG-Biotech, Germany.

2.2.3.2 PCR Enzymes

AmpliTaqGold® polymerase with GeneAmp® 10 PCR× buffer II and $MgCl_2$ solution (Applied Biosystems ABI, USA) was used for the PCR reaction.

The Terminator ready Reaction Mix (3100 BigDye Terminator v3.1 Matrix Standard, Applied Biosystems ABI, USA) was used for the sequencing PCR.

2.2.3.3 dNTP

The DNTP-100A kit (1ml each of 100mM dATP, dCTP, dGTP, dTTP, Sigma, Germany) was used in the PCR reaction.

The working solution of dNTPs was prepared as follows and stored at -20°C:

Distilled water (DW)	950µl
Each dNTP (100mM)	12.5µl
Total (1.25mM)	1000µl

2.2.4 Reagents for Electrophoresis

2.2.4.1 Preparation of 5xTBE Buffer

5xTBE was prepared as follows:

Boric acid (p.A.)	137.5g
Tris (Ultrapure)	270g
EDTA (Biochemica)	20.75g
DW	up to 5000ml

Chemicals were purchased from Applichem, Germany.

2.2.4.2 Ethidium Bromide (EB) Solution

5µl EB (10mg/ml, Applichem, Germany) was added to 100ml agarose solution (working solution: 0.5µg/ml).

2.2.4.3 Preparation of 1.5% Low-Melting Agarose

3.0g low-melting agarose (Invitrogen, Germany) was added to 200ml TBE and heated in a microwave oven for 4 minutes. The bottle was shaken by hand for 2 minutes and cooled down to 60°C. 10µl stock solution of EB was added and 200ml agarose was poured on a plate and allowed to solidify at room temperature for 30 minutes.

2.2.4.4 DNA Markers

The GeneRuler 100bp DNA ladder with 6xLoading Dye (Fermentas, Canada) and the Low DNA Mass ladder (Invitrogen, USA) were used as DNA markers.

2.2.5. Reagents for GeneScan Analysis

2.2.5.1 Chemical Reagents

The following chemicals were used for the PCR-GeneScan analysis:

Urea (Molecular Biology grade, Applichem, Germany)

Acrylamid (Rotiphorese® NF-Acrylamid/bis-solution 40% (29:1), Roche, Germany)

Formamide (P.A., Applichem, Germany)

TEMED (Tetramethylethylenediamine, Sigma, USA)

2.2.5.2. Preparation of 10% Ammonium Persulfate (APS)

APS (Sigma, USA) was dissolved in distilled water (DW) as a 10% stock solution and stored at -20°C.

2.2.5.3. Preparation of 4.5 % Polyacrylamide Gel Solution

The following reagents were used to prepare the polyacrylamide gel solution:

Urea	18g
DW	20ml
5xTBE	10ml
Acrylamide	5.625ml

The polyacrylamide gel solution was filtered using a filter membrane and a vacuum system. The stock solution can be stored at room temperature for 2 weeks. Before use (approximately 40 ml needed for one run), 250µl APS (-20°C) and 30 µl TEMED (-4°C) were added.

2.2.5.4 GeneScan Marker

A Fluorescence Amidite Standard (GeneScan-350 ROX Size Standard, Applied Biosystems, USA) was used as a marker for GeneScan analysis.

2.2.5.5. Preparation of Loading Buffer

The loading buffer was prepared as follows (for 64 lanes):

Formamide	110µl
GeneScan-350 ROX Size Standard	10µl
Dye (loading buffer)	30µl

2.2.6 Reagents for Subcloning

2.2.6.1 PCR Product Purification Kit

The JETsorb gel extraction kit (Genomed, Germany) was used for purification of PCR products.

2.2.6.2 Ligation and Transformation Kit

The TopoTA Cloning® kit (pCR®2.1-Topo® vector) and One Shot® Top10 chemically Competent E.coli (Invitrogen, USA) were used for ligation and transformation.

2.2.6.3 Plasmid DNA Purification Kit

The JETprep plasmid DNA isolation and purification kit (Genomed, Germany) was used for isolation and purification of plasmid DNA.

2.2.6.4 Restriction Enzyme

The EcoRI enzyme (10U/µl, 5000U) with 10x EcoRI buffer (Fermentas, Canada) was used for checking DNA inserts.

2.2.6.5 Preparation of Antibiotics

Ampicillin (Sigma, USA) was prepared as stock solution (50mg/ml) in DW and stored at -20°C. 100µl stock solution was added to 100ml medium as a working solution of 50µg/ml.

2.2.6.6 Preparation of LB Medium and Agar Plates

25g LB medium and 40g LB agar (Roth, Germany) each were dissolved in 1000ml DW and autoclaved at 121°C, 15psi (103kPa) for 20 minutes. 100µl ampicillin stock solution was added to 100ml liquid medium after it cooled down to 60°C. LB agar was

immediately distributed into dishes and allowed to solidify at room temperature. The plates and the LB medium were stored at 4°C.

2.2.6.7 Preparation of X-gal Plates

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, Fermentas, Canada) was dissolved in dimethylformamid (Sigma, USA) as a stock solution (40mg/ml) and kept in light-tight containers at -20°C.

40µl X-gal stock solution was spread on each LB plate and the plate was incubated at 37°C until ready for use.

2.2.7 Reagents for Sequencing

2.2.7.1 Polymer

The Performance Optimized Polymer 3100 pop-4 (Applied Biosystems, USA) was used for sequencing.

2.2.7.2 Buffer

10x Genetic Analyzer buffer with EDTA (Applied Biosystems, USA) was used for sequencing.

2.2.7.3 Loading Buffer

3100 Hi-Di formamide (Applied Biosystems, USA) was used as a loading buffer.

2.2.7.4 Sephadex G50

Sephadex G50 Fine (DNA Grade, Amersham Biosciences AB, Sweden) was dissolved in DW as a 6% working solution and kept at room temperature overnight.

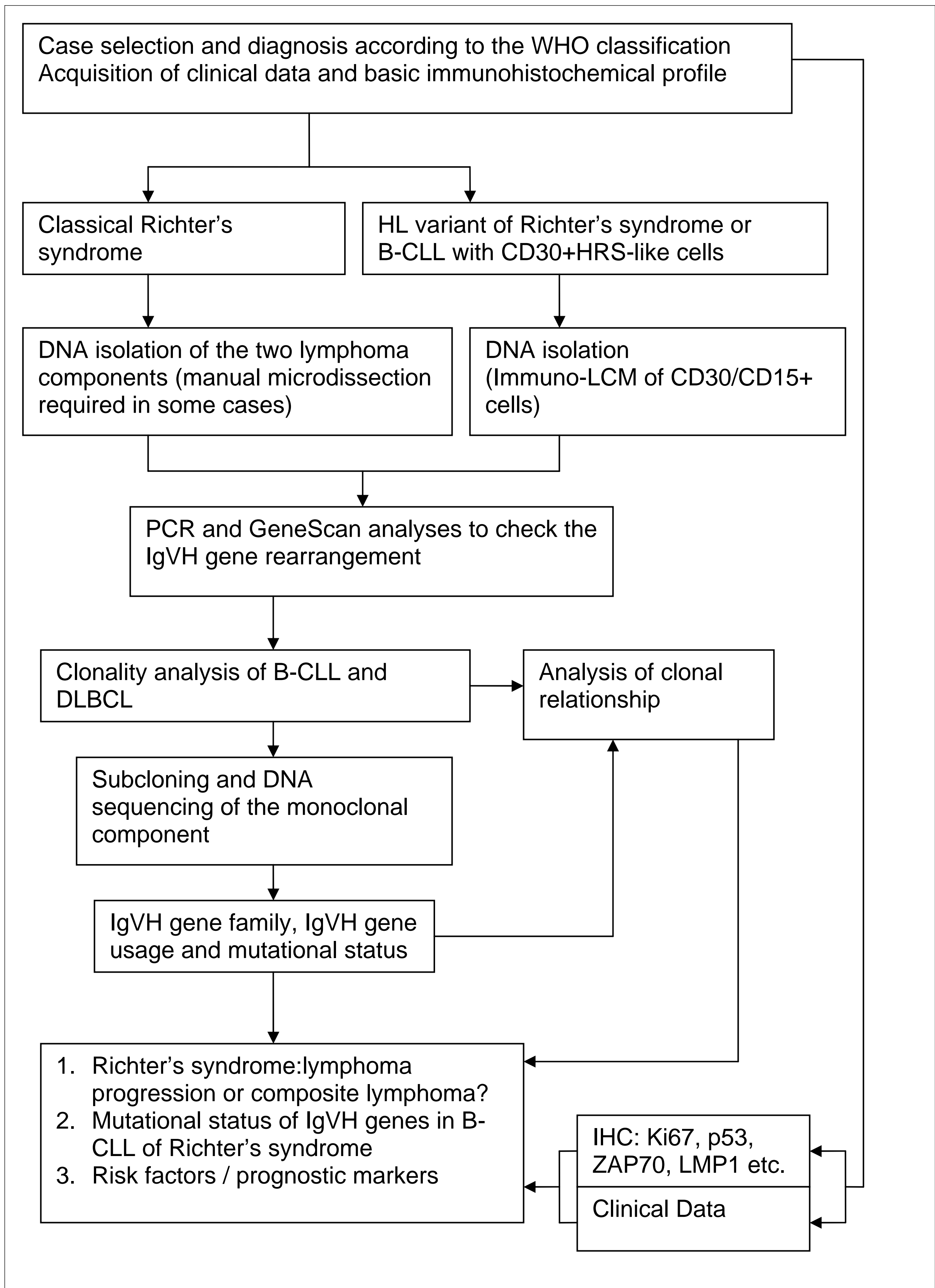
2.3 Equipment

Icebox	LIEBHERR comfort -20°C
	Hereus –80°C, Germany
Microwave Oven	Micromaxx®, Germany
Autoclave	MM Vakulab S3000, Germany
	H+P Labortechnik Varioklav 75S, Germany
Microliter pipettes	Eppendorf , Germany

Pipette controller	Brand Accu-Jet®, Germany
Centrifuge	Eppendorf 5415C/5417R
	Hettich Universal /K2S (cell culture), Germany
Vortexer	Scientific Industries Vortex Genie 2, USA
Shaking incubator	Eppendorf, Germany
Heating Blocks	Liebisch, Germany
Water bath	Heinse-Ziller GFL 1086, Germany
	Kottermann Labortechnik, Germany
PH meter	WTW pH330, Germany
Slide cut machine	JUNG HISTOSLIDE 2000R, Schwabach, Germany
Dry box	TDO66 medite, Germany
Automatic IHC machine	Genesis RSP 200 (TECAN, Germany)
Microscope	Zeiss, Germany
DNA/RNA calculator	Gene Quant pro (Amersham Biosciences, USA)
Photoelectric colorimeter	Herolab, Germany
PCR machine	Eppendorf mastercycler gradient, Germany
	Eppendorf mastercycler personal, Germany
	MWG-BIOTECH primus 96 ^{plus}, Germany
Electropherosis machine	Pharmacia LKB-GPS 200/400 (Pharmacia Biotech, USA)
LCM machine	PixCell® Laser capture microdissection system
	(Arcturus Engineering, USA)
	P.A.L.M laser microbeam microdissection system
	(Leica, Germany)
LCM cap	Capture™ HS LCM Caps
	Capture™ Macro LCM Caps
	(Arcturus Engineering, USA)
GeneScan machine	377-DNA-Sequencer (Applied Biosystems, USA)
Sequencing machine	3100-Avant Genetic Analyzer (Applied Biosystems, USA)

2.4 Methods

The methods are summarized in the following flow chart:


Case selection and diagnosis according to the WHO classification
Acquisition of clinical data and basic immunohistochemical profile
Classical Richter's syndrome
HL variant of Richter's syndrome or B-CLL with CD30+HRS-like cells
DNA isolation of the two lymphoma components (manual microdissection required in some cases)
DNA isolation (Immuno-LCM of CD30/CD15+ cells)
PCR and GeneScan analyses to check the IgVH gene rearrangement
Clonality analysis of B-CLL and DLBCL
Analysis of clonal relationship
Subcloning and DNA sequencing of the monoclonal component
IgVH gene family, IgVH gene usage and mutational status
1. Richter's syndrome:lymphoma progression or composite lymphoma?
2. Mutational status of IgVH genes in B-CLL of Richter's syndrome
3. Risk factors / prognostic markers
IHC: Ki67, p53, ZAP70, LMP1 etc.
Clinical Data

2.4.1 Immunohistochemical Methods

2.4.1.1 Pretreatment of Slides and Heat Induced Epitope Retrieval in Tissue Specimens

Slides were coated with silane or poly-L-Lysine or HistoGripTM. 5-8µm serial sections of paraffin blocks were cut and mounted onto the pretreated slides and then dried at 60ºC for 30 minutes. Slides were submerged into 100% xylene at room temperature for 20 minutes, Xylene was changed and slides were incubated for another 20 minutes to remove the paraffin. Afterwards, slides were hydrated through 100%, 70%, 50% ethanol and distilled water for 2 minutes each and dried in the air. If desired, slides were submerged in peroxidase blocking solution for 10 minutes to perform avidin/biotin blocking and endogenous enzyme quenching and washed three times with PBS. Further, slides were washed with distilled water 3 times for 2 minutes each and put in a slide rack and placed in a 1 liter glass beaker containing 500 ml of 0.01 M citrate buffer. The beaker was placed on a hot plate. The solution was heated and kept boiling for 10 minutes. The beaker was then removed from the hot plate and cooled down for at least 10-20 minutes at room temperature. Slides were rinsed with PBS prior to further immunostaining.

2.4.1.2 Immunohistochemical Staining (IHC)

After performing the peroxidase blocking step, sections were first incubated for 10 minutes with serum blocking solution. Excess blocking solution was drained and blotted away. Second, sections were incubated with the respective primary antibody (see Table 2) for 60 minutes at room temperature and then washed. Third, slides were incubated with a biotinylated secondary antibody for 20 minutes and washed again. Fourth, slides were incubated for 20 minutes with streptavidin-enzyme conjugate and washed. Subsequently, the substrate-chromogen mixture was added and slides were incubated for 5-10 minutes and washed. Finally, slides were counterstained with hematoxylin for 15-30 seconds and mounted with aqueous mounting solution (100%, 95%, 70% ethanol).

2.4.2 Microdissection

2.4.2.1 Manual Microdissection

Tissue areas of interest were gently scraped with a clean knife under the microscope. The dissected tissue was detached from the slide and collected into a 1.5ml Eppendorf-type microtube by using a needle.

2.4.2.2 Immuno-Laser Capture Microdissection (LCM)

Laser Capture Microdissection (LCM) is a technology which allows for obtaining purified cell populations for further investigations.

2.4.2.2.1 Description of Two Types of LCM Systems

The PixCell® system by Arcturus (Arcturus Engineering, USA) and the P.A.L.M laser microbeam microdissection system (Leica, Germany) were used for laser capture microdissection (LCM). In the PixCell® system the laser light is pulsed through specially made microfuge caps which have a contact surface lined with a thermoplastic transfer film. Microdissection is performed by lowering the cap over a desired area of the tissue. Pulses of laser light pass through the collection cap creating heat which causes the membrane to melt and bond the selected region of cells on the membrane surface. The sample is then collected by lifting the cap from the tissue specimen surface. Under the microscope captured cells can be checked and calculated. In the P.A.L.M laser microbeam microdissection (LMM) system, the objective lens of the microscope converge the laser light to produce extremely high-density focal energy. The focused laser beam induces a localised photodecomposition without damage to the surrounding tissue, which can be used to selectively destroy unwanted cells from the specimen. By increasing the laser light intensity and delivering a pulse of laser energy the selected cells are lifted up and out of the section into a waiting collection vessel. Thus, the possibility of contamination is reduced because there is no contact between the specimen and the collecting surface. The two systems were used in our experiments to combine advantages of both techniques. First, the focused laser beam in the P.A.L.M system was used to

destroy the surrounding cells. Then, the PixCell LCM was applied to pick up the cells of interest.

2.4.2.2.2 Preparation of Slides

4µm serial sections were cut, dried and deparaffinized. CD30/CD15 was stained by using the Histostain-SAP Kits with the peroxidase substrate AEC (3-amino-9-ethylcarbazole). Then, the sections were dehydrated sequentially in 70%, 95%, 100% ethanol for 15 seconds each, submerged into 100% xylene twice for 2 minutes and dried at the air.

2.4.2.2.3 Removal of Surrounding Tissue

The entire P.A.L.M (LPC) system was switched on. Firstly, the specimen was visualized under the microscope with the different objectives (4x, 10x, 20x and 40x). Secondly, the laser focus and the laser energy were adjusted until a narrow, clear-cut line was achieved. Finally, the surrounding areas of aimed cells were targeted, destroyed and ejected by the UV laser.

2.4.2.2.4 Capturing the Reed-Sternberg Cells/CD30-Positive Large Cells

The immuno-labelled slides were positioned under the microscope and the tissue areas of interest were found. The special cap (Capture HS LCM cap) was lowered over a desired area of the specimen to create a contact between the membrane and the tissue surface by a swing arm. Then the laser light of the spot diameter (7.5µm = 1 cell) was selected and the intensity was adjusted until the laser beam became the brightest and smallest light spot. When pulses passed through the collection cap, the thermoplastic transfer film was melted and the selected cells were stuck on the membrane. Finally, cells were dissected when the caps were lifted away from the tissue by the swing arm. 20-60 cells were captured in each cap and three caps were obtained in each case.

The film was scraped from the cap with a knife and immersed into a standard microfuge tube (1.5ml) with 10-20µl digestion buffer of 0.4mg/ml proteinase K. A digestion was performed in a 55°C heat bath for 8 hours and the enzyme was heat-inactivated at 95°C for 10 minutes. The extract was used directly for further analyses.

2.4.3 DNA Methods

2.4.3.1 Proteinase K Digestion

5 to 20 10µm sections (according to the size of tissue) were cut and collected in a 1.5ml tube and quickly spun down (14 x 1000 rpm, 30 seconds). 800µl xylene was added, and the samples were vortexed and centrifuged for 5 minutes. This step was repeated once again. Secondly, 800µl ethanol was added, the tube was inverted several times, centrifuged for 5 minutes and dried in a 50°C heat block for 30 minutes. 300µl digestion buffer and 20µl proteinase K (20mg/ml) were then added and the samples were incubated in a shaking water bath (350 rpm) or hot bolt plat at 50°C overnight. Proteinase K was added for several times (according to the results of digestion) and at last proteinase K was then inactivated at 96°C for 10 minutes.

2.4.3.2 Phenol-Chloroform Extraction

Phenol-chloroform extraction was performed according to the method of Herrmann et al. (Herrmann and Frischauf 1987). Briefly, 600µl phenol chloroform was added to the aqueous sample, mixed for 45 seconds and centrifuged at 14.000rpm for 5 minutes. The upper aqueous phase was transferred into a new properly labelled microfuge tube. These two steps were repeated two more times. Subsequently, 500µl of chloroform was added, mixed and spun as before. The upper aqueous layer was transferred into a new microfuge tube and mixed with 1/10 of the volume of 3M sodium acetate, 1ml of 100 % cold ethanol and precipitated overnight at -20°C. The samples were spun at 14.000rpm for 5 minutes and the ethanol was removed. The pellet was washed with 1ml of 70 % ethanol and dried at 37°C (heat block) for 30 minutes. Finally the DNA was dissolved in 100µl distilled water. The DNA concentration was measured by OD analysis and DNA was stored at -20°C.

2.4.4 Polymerase Chain Reaction (PCR)

2.4.4.1 Standard PCR

DNA obtained from whole paraffin-embedded slides by standard phenol chloroform extraction was used to amplify the CDRIII fragment of the IgVH gene by PCR using the primer combination FR3A/LJH-HEX (see Table 3). The reaction consisted of the following components:

PCR reaction mixture:

9.48µl	Distilled water (DW)
2.0µl	10×PCR buffer II
3.6µl	$MgCl_2$ (25mM, final concentration 4.5 mM)
2.5µl	$dNTP_s$ (100mM, final concentration, 156.25µM)
0.12µl	AmpliTaqGold^{Tm}Polymerase (Taq) (5U/µl, final concentration: 0.03U/µl)
0.15 µl	FR3A (50pM, final concentration: 0.375pM)
0.15 µl	LJH-HEX (50pM, final concentration: 0.375pM)
2.0µl	Template
20.0µl	Total

Amplification of the IgVH CDRIII region was carried out by using the following program:

(1) 95 °C 13 min.
(2) 95 °C 30 sec.
56 °C 1 min.
72 °C 1 min.
10 cycles
(3) 91 °C 30 sec.
56 °C 1 min.
72 °C 1 min.
25 cycles
(4) 72 °C 10 min.
(5) 8 °C ∞

2.4.4.2 Semi-nested PCR

Amplification of the CDR II fragment of the IgVH gene in DNA obtained from paraffin-embedded tissues by standard phenol chloroform extraction was completed by a semi-nested PCR approach using the primer combinations FR2A/LJH-HEX and FR2A/VLJH-FAM (see Table 3). The two reaction mixtures and PCR programs were as follows:

PCR reaction mixture (for the first round PCR of the CDR II fragment):

9.48μl	Distilled water (DW)
2.0μl	10×PCR buffer II
3.6μl	$MgCl_2$ (25mM, final concentration 4.5 mM)
2.5μl	$dNTP_s$ (100mM, final concentration, 156.25μM)
0.12μl	AmpliTaqGold^{Tm}Polymerase (Taq) (5U/μl, final concentration:0.03U/μl)
0.15 μl	FR2A (50pM, final concentration: 0.375pM)
0.15 μl	LJH-HEX (50pM, final concentration: 0.375pM)
2.0μl	Template
20.0μl	Total

PCR reaction mixture (for the second round PCR of the CDR II fragment):

11.48μl	Distilled water (DW)
2.0μl	10×PCR buffer II
1.6μl	$MgCl_2$ (25mM, final concentration 2.0 mM)
2.5μl	$dNTP_s$ (10mM, final concentration, 156.25μM)
0.12μl	AmpliTaqGold^{Tm}Polymerase (Taq) (5U/ul, final concentration: 0.03U/ul)
0.15 μl	FR2A (50pM, final concentration: 0.375pM)
0.15 μl	VLJH-FAM (50pM, final concentration: 0.375pM)
0.8μl	Product from the first round PCR
20.0μl	Total

Programs of the IgVH CDRII region PCR were as follows:

FR2A first round (FR2A-1-cyc)

(1) 95 °C 10 min.

(2) 95 °C 30 sec.

60 °C 45 sec.

72 °C 45 sec.

30 cycles

(3) 72 °C 10 min.

(4) 8 °C ∞

FR2A second round (FR2A-2-cyc)

(1) 95 °C 5 min.

(2) 95 °C 30 sec.

65 °C 30 sec.

72 °C 1 min.

5 cycles

(3) 95 °C 30 sec.

64 °C 30 sec.

72 °C 1 min.

5 cycles

(4) 95 °C 30 sec.

63 °C 30sec.

72 °C 1min.

18 cycles

(5) 72 °C 10min.

(6) 8 °C ∞

Amplification of the CDR III fragment of the IgVH gene from DNA extracted from laser captured tissue was carried out by seminested PCR using the primer combinations FR3A/JH-HEX and FR3A/VLJH-FAM.

PCR reaction mixture (for the first round PCR of the CDR III fragment):

12.25µl	Distilled water (DW)
2.5µl	20mM PCR buffer with $MgCl_2$
4.0µl	$dNTP_s$ (200mM)
0.25µl	AmpliTaqGold^{Tm}Polymerase (Taq) (5U/µl, final concentration: 0.05U/µl)
0.5 µl	FR3A (20pM)
0.5 µl	JH (20pM)
5.0µl	Template
25.0µl	Total

PCR reaction mixture (for the second round PCR of the CDR III fragment):

18.875µl	Distilled water (DW)
2.5µl	PCR buffer (20mM) with $MgCl_2$
0.5µl	$dNTP_s$ (200µM)
0.125µl	AmpliTaqGold^{Tm}Polymerase (Taq) (5U/µl, final concentration: 0.025U/µl)
0.25 µl	FR3A (20pm)
0.25 µl	VLJH-FAM (20pm)
2.5µl	Product from the first round PCR
25.0µl	Total

The program for the IGVH CDRIII PCR was as follows:

FR3A first round (FR3A-1-cyc)

(1) 95 °C 10 min.

(2) 94 °C 1 min.

56 °C 1 min.

74 °C 1 min.

40 cycles

(3) 74 °C 7 min.

(4) 8 °C ∞

FR3A second round (FR3A-2-cyc)

(1) 95 °C 10 min.

(2) 94 °C 1 min.

56 °C 1 min.

74 °C 1 min.

25 cycles

(3) 74 °C 7 min.

(4) 8 °C ∞

2.4.4.3 PCR for Subcloning

The same PCR reactions and programs were used as described with minor modifications. Primers without fluorescence labelling were used and the volume of PCR reactions was 50-60µl.

2.4.4.4 Sequencing PCR

The sequencing PCR was performed in a 10µl system using the primers M13 (forward/reverse) (see Table 3). The amount of template DNA was 5ng.

PCR mixture consisted of the following components:

Terminator Ready Reaction Mix	2µl
Template	Xµl
Primer (M13 forward/reverse)	1µl
Distilled water	add to total volume of 10µl

The program was as follows:

(1) 96 °C 1 min.
(2) 96 °C 10 sec.
50 °C 5 sec.
60 °C 4 min.
35 cycles
(3) 8 °C ∞

2.4.5 Electrophoresis Methods

2.4.5.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis is the easiest and most common way of separating and analyzing DNA. About 50-60µl of total PCR products mixed with 10µl 6x loading buffer were loaded on the 1.5% low melting molecular agarose gel. The gel was electrophoresed in 1xTBE solution at 120-150V for 1 hour. The band was cut out under low wave UV for further purification and subcloning.

The 10µl products of restriction enzyme (EcoRI) digestion were loaded on the 1.5% agarose gel and run at 120V for 30-45 minutes, using a reference of a 100bp DNA ladder (Invitrogen, Germany) on the same gel. The fragment size was determined and the quantity of DNA was estimated by comparison on the UV illuminator.

2.4.5.2 PCR-based GeneScan Analysis

The amplified products were electrophoresed on 4.5% polyacrylamide gels in a 377-DNA-Sequencer (Applied Biosystems, USA).

2.4.5.2.1 Preparation of the Gel Cassette

The electrophoresis glass-plates with the 0.4mm spacers were washed and cleaned. After air-drying, gel-running plates with spacers were fixed with clippers and installed tightly. The prepared gel solution was poured between the electrophoresis plates carefully, slowly and continuously. Then plates were left on frames horizontally without vibrating for 30-45 minutes. The comb was pulled out and the outsides of the electrophoresis plates were washed and dried. Afterwards the plates were installed and fitted in the chamber of GeneScan and fixed tightly. The upper and lower basins of buffer in the GeneScan machine were washed and cleaned.

2.4.5.2.2 Running of GeneScan

The instrument was set up. By clicking the option NEW/GENESCAN RUN, the ABI PRISM software was activated and a new run was created. GS run 36D-2400 and XL Scan were applied as run mode. 377-4.5% FHNR Gel's Matrix file, 64 lanes, 36 cm distance and 3 hours for collecting time were chosen as run parameters. The glass-plates containing the gel were checked to ensure no peak was produced due to the fluorescence on the plates or the gel by clicking the option PLATE CHECK. Then the upper and lower basins of gel-running chamber were filled fully with 1xTBE buffer. The 0.4mm comb was inserted and the gel was pre-run 10 minutes to balance the temperature. A mixture of 2µl loading buffer and 1.9µl PCR products was denatured at 98°C for 7 minutes, and immediately transferred on ice. 1.2µl mixture was loaded onto the gel for the first 32 lanes and the gel was run 2 minutes. The door of the machine was opened, another 32

samples were continued to load on the lanes. The gel was run for 1.5 hours for the IgVH gene CDRIII fragment and for 2 hours for the the IgVH gene CDRII fragment.

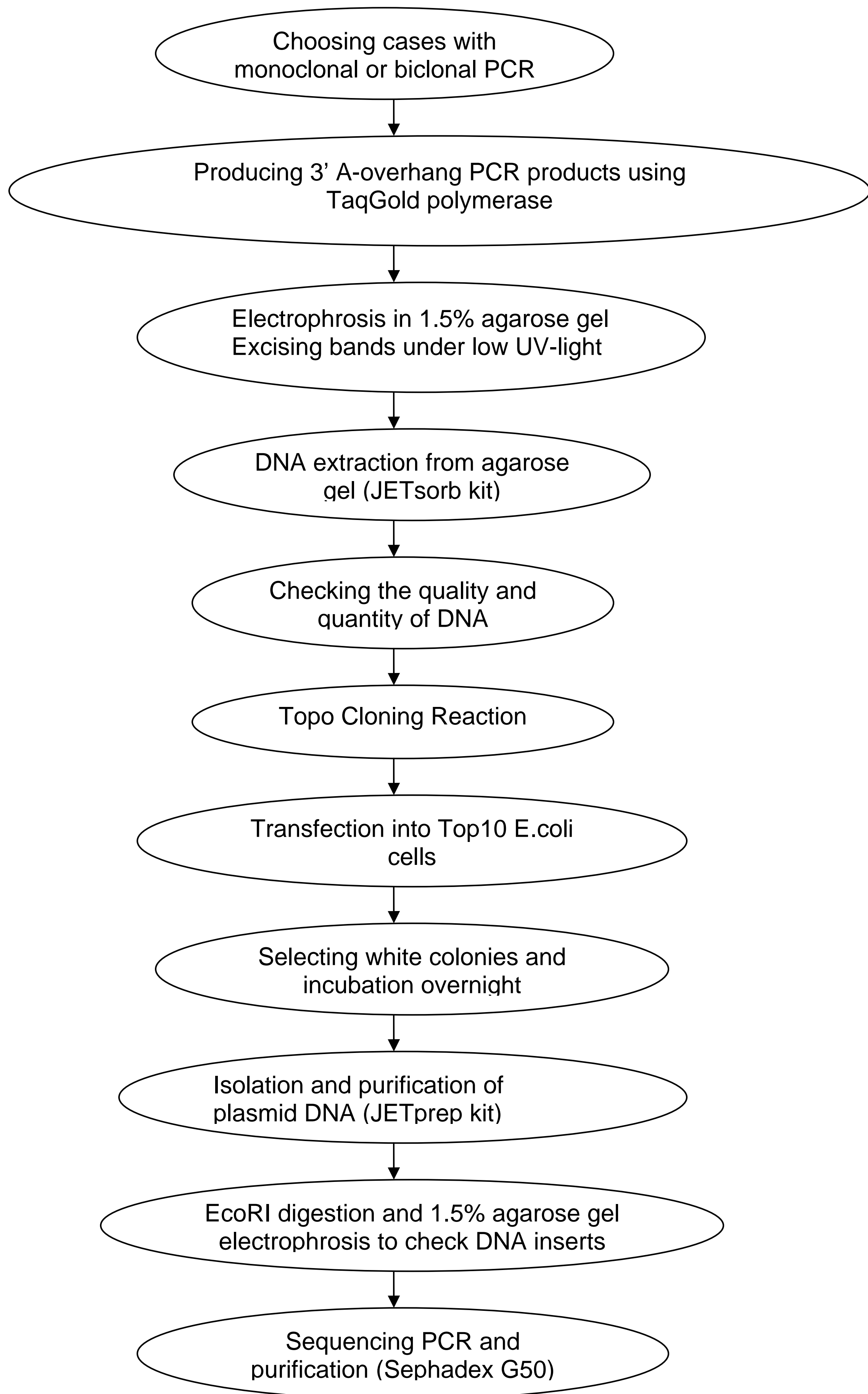
2.4.5.2.3 Analysis of Results

During electrophoresis the fluorescence intensity was recorded as a function of time and wavelength. They were colour-coded and displayed as peaks in the electrophoresis. Once the electrophoresis was completed, the gel image was checked for any obvious artifacts. Lanes were tracked and the traces were verified after gel contrast was adjusted. Then the data from different lanes were extracted, the analysis parameters were set and the local southern method was used as the size calling method. For the peak detection thresholds, 50(rfus) was usually used as the default. Finally, the analysis control window was opened and the size standard was assigned to all sample files. For GeneScan-350 (ROX) size Standard, the size standards were defined at 50, 75, 100, 139,150,160, 200, 250,300, 340 and 350bp and, afterwards, the appropriate dye colours (red for standard, green for FR3A/LJH-HEX, and blue for FR2A/VLJH-FAM) were highlighted for each sample and analysed. Then the result control window was opened and the evaluation of the sizes of obtained fragments was performed.

According to the number and size of peaks (products), there were five possibilities: If one or two strong, dominant peaks within expected size ranges (between 75-150bp for FR3A fragment and between 200-350bp for FR2A fragment) appeared in same size (less than 1bp difference) more than twice, it was regarded as a monoclonal (M) or biclonal (Bi) pattern; if a strong peak appeared on a background of multiple much weaker peaks, it was regarded as a monoclonal pattern with polyclonal background (MPC). When three or more peaks (3-9) were obtained within expected size ranges, it was considered as an oligoclonal pattern (Olig.). Another possibility was a ladder of 10-20 peaks showing a Gaussian distribution over the expected size range (3bp intervals for IgVH); this pattern was considered polyclonal (Poly.). When less than 10 peaks appeared with a random distribution of different intensities and variations between duplicate experiments, the product was considered incomplete (IP). The last pattern was no signal (NS) result without peaks or only weak peaks outside the expected size range.

2.4.6 Subcloning and Sequencing

A flow chart of total process is shown on the following page:


Choosing cases with
monoclonal or biclonal PCR
Producing 3’ A-overhang PCR products using
TaqGold polymerase
Electrophrosis in 1.5% agarose gel
Excising bands under low UV-light
DNA extraction from agarose
gel (JETsorb kit)
Checking the quality and
quantity of DNA
Topo Cloning Reaction
Transfection into Top10 E.coli
cells
Selecting white colonies and
incubation overnight
Isolation and purification of
plasmid DNA (JETprep kit)
EcoRI digestion and 1.5% agarose gel
electrophrosis to check DNA inserts
Sequencing PCR and
purification (Sephadex G50)

2.4.6.1 DNA Purification

The PCR products were electrophoresed by a 1.5% agarose gel and extracted by using the JETsorb gel extraction kit. Following the manufacturer's protocol, to each 100mg gel slice 300µl buffer A1 and 10µl JETsorb suspension were added to dissolve the agarose. Subsequently, the assay was mixed and incubated at 50°C for 15minutes to bind DNA, and mixed every 3 minutes during incubation. After centrifuging (30 seconds, >10,000rpm), the supernatant was removed and the pellet was washed once with 300µl of buffer A1 (high salt buffer). After centrifuging (30 seconds, >10,000rpm), the supernatant was removed and the pellet was washed with 300µl of buffer A2 (low salt buffer). The assay was centrifuged as before and the supernatant was removed again. Low salt washing was repeated once more. The tubes were incubated for 10 minutes at 50°C with the caps open to dry till the pellets became white. 30µl water was added and the mix was incubated for 5 minutes at 50°C to elute DNA. After centrifugation, the supernatant was removed into a new cap. The quantity and quality of DNA were checked by electrophoresis on a 1.5% agarose gel (120V, 30 minutes).

2.4.6.2 Ligation and Transformation

Topo cloning reaction system (for chemically competent E. coli) consisted of:

Fresh PCR product	0.5-4µl
Salt Solution	1µl
Topo vector	1µl
Sterile water	add to a total volume of 6µl

The reaction was mixed gently and incubated for 5 minutes at room temperature. 2µl Topo cloning reaction were added into the tube with one shot chemically competent E. coli and then mixed gently, incubated on ice for 20 minutes. Then the cells were heat-shocked for 30 seconds at 42°C without shaking and immediately transferred to ice for 2 minutes. After 250µl of room temperature S.O.C. medium had been added, the tubes were shaken with 200 rpm at 37°C for 1 hour. 25µl from each transformation was spread on a prewarmed selective plate with X-gal, and then plates were incubated at 37°C. Blue/white screening was performed after 12 hours in case of ampicillin selection.

Finally, 5-6 white colonies were picked up and transferred into tubes with 3ml medium. They were incubated and shaken at 37°C overnight.

2.4.6.3 Purification of Plasmid DNA

Isolation and purification of plasmid DNA was performed by using the JETprep plasmid DNA isolation and purification kit. After 3ml overnight culture of bacterial cells had been centrifuged (4,000rpm, 20 minutes), the supernatant was removed and bacterial cells were harvested. 200µl of solution D1 was added to the pellet, samples were mixed till the suspensions were homogeneous. Then 200µl of solution D2 was added and mixed gently without vortexing and afterwards incubated at room temperature for 5 minutes. 200µl of solution D3 was added and mixed gently without vortexing again. The supernatant was transferred with a pipette into a fresh tube immediately after centrifugation (>10,000rpm, 10 minutes). 500µl of solution D4 was added to bind DNA, mixed gently and incubated at room temperature for 5 minutes. During incubation, the assay was mixed once. Then the supernatant was removed again after centrifugation (>10,000rpm, 30seconds). The pellet was washed with 600µl of reconstituted solution D5, the resin was collected by centrifuging as before and the supernatant was removed. Washing with solution D5 was repeated once. The JETprep resin dried under vacuum till it turned white (vacuum chamber 6 minutes), 30µl water were added and incubated for 5 minutes at 60°C to elute plasmid DNA. Finally, the supernatant was transferred into a new tube immediately after centrifugation as before.

2.4.6.4 Restriction Enzyme Digestion

The digestion reaction was prepared as follows:

Restriction enzyme (EcoRI)	0.2 µl
Digestion buffer	2 µl
DW	16.8 µl
Template	1 µl
Total	20 µl

The digestion system was mixed and incubated at 37°C for 1 hour. The products were then visualized in the presence of a marker (Low DNA Mass ladder) in 1.5% agarose gels at 120V for 30 minutes.

2.4.6.5 Purification of Sequencing PCR Products

Filter tips were cut and filled with 300µl Sephadex G50. The tubes with cut filter tips were centrifuged at 3000 rpm for 3 minutes. Then filter tips with Sephadex G50 were removed to new caps and 10µl product of sequencing PCR was added. After centrifuging at 3000 rpm for 3 minutes, approximately 7-9µl purified DNA was obtained for sequencing.

2.4.6.6 Automated DNA Sequencing and Analysis

Automated DNA sequencing is based on the method of Sanger and relies on the use of 4 fluorescent dyes as labels for the reactions (Sanger, Nicklen et al. 1977). The dyes are either incorporated into the extension primer (dye primer) or into the dideoxynucleotides (dye-deoxy terminator) using polymerase chain reaction (PCR). 2', 3'-dideoxyribonucleoside triphosphates (ddNTP) are analogues of dNTP. The incorporation of these analogues can block further extension of the new chain because of the lack of the 3'-hydroxyl terminus which is needed to form the next phosphodiester bond. The labelled DNA fragment is electrophoresed through the capillary filled with a separation matrix. Fluorescence from the dye labelled reaction products was excited by light from the laser, and the resulting data was recorded as a four colour raw data plot.

2.4.6.6.1 Preparation of the Instrument

The polymer blocks were attached to the instrument; the syringes were prepared and installed, polymer 3100 pop-4 was added and all bubbles in the polymer blocks were removed. The reservoirs were filled with 5ml 10x Genetic Analyzer buffer plus 45ml water. Before each run, the polymer and buffer were checked to determine whether they needed to be changed or refilled. The sequencing analysis plate record was created and completed.

2.4.6.6.2 Denaturing and Loading of Samples

20µl Hi-Di [Tm] formamide was added to the purified DNA and denatured at 50°C for 3 minutes. A total of 30µl product was added onto a 96-well sample plate. The plate was assembled with a base plate, sample plate, plate septa and plate retainer and placed into the autosampler. Then the 3100-avant Data collection Software was started and the run was performed in 40 minutes for four samples in 36 cm array-length.

2.4.6.6.3 Analysis of Results

Sequences were analyzed using version 5.1 3100/3100-avant sequencing analysis software (Applied Biosystems, USA). The data was further analysed by using the software CHROMAS version 2.3 and DNAMAN (Lynnon, USA). The analyses of IgVH gene usage and mutational status were performed using web-based analysis tools (http://www.ncbi.nlm.nih.gov/blast/Igblast and http://imgt.cines.fr/textes/vquest).

Chapter 3

Results

To analyze the clonal relationship and the mutational status of the IgVH genes in Richter's syndrome, 48 cases were collected from the Institutes of Pathology at the Universities of Wuerzburg, Munich (both Germany) and Innsbruck (Austria) as well as from the files of the Laboratory of Pathology, National Cancer Institute, NIH (USA). The cases were carefully reviewed and classified according to the WHO classification (Jaffe 2001). 34 cases were diagnosed as classical Richter's syndrome (B-CLL and DLBCL), 6 cases were classified as the HL variant of Richter's syndrome (B-CLL and classical HL) and additional 8 cases showed B-CLL with scattered CD30-positive HRS-like cells.

3.1 Classical Richter's Syndrome: B-CLL and DLBCL

3.1.1 Clinical Characteristics and Histology

Among the 34 cases of classical Richter's syndrome, males were more often affected than females (ratio 2.4:1), and the median age at diagnosis was 66 years (range 25-86 years). The major localization of B-CLL was in the lymph nodes (LN) (19/34 cases, 55.9%), including lymph nodes of the gastrointestinal region (4/19 cases, 21.1%). Bone marrow was affected in 10/34 cases (29.4%), the spleen in 3/34 cases (8.8%), the skin in 1/34 cases (2.9%) and the tonsil in 3/34 cases (8.8%). In cases 3 and 7 biopsies were taken from two different localizations (see Table 4). DLBCL were mainly localized in lymph nodes (20/34 cases, 58.8%), and other localizations were in the gastrointestinal tract (5/34 cases, 14.7%), bone marrow (4/34 cases, 11.8%), skin (2/34 cases, 5.9%), liver (1/34 cases, 2.9%) and in the tonsil (3/34, 8.8%). In case 19 two anatomic sites were biopsied (see Table 4). Different sites of involvement by B-CLL and DLBCL occurred in 19/34 (55.9%) cases.

Histology was evaluated in hematoxylin and eosin (HE) and Giemsa stains in all cases. HE stain is the most widely used staining method in diagnostic histopathology; hematoxylin stains nuclei blue to dark-blue and eosin stains the cytoplasm pink to red

(Figures 1C and D). The Giemsa stain is a specific staining method that labels phosphate groups of DNA and thus highlights nuclear and nucleolar details (Figure 1A and B). Morphologically, two variants of DLBCL occurring in classical Richter's syndrome can be distinguished. The immunoblastic variant (IB) is characterized by atypical large tumor cells with prominent central nucleoli in more than 90% of tumor cells (Figure 1B). This variant occurred in 18.8% (6/32 cases) of cases in classical Richter's syndrome in our series. In contrast, the centroblastic variant (CB) is characterized by large tumor cells with fine chromatin and 2-4 membrane bound nucleoli (Figure 1D). This variant was present in 81.2% (26/32 cases) of cases in our series. Among clonally related cases, the immunoblastic variant was more common (28.8%, 5/18 cases). Figure 1 presents morphological details in two cases with classical Richter's syndrome.

3.1.2 Immunophenotypic Findings

Immunophenotypic analyses were performed on paraffin-embedded specimens from 32 patients of classical Richter's syndrome, including 24 cases that had molecular data available and 8 cases without such data. The staining intensity was initially graded as negative (-), weakly positive (+), moderately positive (++) and strongly positive (+++) according to the percentage of positive cells and the degree of expression (Figure 2). Figure 3 shows representative stainings for ZAP70, p53 and IRF-4. In the bar graph analysis displayed in Figure 4, the initial staining categories (+), (++) and (+++) for the markers CD5, CD23, ZAP70, p53 and IRF-4 were combined and scored as positive.

As a marker demonstrating B-cell origin, CD20 was detected in all cases of B-CLL and DLBCL (not shown in Figure 4). CD5 is a signal transduction molecule present on the surface of most T-cells. It is also detectable on a small subset of circulating B-cells. CD23 is a membrane glycoprotein that acts as a receptor for IgE. The simultaneous expression of CD5 and CD23 is a characteristic of B-CLL and therefore most useful in distinguishing B-CLL from other small lymphocytic B-cell malignancies. CD5 and CD23 expression was detected in 28 cases of B-CLL, in 4 cases this information was unavailable. The DLBCL components in Richter's syndrome retained CD5 expression in

9/28 cases (32.2%), while CD23 expression was retained in only 4/28 cases (14.3%) (Figure 4).

Zeta-associated protein 70 (ZAP70), a member of the tyrosine kinase family with a critical role in T-cell receptor signalling, is normally expressed in T-cells and natural killer (NK) cells, but not in normal B-cells. 14/21 cases (66.7%) of B-CLL were positive for ZAP70 staining (Figure 3A, Figure 4), but only 3 cases of DLBCL expressed ZAP70 at the same time (Figure 3B, Figure 4).

The tumor suppressor gene p53 can frequently be detected immunohistochemically, when an underlying p53-mutation is present (due to a longer half-life of the mutated p53 protein). In our series, the immunohistochemical expression of p53 could be studied in 31 cases of Richter's syndrome and both, B-CLL and DLBCL were positive in 10/31 cases (32.3%) (Figure 3C and D, Figure 4). Among the remaining 21 cases, in which the B-CLL was negative for p53, the corresponding DLBCL showed p53-positivity in 15 cases (71.4%). In the remaining 6 cases, B-CLL and DLBCL were negative for p53 (Figure 4).
IRF-4, a member of the interferon regulatory factor (IRF) family, is known to play a role in the control of B-cell proliferation and differentiation. In 23 cases that could be evaluated for IRF-4 expression, the DLBCL expressed IRF-4 in 19 cases (82.6%). In 8 of these cases (34.8%), IRF-4 was also positive in the B-CLL component (Figure 3E and F, Figure 4).

Finally, Bcl-6 that serves as a transcriptional regulatory protein and is normally expressed in germinal center lymphocytes, was not detected in B-CLL and was found to be positive in 12/32 DLBCL cases (37.5%). CD10, also a germinal center cell marker that is positive in the majority of follicular lymphomas, was detected in only 2/32 cases (6.25%) of B-CLL and in 7/32 cases (21.9%) of DLBCL.

No significant relationship between the expression of ZAP70, p53 and IRF-4 could be detected in B-CLL of classical Richter's syndrome by Fisher's exact test (see Table 5).

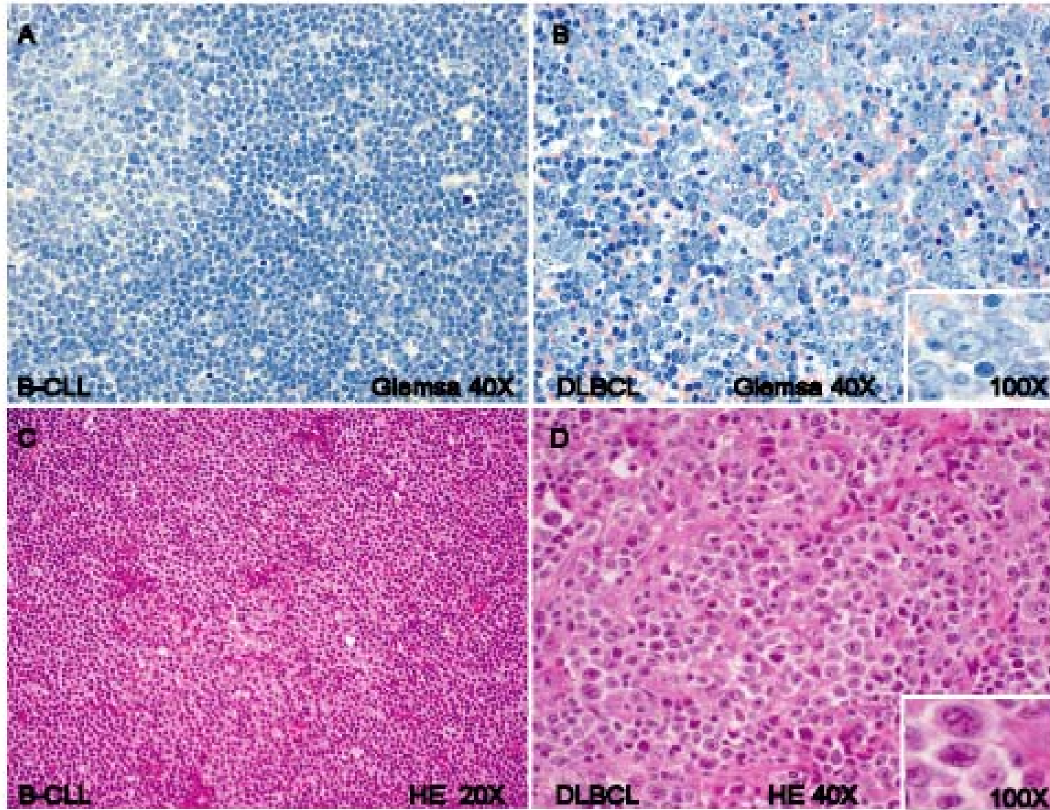


Figure 1: Morphology of Classical Richter's Syndrome.
A and B: Giemsa staining of B-CLL and DLBCL in a case of Richter's syndrome. In **A** (B-CLL), a pseudofollicle (pale area) is surrounded by small B-CLL lymphocytes (darker area). In **B** (DLBCL), morphology shows the immunoblastic variant, in which immunoblastic tumor cells show prominent central nucleoli.
C and D: H&E staining of B-CLL and DLBCL in another case of Richter's syndrome. In **C**, typical morphology for B-CLL is seen, and in **D**, the centroblastic variant of DLBCL can be recognized.

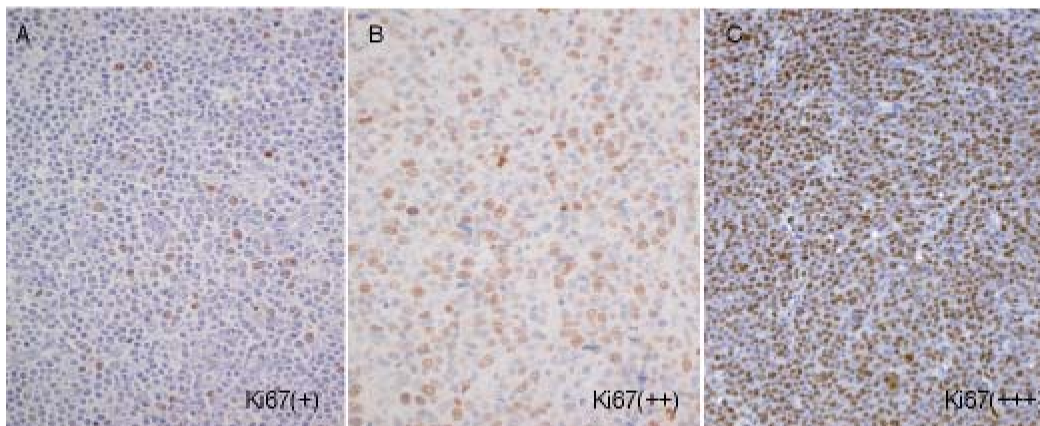


Figure 2: Varying degrees of Ki-67 expression: Representative examples (+, ++, +++) are presented.

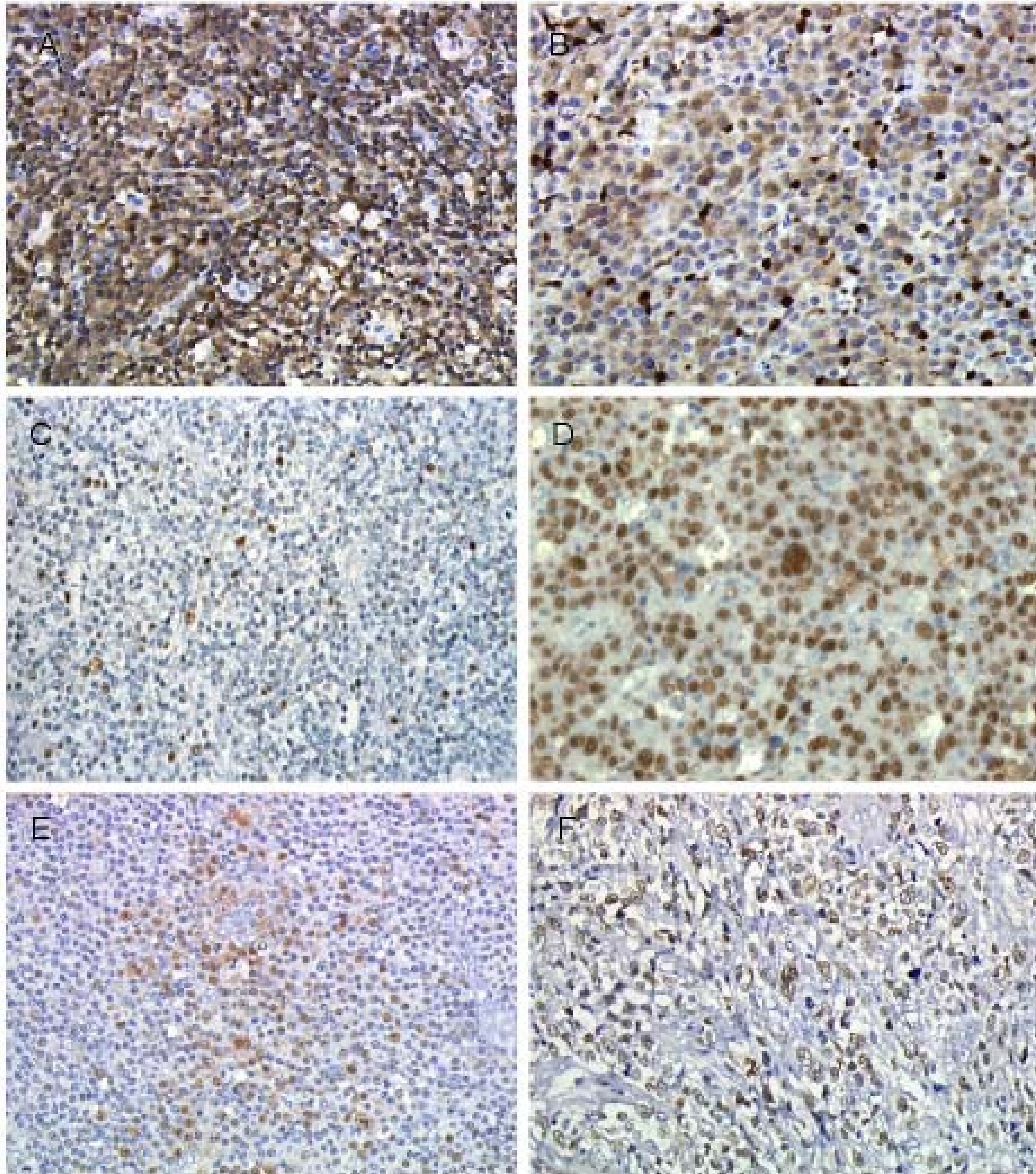


Figure 3: Immunohistochemical Staining of Classical Richter's Syndrome.
ZAP 70 expression in B-CLL (**A**) and partially in DLBCL (**B**). Scattered p53 expression in B-CLL (**C**) and strong staining in DLBCL (**D**). IRF-4 expression in B-CLL (**E**) and DLBCL (**F**).

Figure 4: Immunohistochemical results for CD5, CD23, ZAP70, p53 and IRF-4 in B-CLL and DLBCL in classical Richter's syndrome (**A**). In **B**, data is provided for the clonally related cases only.

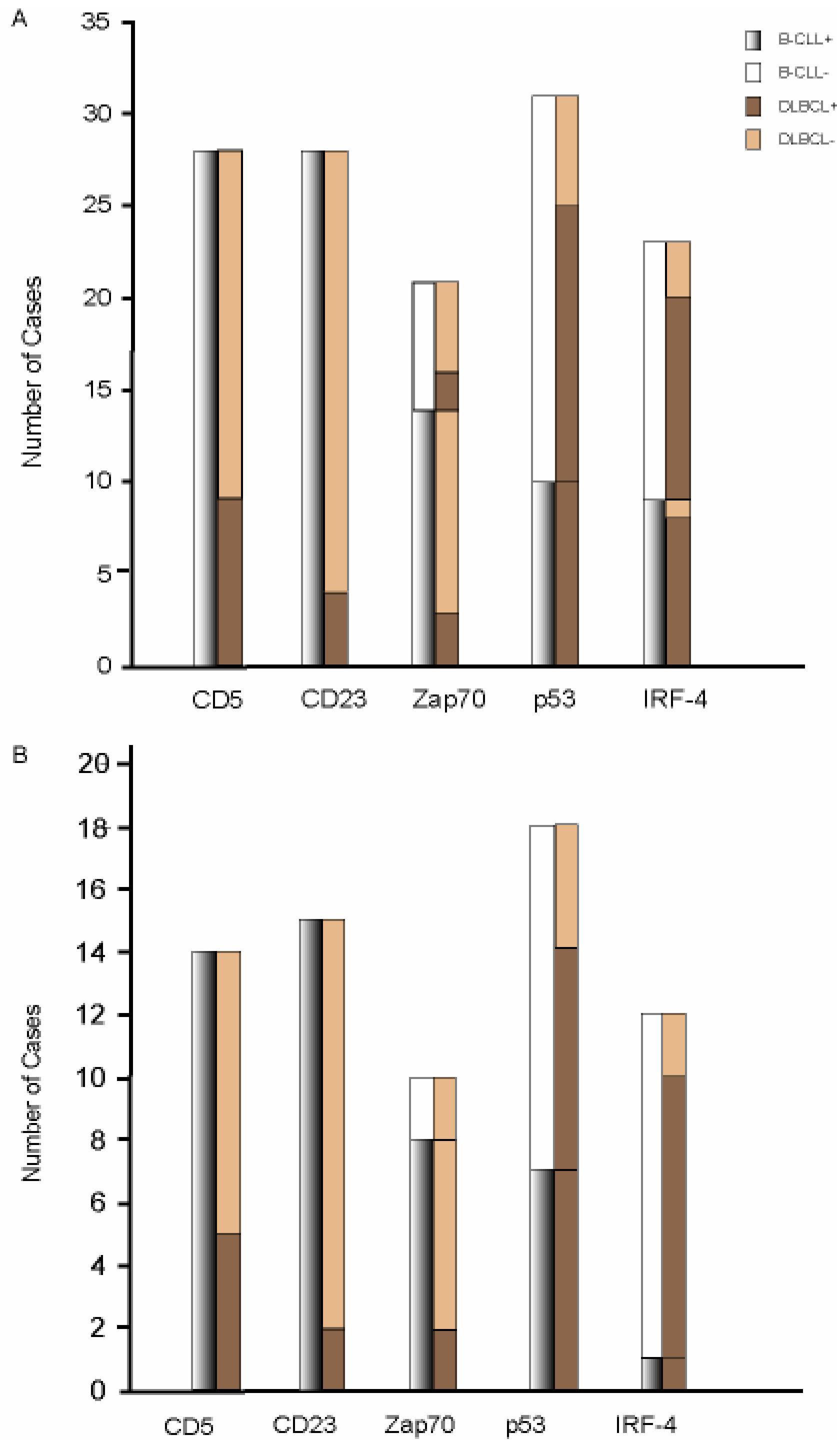


Table 4: Classical Richter's Syndrome: Molecular Analysis of B-CLL and DLBCL

No.	Sex	Diagnosis	IHC	Age at Diagnosis	Localization	IgVH gene	Homology(%)	Clonality
1	male	B-CLL	+	63	LN (retroperit.)	VH3-74	99	Related
		DLBCL	+	63	LN (retroperit.)	VH3-74	99	
2	male	B-CLL	+	57	LN (supraclav.)	VH3-74	98	Related
		DLBCL	+	57	LN (cervical)	VH3-74	98	
3	male	B-CLL	+	65	LN; spleen	VH3-9	99	Related
		DLBCL	+	66	LN (axilla)	VH3-9	99	
4	male	B-CLL	+	76	LN (axilla)	VH1-2	99	Related
		DLBCL	+	76	LN (axilla)	VH1-2	99	
5	male	B-CLL	+	69	BM	VH3-15	98	Related
		DLBCL	+	70	LN	VH3-15	98	
6	female	B-CLL	+	69	BM	VH4-39	98	Related
		DLBCL	+	69	BM	VH4-39	98	
7	female	B-CLL	+	67	BM,spleen	VH1-69	99	Related
		DLBCL	+	68	LN (cervical)	VH1-69	99	
8	female	B-CLL	+	54	BM	VH1-2	99	Related
		DLBCL	+	54	BM	VH1-2	99	
9	male	B-CLL	+	78	LN (axilla)	VH3-66	98	Related
		DLBCL	+	78	LN (axilla)	VH3-66	98	
10	male	B-CLL	+	77	LN (cervical)	VH3-23	98	Related
		DLBCL	+	77	LN (cervical)	VH3-23	98	
11	male	B-CLL	+	71	Tonsil	VH3-21	98	Related*
		DLBCL	+	71	Tonsil	No data	No data	
12	male	B-CLL	+	60	LN (axilla)	VH3-23	97	Related*
		DLBCL	+	60	LN (axilla)	No data	No data	
13	male	B-CLL	+	69	LN (mesent.)	VH3-11	92	Related
		DLBCL	+	69	Duodenum	VH3-11	92	
14	male	B-CLL	+	63	LN (cervical)	VH3-30	90	Related*
		DLBCL	+	63	LN (cervical)	No data	No data	
15	male	B-CLL	+	74	LN (stomach)	VH3-21	85	Not related
		DLBCL	+	74	Stomach	VH3-74	98	
16	male	B-CLL	+	74	LN (cervical)	VH3-9	98	Not related
		DLBCL	+	78	Colon	VH1-24	83	
17	female	B-CLL	+	45	BM	VH3-23	94	Not related
		DLBCL	+	45	LN (cervical)	VH2-5	99	
18	male	B-CLL	+	59	BM	VH3-48	95	Not related
		DLBCL	+	62	LN	VH4-39	98	
19	female	B-CLL	+	71	BM	VH3-21	95	Not related
		DLBCL	+	71	BM,skin	VH3-23	95	

20	male	B-CLL	+	68	LN (pericolic)	VH3-9	98	No data
		DLBCL	+	68	Colon	No data	No data	
21	male	B-CLL	+	86	BM	No data	No data	Related*
		DLBCL	+	86	LN (axilla)	VH1-69	98	
22	male	B-CLL	-	62	BM	No data	No data	No data
		DLBCL	-	62	LN	VH4-61	98	
23	male	B-CLL	-	59	Spleen	No data	No data	No data
		DLBCL	-	59	Liver	VH4-39	98	
24	male	B-CLL	+	66	LN (perigastric)	VH3-49/VH4-61	85/89	Related*
		DLBCL	+	66	Stomach	No data	No data	
25	female	B-CLL	+	66	LN (supraclav.)	No data	No data	Related*
		DLBCL	+	66	LN (supraclav.)	No data	No data	
26	male	B-CLL	+	67	LN (inguinal)	No data	No data	Related*
		DLBCL	+	67	LN (inguinal)	No data	No data	

*Comparison of the CDR3 regions of IgVH genes

Table 5: Relationship between ZAP70, p53 and IRF-4 expression in B-CLL of Classical Richter's Syndrome

		ZAP70 +	ZAP70 -	Total
p53	+	4	6	10
	-	14	3	17
	Total	18	9	27
		NS		

		ZAP70 +	ZAP70 -	Total
IFR-4	+	5	4	9
	-	7	4	11
	Total	12	8	20
		NS		

		p53 +	p53 -	Total
IFR-4	+	3	6	9
	-	5	9	14
	Total	8	15	23
		NS		

Fisher's exact test
NS: No significance

3.1.3 Results of PCR-GeneScan Analysis and Clonal Relationship of B-CLL and DLBCL

The results are summarized in Table 4. Monoclonal rearrangement of the IgVH gene was demonstrated in 84.6% (22/26 cases) and 80.8% (21/26 cases) of B-CLL in classical Richter's syndrome using the FR3A and FR2A PCR-GeneScan assays, respectively. Regarding DLBCL cases in classical Richter's syndrome, the FR3A assay detected clonal IgVH gene rearrangements in 24/26 cases (92.3%), and the FR2A assay in 19/26 cases (73.1%) (Figure 5).

Following the PCR-based GeneScan assay, the exact size of the CDR3 or CDR2 fragments of the IgVH genes in B-CLL and DLBCL were compared. The bands of the CDR3 fragments in B-CLL and DLBCL had the same size in 17/26 cases (65.4%) and the size was different in 4/26 cases (15.4%). The remaining cases were not evaluable. Comparing the CDR2 fragment sizes of the two lymphoma components, the same size was observed in 11/26 cases (42.3%) and the size was different in 5/26 cases (19.2%) (Figure 6). Again, the remaining cases could not be evaluated.

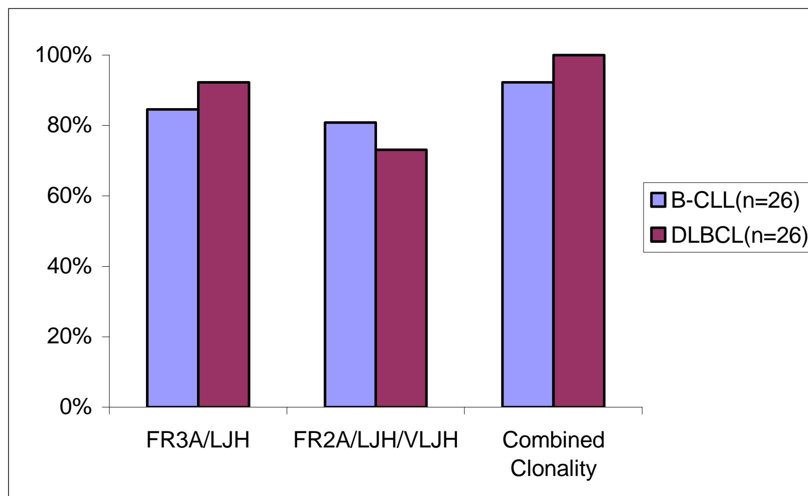


Figure 5: Clonal IgVH Gene Arrangements in Classical Richter's Syndrome

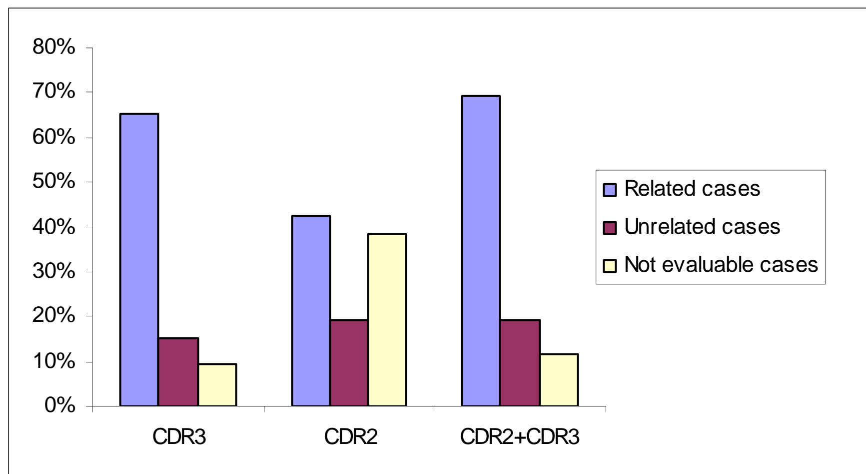


Figure 6: Relationship between B-CLL and DLBCL based on GeneScan Analysis of the IgVH Gene

3.1.4 IgVH Gene Sequencing Results

3.1.4.1 Clonality Analyses based on IgVH Gene Sequences

Nucleotide sequencing of the IgVH gene was performed in 26 samples. Among these cases, B-CLL and DLBCL showed the same nucleotide sequence of the CDR3 region in 7 cases and the same nucleotide sequence of the CDR2 region in 11 cases, whereas different nucleotide sequences of the CDR2 region were found in 5 cases (Figure 7). Only one lymphoma component (B-CLL or DLBCL) yielded nucleotide sequences of the IgVH genes in 3 cases (cases 20, 22 and 23, see Table 4). Therefore, in these cases clonal relationship could not be determined.

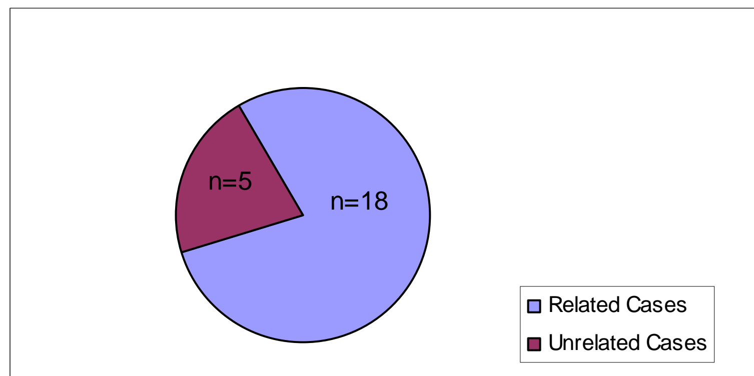


Figure 7: Clonal Relationship between B-CLL and DLBCL
Based on IgVH Gene Sequences in Classical Richter's Syndrome

3.1.4.2 IgVH Gene Usage in B-CLL and DLBCL

Nucleotide sequencing of the CDR2 region of the IgVH gene was performed in 21 samples of B-CLL and 19 samples of DLBCL. Compared to the closest germ line sequence in Genbank using IgBlast and Imgt (see Methods), VH3 family genes were found to be the most commonly used, followed by VH1 and VH4 family genes at somewhat lower frequencies. VH2 was used only once in DLBCL (Table 4, Figure 8). VH3-21, VH3-9 and VH3-23 were the most commonly used IgVH genes in B-CLL, whereas VH3-74 and VH4-39 were the most frequent used IgVH genes in DLBCL (Figure 9).

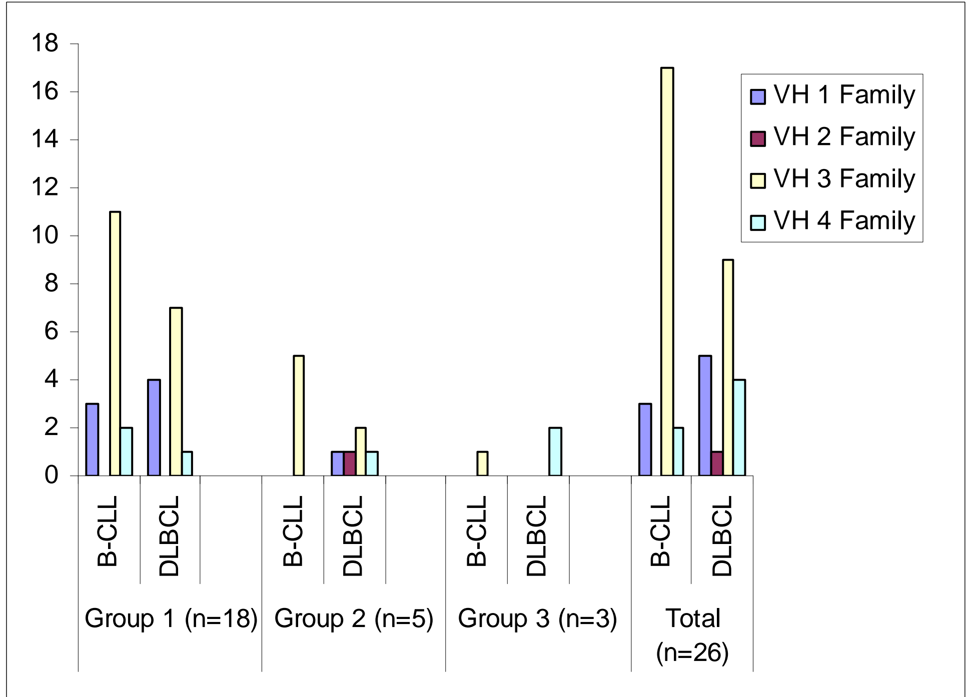


Figure 8: IgVH Gene Family in B-CLL and DLBCL in Classical Richter's Syndrome.
Group1: Clonally Related Cases; Group 2: Clonally Unrelated Cases; Group 3: Cases not Evaluated.

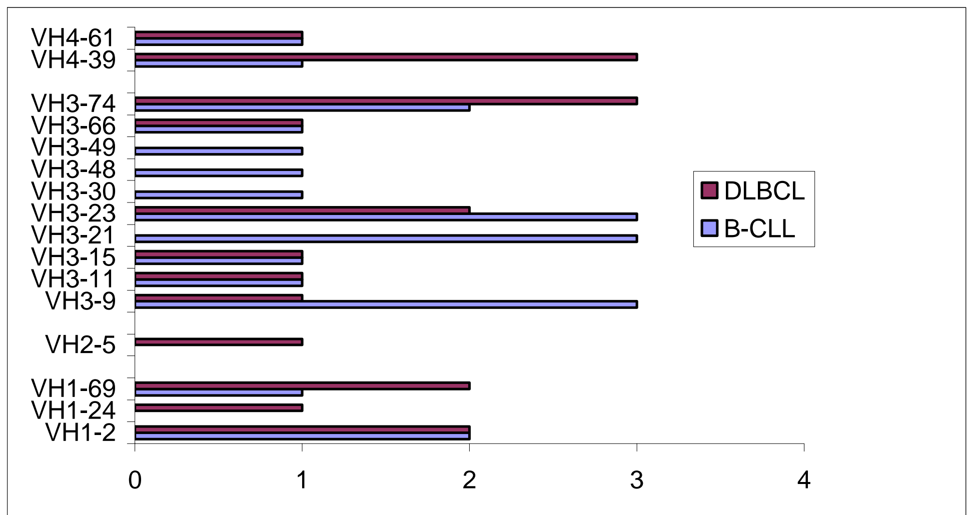


Figure 9: IgVH Gene Usage in B-CLL and DLBCL in Classical Richter's Syndrome

3.1.4.3 Mutational Status of IgVH Genes

Since the nucleotide sequence of the CDR3 region is too short to analyze the mutational status of the respective IgVH gene, this analysis could only be performed in 21 samples of B-CLL and 19 samples of DLBCL, in which the sequence of CDR2 region could be obtained. 61.9% (13/21 cases) of B-CLL cases and 84.2% (16/19 cases) of DLBCL cases carried IgVH genes that differed less than 2% from the most similar germ line IgVH gene (Table 4). Thus, these cases were considered to carry 'unmutated' IgVH genes.

Out of 18 clonally related cases, sequences of the CDR2 region were available in 15 samples of B-CLL and in 12 samples of DLBCL. Among clonally identical cases, 11 (73.3%) B-CLL patients carried unmutated IgVH genes and 4 (26.7%) B-CLL patients carried mutated IgVH genes. In contrast, in 5 cases with different IgVH gene sequences, 4 (80%) cases of B-CLL carried mutated IgVH genes (Table 4).

3.1.4.4 Relationship between IgVH Gene Status and Expression of ZAP70

Using Fisher's exact test, there were no significant relationships between the mutational status of the IgVH genes and the expression of ZAP70, p53 and IRF-4 in B-CLL with classical Richter's syndrome (Table 6).

Table 6: Relationship between IgVH gene mutational status, ZAP70, p53 and IRF-4 expression in classical Richter's syndrome.

		ZAP70					p53					IRF-4		
		+	-	Total			+	-	Total			+	-	Total
IgVH Gene	+	3	3	6	IgVH Gene	+	3	4	7	IgVH Gene	+	1	3	4
	-	10	1	11		-	5	9	14		-	2	7	9
	Total	13	4	17		Total	8	13	21		Total	3	10	13
		NS					NS					NS		

Fisher's exact test
NS: No significance
IgVH Gene (+): mutated IgVH gene; IgVH Gene(-): unmutated IgVH gene

3.1.5 Clinical Outcome

Follow-up data was obtained from 17 patients with classical Richter’s syndrome. The duration of B-CLL before DLBCL transformation varied from 1 to 115 months (mean: 53.5 months) and the survival time after DLBCL transformation varied from 1 to 47 months (median: 7 months).

Survival curves were plotted according to the Kaplan-Meier method (Figure 10). Survival analysis based on the IgVH gene mutational status did not show significant differences between mutated and unmutated B-CLL subgroups. Furthermore, there was no significant difference in overall survival between clonally related and unrelated groups of classical Richter’s syndrome. Likewise, no significant survival differences were detected between ZAP70-, p53- and IRF-4-positive and negative cases.

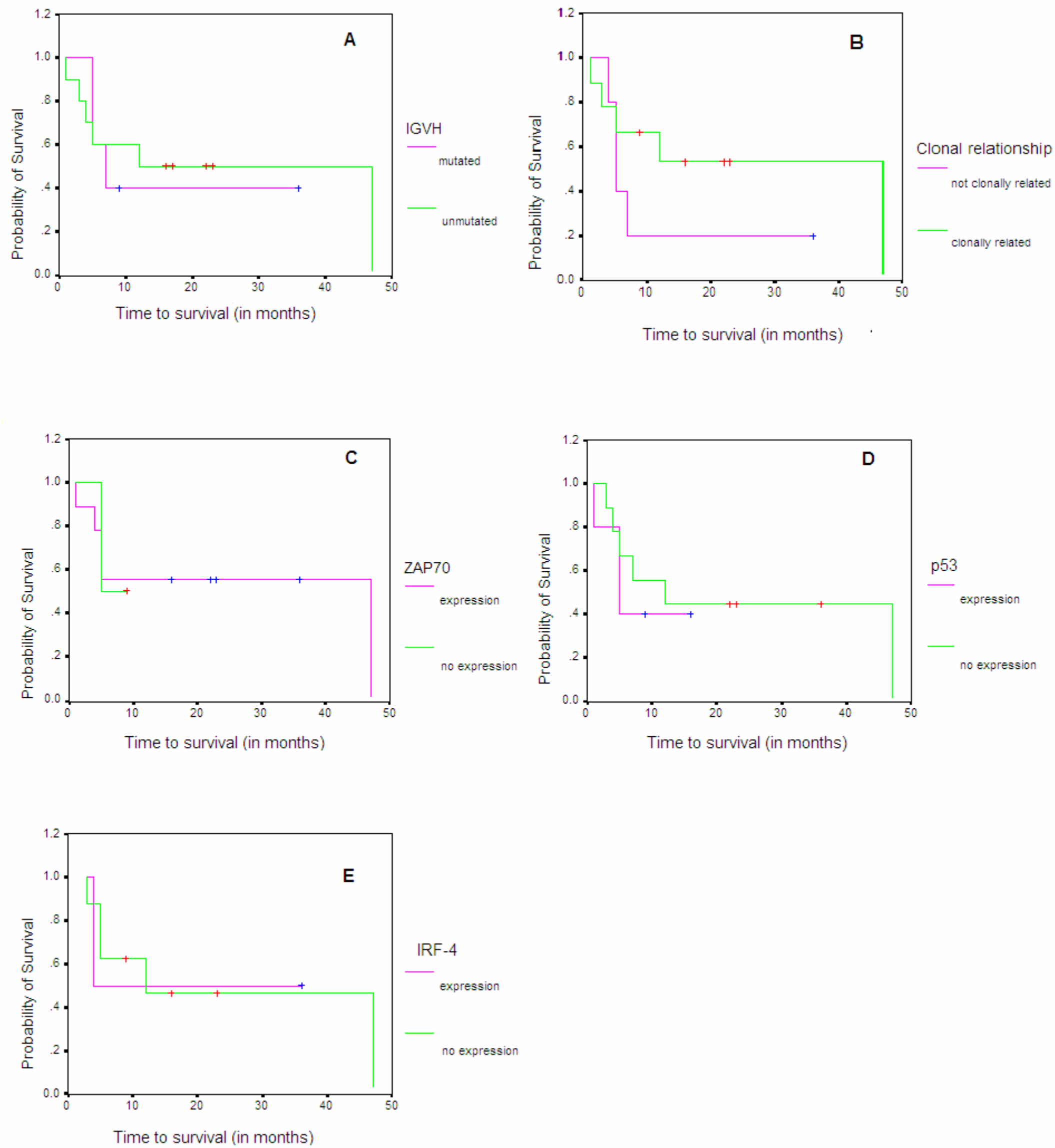


Figure 10: Survival analysis of patients with classical Richter's syndrome. No survival differences were observed between IgVH-mutated and unmutated cases (**A**), clonally related and unrelated cases (**B**), ZAP70-positive and –negative cases (**C**), p53-positive and –negative cases (**D**) and IRF4-positive and –negative cases (**E**).

3.2 Richter's Syndrome: B-CLL and Hodgkin's Lymphoma

3.2.1 Histology and Immunohistochemical Findings

Among 6 cases of Richter's syndrome with the Hodgkin's Lymphoma (HL) variant and 8 cases of B-CLL with scattered CD30-positive HRS-like cells, the proportion of males versus females was 1:1. The median age was 67 (range 54-74) and 63.5 years (range 59-96), respectively. All biopsies were taken from lymph nodes. Hodgkin/Reed-Sternberg (HRS) /HRS-like cells and corresponding B-CLL presented in the same anatomical sites (Table 8).

In 6 cases of Richter's syndrome with the HL variant, classical Hodgkin/Reed-Sternberg (HRS) cells were seen in a polymorphous background of small lymphocytes, epithelioid histiocytes, neutrophils, eosinophils, plasma cells and small blood vessels as features of classical HL (Figure 11B), whereas in additional 8 cases only CD30-positive HRS-like large cells were noted on the background of small B-CLL tumor cells (Figure 11C-F).

Immunophenotyping was performed in 4 cases of Richter's syndrome with the HL variant and in 8 cases of B-CLL with CD30-positive HRS-like cells. The results are given in Table 7. B-CLL cells expressed CD5, CD23 and CD20 in all 4 cases of Richter's syndrome with the HL variant and in all 8 B-CLL cases with HRS-like cells, whereas CD15, CD30, LMP1 and p53 were negative. All HRS and HRS-like cells expressed CD30, but were negative for CD5 and CD23. HRS cells were CD20 negative (4/4) and paritally positive for CD15 (2/4, 50%), LMP1 (2/4, 50%) and p53 (2/4, 50%). HRS-like cells expressed CD20 in 5/8 (62.5%) cases, p53 in 3/8 (37.5%) cases, CD15 and LMP1 in 2/8 (22.3%) cases. IRF-4 was expressed in the majority of HRS cells (3/4 cases, 75%) and HRS-like cells (5/8, 62.5 %,) (Table 7).

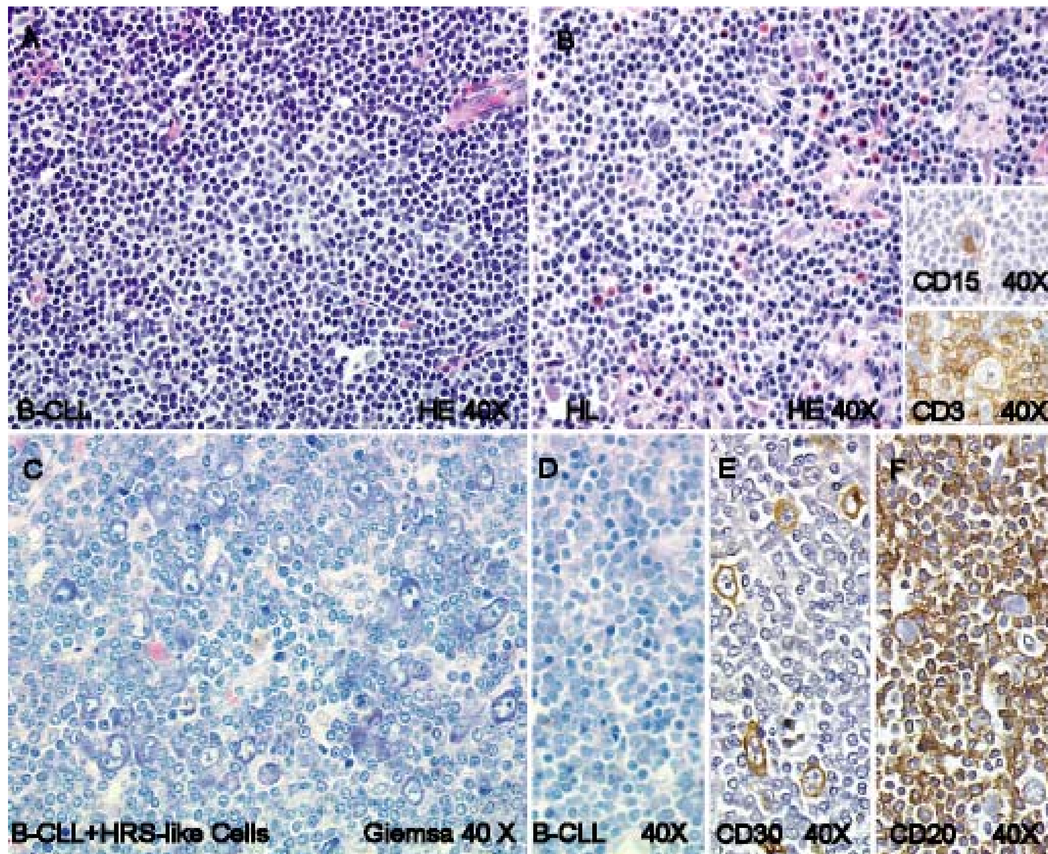


Figure 11: Morphology of Richter's syndrome with the HL variant (**A, B**) and B-CLL with CD30-positive HRS-like cells (**C- F**).

A and B: H&E staining of B-CLL (**A**) and HL (**B**) in one case. High magnification reveals typical HRS-cells expressing CD15 that are surrounded by CD3-positive T-cells (**B**).

C and D: Giemsa staining of HRS-like cells embedded in the background of small B-CLL cells. HRS-like cells are positive for CD30 (**E**) and are surrounded by CD20-positive B-CLL cells (**F**).

Table 7: Immunophenotype of B-CLL and HRS/CD30-positive HRS-like cells

Case	Diagnosis	CD5	CD20	CD23	CD15	CD30	LMP1	EBNA	ZAP70	p53	IRF-4
27	B-CLL	++	++	++	-	-	-	-	-	-	-
	Hodgkin's Lymphoma	-	-	-	++	+++	+	-	+	+	++
28	B-CLL	++	+	+	-	-	-	-	-	-	-
	Hodgkin's Lymphoma	-	-	-	-	++	++	-	-	+	++
29	B-CLL	++	++	++	-	-	-	-	-	-	-
	Hodgkin's Lymphoma	-	-	-	+	+	-	-	-	-	+
32	B-CLL	++	++	++	-	-	-	n.d.	-	-	n.d.
	Hodgkin's Lymphoma	-	-	-	-	+++	-	n.d.	-	-	n.d.
33	B-CLL	++	++	++	-	-	-	-	+	-	-
	HRS-like cells	-	+	-	-	++	+++	-	-	+	+
34	B-CLL	++	+++	++	-	-	-	-	n.e.	-	-
	HRS-like cells	-	-	-	-	+++	-	-	n.e.	+	+
35	B-CLL	++	++	+	-	-	-	-	-	-	-
	HRS-like cells	-	+	-	++	++	-	-	-	-	++
36	B-CLL	++	++	++	-	-	-	-	-	-	-
	HRS-like cells	-	-	-	+++	+++	++	-	-	+	++
37	B-CLL	++	++	++	-	-	-	-	+	-	+
	HRS-like cells	n.e.	+	n.e.	-	++	-	-	n.e.	-	n.e.
38	B-CLL	++	++	+	-	-	-	n.d.	n.d.	-	n.d.
	HRS-like cells	n.e.	++	n.e.	-	++	-	n.d.	n.d.	-	n.d.
47	B-CLL	+	+++	+	-	-	-	-	-	-	-
	HRS-like cells	-	-	-	-	+++	-	-	-	-	++
48	B-CLL	++	++	+	-	-	-	-	-	-	-
	HRS-like cells	n.e.	++	n.e.	-	+	-	-	-	-	-

n.d.: not done
n.e.: not evaluable

3.2.2 IgVH Gene Analysis of B-CLL in the HL and CD30-Positive HRS-like Variants of Richter's Syndrome

Polymerase chain reaction (PCR) and sequencing of the CDR2 region of the IgVH gene was successfully performed in 6 B-CLL cases of Richter's syndrome with the HL variant and in 6 cases of B-CLL with CD30-positive HRS-like cells.

All B-CLL cases with the HL variant carried VH3 and VH4 IgVH family genes (Figure 12). In these cases, VH3-48 (2/6, 33.3%) and VH4-34 (2/6, 33.3%) were predominantly found. In the cases of B-CLL with a CD30-positive HRS-like cells, the VH3 family was the most commonly used IgVH gene (4/6 cases) followed by the VH4 family (Figure 12).

In most cases, B-CLL cells carried mutated IgVH genes (5/6 cases with the HL variant and 5/6 cases in the CD30-positive HRS-like variant).

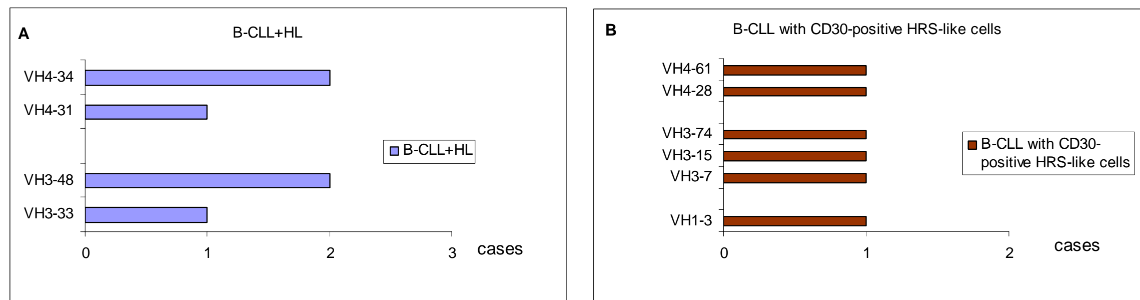


Figure 12: IgVH gene usage of B-CLL cells with the HL variant (**A**) and in the CD30-positive HRS-like variant (**B**)

Table 8: Richter's syndrome: Molecular analysis of B-CLL and Hodgkin's lymphoma (HL)/HRS-like cells

No.	Sex	Diagnosis	IHC	Age	Localization	Ig VH gene	Homology (%)	Clonality
27	male	B-CLL+HL	+	54	LN (axilla)	VH4-31	95	Not Related
28	male	B-CLL+HL	+	67	LN (cervical)	VH3-48	95	Not Related
33	male	B-CLL+HRS-like cells	+	63	LN	VH3-15	100	Not Related
34	male	B-CLL+HRS-like cells	+	64	LN	VH3-7	93	Related
29	female	B-CLL+HL	+	67	LN (mediastinal)	VH4-34	93	No data
30	female	B-CLL+HL	-	67	LN (mediastinal)	VH4-34	92	No data
31	female	B-CLL	-	74	BM	VH3-48	95	
		B-CLL+HL	-	81	LN	VH3-48	95	No data
32	male	B-CLL+HL	+	67	LN	VH3-33	99	No data
35	female	B-CLL+HRS-like cells	+	96	LN	VH4-61	91	No data
36	male	B-CLL+HRS-like cells	+	59	LN (cervical)	VH3-74	95	No data
37	female	B-CLL+HRS-like cells	+	89	LN	VH1-3	97	No data
38	female	B-CLL+HRS-like cells	+	73	LN (inguinal)	VH4-28	91	No data

3.2.3 Immuno-Laser Capture Microdissection and Clonality Analysis in the HL variant of Richter's Syndrome

Sequencing of the CDR3 region of the IgVH gene in HRS cells or in CD30-positive HRS-like cells was successfully performed in 4 B-CLL cases with CD30-positive large cells after the application of immuno-laser capture microdissection and seminested-PCR (Figure 13).

Both Richter's syndrome cases with B-CLL and the HL variant were clonally unrelated as was one case with B-CLL and CD30-positive HRS-like cells. However, in one case, CD30-positive HRS-like cells were clonally identical to the B-CLL cells (case 34).

Interestingly, in our three clonally distinct cases HRS or HRS-like cells expressed LMP1, whereas in the identical case HRS-like cells did not express LMP1(Table 7).

3.2.4 Clinical Outcome

Follow-up data were obtained from 3 patients with Richter's syndrome with the HL variant and 4 patients with B-CLL with CD30-positive HRS-like cells. Among B-CLL patients with HL transformation, one patient died after 12 months following the diagnosis of Richter's syndrome and 2 patients were alive after 15 and 28 months, respectively. Among patients with B-CLL and CD30-positive HRS-like cells, one patient died after 1 month and the remaining patients were alive after 14, 52 and 73 months.

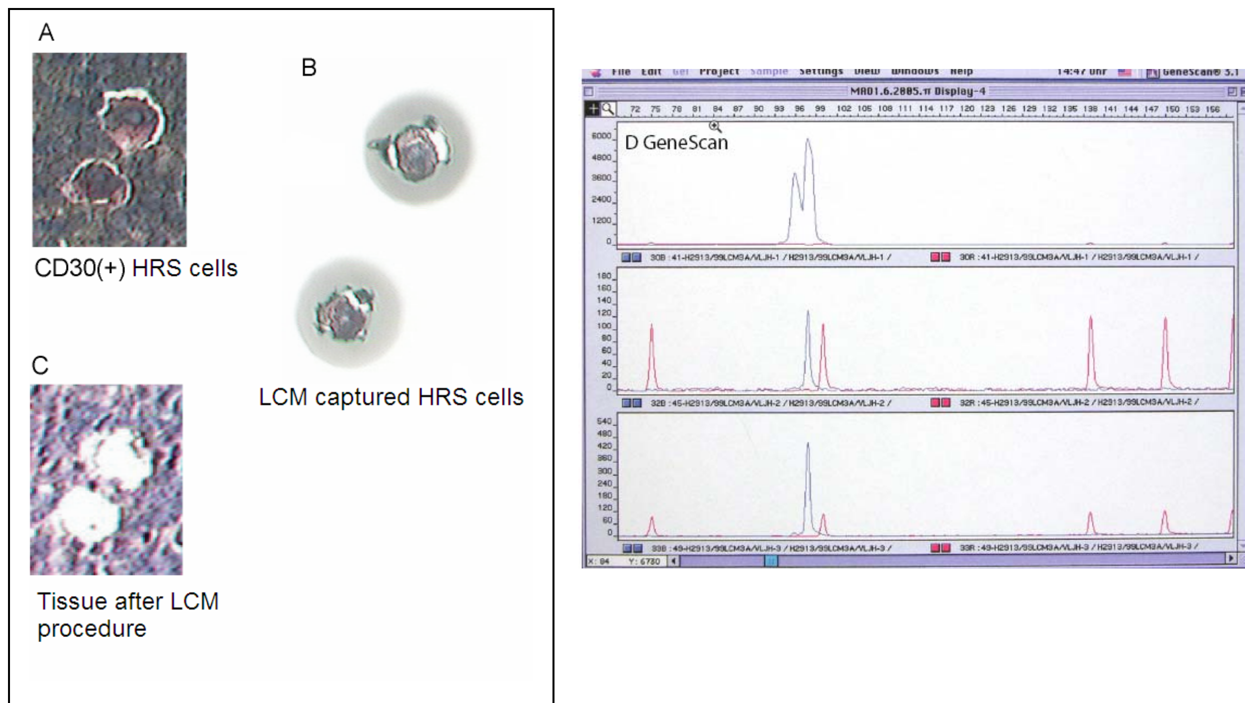


Figure 13: Immuno-laser capture microdissection (LCM): CD30 immunostaining of HRS cells (**A**), two HRS cells captured by PixII (**B**), tissue section after LCM of HRS cells (**C**) and GeneScan for the bands of semi-nested PCR of the CDR3 region of the IgVH gene (**D**).

3.3 Summary of Clinical Data in Richter's Syndrome Patients

Considering all 40 patients with Richter's syndrome that were included in the current study, the male to female ratio was 2.1:1; the median age at diagnosis was 67 years and the median survival time was 10.5 months. Among 34 patients with classical Richter's syndrome, the ratio of males to females was 2.4:1 with a median age at diagnosis of 66 years and a median survival time of 7 months. In 6 patients with the HL variant of Richter's syndrome and additional 8 patients with B-CLL and CD30-positive HRS-like cells, the male to female ratio was 1:1 and the median age at diagnosis was 67 and 63.5 years, respectively. Median survival was 21 and 33 months, respectively. There was no statistically significant difference in survival time between the two subtypes of Richter's syndrome (Table 9).

The IgVH mutation status was associated with the expression of ZAP70 ($p<0.05$), whereas no significant relationship was detected between IgVH mutational status and p53 or IRF-4 expression in B-CLL cells when all Richter's syndrome patients were considered. Moreover, there was no statistical correlation between p53, ZAP70 and IRF-4 expression (Table 10). Considering all Richter's syndrome patients, there was no statistical survival difference between IgVH-mutated and unmutated cases or between clonally related and unrelated cases. The ZAP70, p53 and IRF-4 status also had no impact on the clinical survival (Figure 14).

Table 9: Clinical characteristics in different subtypes of Richter's syndrome.

Subtype	M:F	Age at Diagnosis	Range	Survival Time (Months)	Range
B-CLL+DLBCL/HL(n=40)	27:13	67	25-86	10.5	1-47
B-CLL+DLBCLL(n=34)	24:10	66	25-86	7	1-47
B-CLL+HL(n=6)	3:3	67	54-74	21	15-28
B-CLL+CD30(+)HRS-like cells(n=8)	4:4	63.5	59-96	33	1-73

Table 10: Relationship between the IgVH gene mutational status and ZAP70, p53 and IRF-4 expression in all Richter's syndrome patients

		ZAP70					p53					IRF-4		
		+	-	Total			+	-	Total			+	-	Total
IgVH Gene	+	3	8	11	IgVH Gene	+	3	8	11	IgVH Gene	+	1	6	7
	-	10	2	12		-	4	10	14		-	2	7	9
	Total	13	10	23		Total	7	18	25		Total	3	13	16
		S					NS					NS		

		ZAP70					ZAP70					p53		
		+	-	Total			+	-	Total			+	-	Total
p53	+	4	6	10	IRF-4	+	5	4	9	IRF-4	+	3	6	9
	-	14	7	21		-	7	7	14		-	5	12	17
	Total	18	13	31		Total	12	11	23		Total	8	18	26
		NS					NS					NS		

Fisher's exact test (S: Significance $P < 0.05$; NS: No Significance)
IgVH Gene (+): Mutated IgVH gene; IgVH gene (-): unmutated IgVH gene

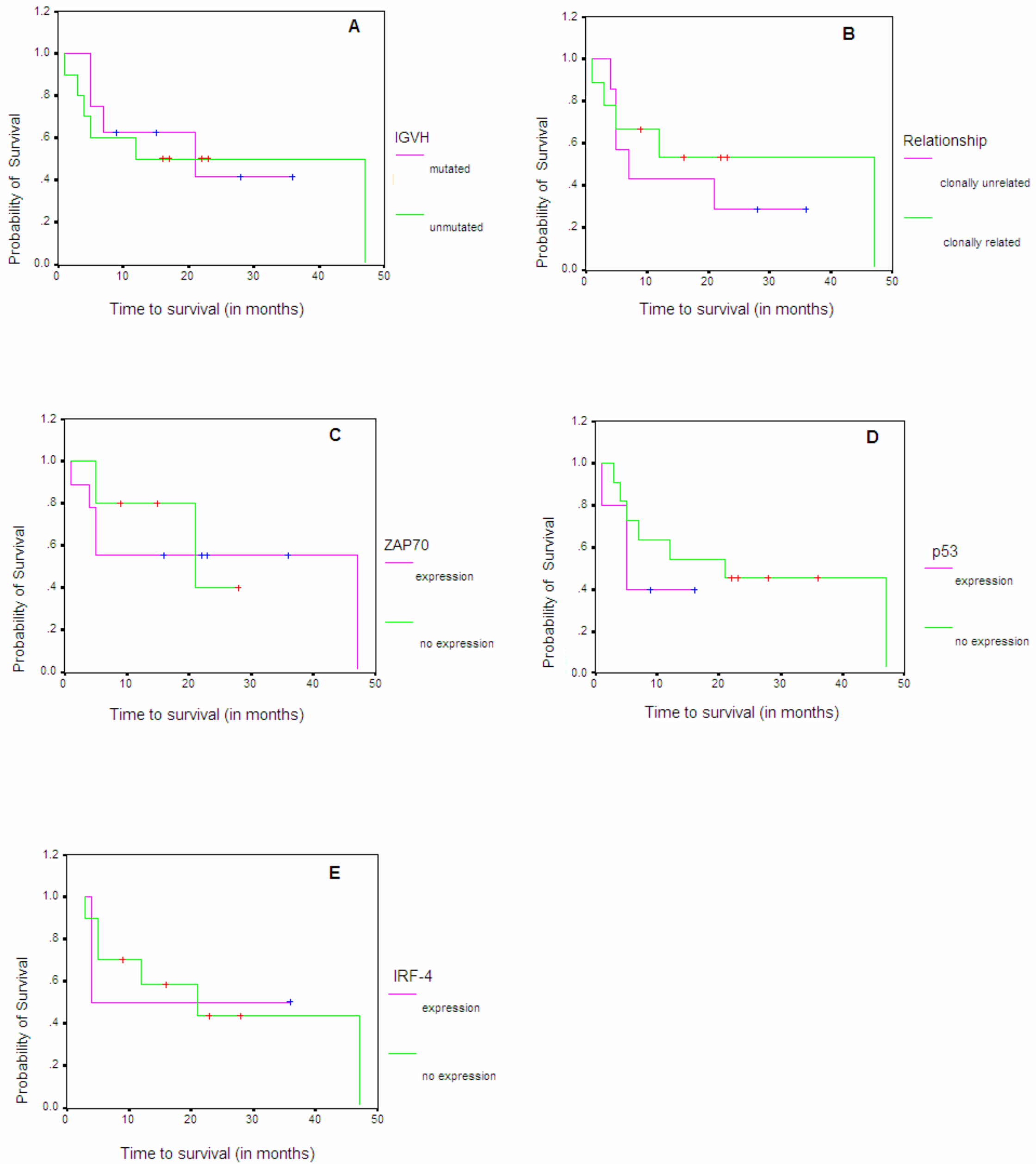


Figure 14: Survival analysis of all patients with Richter's syndrome. No survival differences were observed between IgVH-mutated and unmutated patients (**A**). Clonal relationship (**B**), ZAP70 expression (**C**), p53 expression (**D**) and IRF-4 expression (**E**) are also not associated with survival.

Chapter 4

Discussion

4.1 Classical Richter's Syndrome (B-CLL and DLBCL)

4.1.1 Morphological, Immunohistochemical and Clinical Findings in Classical Richter's Syndrome

In accordance with published literature, our results show that Richter's syndrome occurs more frequently in elderly people with a median age of 67 years, and with males more often affected than females (2.4:1). Both findings are similar to those found in conventional B-CLL (Jaffe 2001). It has been reported that patients who are younger than 55 years may have a 5-fold higher incidence of Richter's syndrome in comparison to patients older than 55 years, but it was claimed that this may be due to the fact that older patients with advanced and unresponsive disease are less likely to undergo tissue biopsies (5.9% vs. 1.2%; $P < .00001$) (Mauro, Foa et al. 1999). Our series included only five patients whose primary diagnosis of B-CLL was established before the age of 55 years; this difference may be explained with a potential referral bias to our institution, since a reference center for lymph node diseases is much more likely to receive B-CLL cases for consultation with predominant lymph node involvement which represent only a subset of all B-CLL cases.

In our study, DLBCL as part of classical Richter's syndrome was diagnosed most frequently in lymph nodes (20/34) and the gastrointestinal (GI) tract (5/34). Other localizations included bone marrow (4/34), skin (2/34), liver (1/34) and tonsil (3/34) (see Table 4). Previous reports about the localization of Richter's syndrome also included the central nervous system (CNS)(Agard, Hamidou et al. 1999; Robak, Gora-Tybor et al. 2004; Schmid, Diem et al. 2005), eye (Hattenhauer and Pach 1996), testis (Houdelette, Dumotier et al. 1989), and lung or kidney (Foucar and Rydell 1980).

It has been suggested that Richter's syndrome occurring in the gastrointestinal tract may be clinically different from Richter's syndrome at other localizations, since a superior survival time (median survival of 22 months) was reported (Parrens, Sawan et al. 2001). This observation is confirmed in our study, in which the median survival of the five Richter's syndrome cases involving the GI-tract was markedly better as compared to cases of other localizations (28 months vs. 5 months). A possible explanation is that DLBCL cases occurring in the GI-tract may show an extended period of localized involvement only thus enabling complete surgical resection (Parrens, Sawan et al. 2001). Conflicting data exists about the clonal relationship of B-CLL and DLBCL in cases involving the GI-tract. In an initial study investigating 4 cases of Richter's syndrome in the stomach, B-CLL and DLBCL were clonally different in 3 cases, while no data could be obtained from the fourth case (Ott, Ott et al. 1995). This finding led to the conclusion that Richter's syndromes in the GI-tract may represent true secondary neoplasms in the majority of cases. However, clonally related cases were also reported recently (Parrens, Sawan et al. 2001), and this is in line with our observations. In our series, two out of five patients with Richter's syndrome involving the GI-tract carried the same monoclonal rearrangement of IgVH gene in the B-CLL and DLBCL as demonstrated by PCR analysis and subsequent sequencing. In contrast, two samples showed a different rearrangement of the IgVH gene, whereas in the fifth tumor sample only the B-CLL component could be successfully analyzed leaving the clonal relationship to the DLBCL component unresolved (see Table 4, case 20). In summary, accumulating evidence suggests that cases of Richter's syndrome involving the GI-tract can be clonally related or unrelated to the preceding B-CLL.

Very few cases of Richter's syndrome in the literature have been thoroughly characterized morphologically and immunophenotypically and this prompted us to investigate these features in greater detail. In Richter's original report (Richter 1928), DLBCL cases showed immunoblastic features and it appears from sparse reported data that the occurrence of immunoblastic morphology may be more frequent than observed in *de novo* DLBCL cases, in which the frequency of immunoblastic morphology is around 11% (Diebold, Anderson et al. 2002). We were able to characterize 32 of 34 DLBCL

cases morphologically, and our data clearly supports the notion that immunoblastic morphology is more frequent than among *de novo* DLBCL. In particular, 6 of our cases were of immunoblastic subtype (19%), while the remainder showed centroblastic morphology. Considering only DLBCL cases that demonstrated clonal evolution from the preceding B-CLL, the frequency of immunoblastic morphology increased to 38% (5/13 cases). We therefore conclude that immunoblastic morphology is indeed more prevalent in DLBCL cases of Richter's syndrome as compared to *de novo* DLBCL.

Since immunohistochemical data is also sparse in the literature of Richter's syndrome we applied a broad immunohistochemical panel in those cases with sufficient material available. In previously published series including low numbers of patients it was suggested that CD5 and CD23 expression is frequently lost in DLBCL cases of Richter's syndrome. CD5 and CD23 are classical markers of B-CLL that distinguish this B-cell malignancy from other indolent B-cell lymphomas. CD5 is a signal transduction molecule present on the surface of most thymocytes and mature peripheral T-cells. It is also detectable in a small subset of circulating B-cells and is typically positive in B-CLL cells. CD23, also present on the surface of B-CLL cells, is a membrane protein that usually exhibits weak binding of IgE and regulates cytokine release from monocytes.
In our series, 28 cases of Richter's syndrome were evaluable for CD5 and CD23 expression. While CD5 and CD23 were detectable in all 28 cases of B-CLL, DLBCL cases showed expression of CD5 in 32% and of CD23 in 14% only. Importantly, these frequencies were not substantially different between clonally related and unrelated cases.
We next addressed the question if CD5-positive DLBCL were clinically different from CD5-negative DLBCL in Richter's syndrome. Among nine DLBCL cases with CD5-expression, the ratio of male to female patients was 1:3, and the median age at the diagnosis of Richter's syndrome was 65.9 years (range: 45-83). Two patients died 1 and 5 months after diagnosis, and two patients were alive after 22 and 23 months, respectively. From the remaining patients, clinical follow-up was not available. In our series, survival times were not different between CD5-positive and CD5-negative DLBCL in Richter's syndrome suggesting that CD5 may not be useful as a marker that identifies patients with

a particularly good or poor clinical course. Although CD5-expression is present in a subset of DLBCL cases in Richter's syndrome, this feature can not be taken as an evidence of an underlying B-CLL, since CD5-expression is also observed in *de novo* DLBCL (Yamaguchi, Seto et al. 2002; Kong, Cho et al. 2004). Interestingly, the clinical characteristics of the CD5-positive DLBCL cases in Richter's syndrome in our series appear to be similar to those of *de novo* CD5-positive DLBCL which have been reported to be characterized by elderly onset, a female predominance and aggressive clinical features (Yamaguchi, Seto et al. 2002; Kong, Cho et al. 2004). However, on the molecular level, these two subgroups of CD5-positive DLBCL show marked differences. According to genetic analyses, the vast majority of *de novo* CD5-positive DLBCL have mutated IgVH genes and show similar underlying genetic alterations in comparison to mutated B-CLL cases suggesting similar progenitor cells in both entities (Katzenberger, Lohr et al. 2003). In our study, six cases of CD5-positive DLBCL in Richter's syndrome could be molecularly analyzed. Five cases were clonally identical to the corresponding B-CLL, which carried unmutated IgVH genes in four cases and mutated IgVH genes in one case. The finding of predominantly unmutated IgVH genes in these cases is in clear contrast to the findings in CD5-positive *de novo* DLBCL and suggests a different pathogenesis between these DLBCL subgroups.

CD23 expression was detected in only four DLBCL cases in our series. Three of these tumors carried unmutated IgVH genes regardless of whether they were clonally related to the preceding B-CLL or not. The clinical course of these patients was poor and three patients died one, three and five months after diagnosis, respectively. CD23 expression is also detected in a subset of *de novo* DLBCL (Calaminici, Piper et al. 2004), in which it was reported to be a prognostically favorable marker together with CD40 (Linderoth, Jerkeman et al. 2003). Although the number of patients is low, our results indicate that the biology and clinical behavior of CD23-positive DLBCL in Richter's syndrome might be different from that of *de novo* CD23-positive DLBCL.

ZAP70, a tyrosine kinase associated with T-cell receptor signaling, was recently identified as a surrogate marker for B-CLL cases with unmutated IgVH genes (Wiestner,

Rosenwald et al. 2003) and may therefore serve as a good prognostic marker in this disease. While ZAP70 expression has been widely investigated in conventional B-CLL, no data exists so far in the literature regarding ZAP70 expression in B-CLL and DLBCL cases in Richter's syndrome. In our series, immunohistochemistry revealed ZAP70 expression in 66.7% cases of B-CLL (14/21 cases, see Figure 3) in classical Richter's syndrome. However, no statistically significant relationship between ZAP70 expression and the IgVH gene mutational status was found in these cases (see Table 6). In addition, no survival differences were detected between B-CLL subgroups with and without ZAP70 expression. These findings are not surprising given the highly selected study of patients with Richter's syndrome, in which the transformation event is likely to override any prognostic effect that ZAP70 may exhibit in conventional B-CLL. However, considering all cases of Richter's syndrome including the HL variant of Richter's syndrome, the relationship between ZAP70 expression and unmutated IgVH genes was significant ($P<0.05$) (see Table 10). Whether ZAP70 expression may serve as a marker of unmutated IgVH genes in B-CLL cases of Richter's syndrome, will have to be investigated in future larger series.

It is interesting to note that ZAP70 expression was detected by immunohistochemistry in six DLBCL cases of Richter's syndrome in our series (see Figure 3) which is in contrast to published series of conventional DLBCL (Admirand, Rassidakis et al. 2004; Carreras, Villamor et al. 2005). Five of these cases carried unmutated IgVH genes, but it should be mentioned that the ZAP70 expression was usually weaker as compared to ZAP70-positive B-CLL cases.

The role of the tumor suppressor p53 has been widely studied in B-CLL and alterations of p53 as detected by immunohistochemistry or on the genetic level have been associated with adverse clinical behaviour (Dohner, Fischer et al. 1995; Cordone, Masi et al. 1998). p53 is activated by DNA damage and induces cell cycle arrest and apoptosis. In case of p53 deficiency, either by mutation or genetic deletion, sensoring of DNA damage in affected cells is dysfunctional and genetic alterations are allowed to accumulate (Lane 1992). p53 mutations have been found in approximately 50% of human malignancies,

thus representing the most frequent genomic abnormality in human cancer (Hollstein, Sidransky et al. 1991). Since most of the p53 gene mutations result in an abnormally prolonged half-life of the p53 protein, increased immunohistochemical expression of the p53 protein has been used as a surrogate marker for underlying p53 mutations (Bartek, Bartkova et al. 1991). In B-CLL, p53 expression or mutations were reported to occur at a frequency of 10-15%, and these events are strongly associated with advanced clinical stage, progressive disease, poor response to therapy, and short survival (Cordone, Masi et al. 1998; Giles, Bekele et al. 2003). As expected based on the selective study of Richter's syndrome cases, the frequency of p53 protein expression in B-CLL in our series was higher (32.3%) than in conventional B-CLL cases. Since the occurrence of Richter's syndrome in general is associated with short survival times, we did not observe a significant difference in survival between p53-negative and p53-positive B-CLL cases (see Figure 14). Strikingly, high p53 expression was detected in 80.6% of our DLBCL cases in Richter's syndrome. When considering only the 18 clonally related cases, p53 expression in the DLBCL components was observed in a similar frequency (see Figure 4). We therefore conclude that alterations of the tumor suppressor p53 may play an important role in B-CLL cases that show a clonal transformation to DLBCL in Richter's syndrome. The finding that p53 expression is also observed in the vast majority of DLBCL that occur as a secondary, clonally not related neoplasm points to an important role of the p53 pathway also in these cases.

We decided to study the expression of IRF-4, a member of the interferon regulatory factor (IRF) family, in our cases of Richter's syndrome, since IRF-4 expression had been suggested as an unfavourable prognostic factor in B-CLL (Ito, Iida et al. 2002). IRF-4 is known to play a role in the control of B-cell proliferation and differentiation and cooperates with PU.1, STAT6 and Blimp-1 (Iida, Rao et al. 1997; Brass, Zhu et al. 1999; Gupta, Jiang et al. 1999; Gupta, Anthony et al. 2001). IRF-4 is mainly expressed in plasma cells, but also in a small subset of germinal center B-cells in normal lymphoid tissue. In lymphomas, IRF-4 is strongly expressed in lymphoplasmacytoid lymphoma, multiple myeloma, HRS cells in HL. In line with the fact that IRF-4 is a marker of postgerminal center B-cell derivation, IRF-4 expression is not detected in mantle cell

lymphoma (MCL) or follicular lymphoma (FL)(Falini, Fizzotti et al. 2000; Tsuboi, Iida et al. 2000). In DLBCL, IRF-4 expression is strongly associated with the activated B-like (ABC-like) subtype and predicts poorer outcome as compared to germinal center B-like (GCB-like) subset (Berglund, Thunberg et al. 2005).

We confirmed the heterogeneous expression pattern of IRF-4 in our B-CLL cases, in which IRF-4 expression was detected immunohistochemically in 39.1% of cases. In this series, IRF-4 expression was not significantly associated with the IgVH mutational status or survival time (see Table 6, Figure 10), but, again, our series consisted of cases of Richter's syndrome exclusively and did not include conventional B-CLL cases. Interestingly, 82.6% of DLBCL cases in our series showed IRF-4 expression (see Figures 3 and 4), which is a much higher frequency as compared to *de novo* DLBCL. This may indicate that the majority of DLBCL cases in Richter's syndrome belong to the ABC-like DLBCL category which may explain, at least in part, the inferior survival of these patients.

In conclusion, DLBCL in classical Richter's syndrome have a tendency to lose the expression of the B-CLL markers CD5 and CD23 despite of their frequent clonal relationship. Their high rate of p53 and IRF-4 expression may indicate that DLBCL in Richter's transformation are molecularly distinct from conventional, *de novo* DLBCL.

4.1.2 78% of B-CLL and DLBCL in Richter's Syndrome Are Clonally Identical

Various PCR approaches have been used in the past to demonstrate a monoclonal B-cell population in lymphoid malignancies. The detection rate of monoclonal B-cells in lymphoma specimens shows considerable variation, depending on the lymphoma entity, the PCR strategy employed and the available tissue (paraffin-embedded vs. fresh frozen tissue) (Diss, Pan et al. 1994). Diss et al. (Diss, Pan et al. 1994) reported equivalent detection rates (81%) of clonal B-cell populations in paraffin embedded tissue sections and fresh frozen material when using FR3A primers. However, for the FR2A assay the

success rate dropped considerably in paraffin-embedded tissues (67%) as compared to fresh frozen tissue specimens (86%) (Diss, Pan et al. 1994). The main reason for the significantly lower detection rate with the FR2A assay in paraffin-embedded material is the severe degradation of DNA by the fixation process in paraffin-embedded tissue. While the length of the PCR amplicon in the FR3A assay is between 70 and 120bp, the FR2A assay produces amplicons between 220 and 280bp. Degraded DNA may therefore amplify well in the FR3A assay, but may lack sufficient length to produce results in the FR2A assay. Essop et al. (Essop, Blakolmer et al. 1997) published a series of 38 low grade and 31 high grade lymphomas in which they applied the FR2A and FR3A assays in paraffin-embedded lymphoma tissues. Combining the results for both assays, the success rate was 89% for low grade B-cell lymphomas and 97% for high grade lymphomas. However, in other studies the detection rate of monoclonal B-cell populations in lymphoma specimens was lower (Diss, Peng et al. 1993; Lombardo, Hwang et al. 1996). In DLBCL, the detection of clonal rearrangements of the IgVH genes varied from 17% to 70% with the FR3A assay in these studies. The low detection rate of monoclonal IgVH gene rearrangements in DLBCL in some studies may be attributed to the presence of extensive somatic hypermutation and unusual rearrangements of the IgVH gene locus, such as inversions or minimal deletions. DLBCL and FL which are considered to originate from germinal center or post-germinal center B-cells often carry a high load of somatic hypermutations which may affect binding of the FR2A and FR3A primers.

The results from our study are summarized in Figure 5 and are in line with previous reports. It is noteworthy that the detection rate of monoclonal rearrangements of the IgVH genes was high in our series in both, B-CLL and DLBCL. This may be due to the utilization of both the FR2A and FR3A assays, the sensitive GeneScan method and the high rate of unmutated IgVH genes in the B-CLL and DLBCL specimens in our series (see below).

As discussed in the Introduction section, there are different methods to detect whether the DLBCL is clonally related to the pre-existing B-CLL in Richter's syndrome. Comparing these different methods (immunoglobulin light chain detection by immunohistochemistry,

Southern hybridization of the rearranged heavy or light chains, PCR-based GeneScan and sequencing), matched CDR3 regions of IgVH genes in both components are considered as the most convincing proof of clonal relationship, since every B-cell clone is characterized by a unique sequence in this genomic region.

Since the precise length of the PCR product can be calculated and analyzed by the GeneScan software, same-sized bands in the FR2A or FR3A assays in both the B-CLL and DLBCL suggest clonal relationship. In our study, utilizing GeneScan results for the CDR3 region, same-sized bands in the two components were found in seventeen cases, and different bands were found in four cases. In the GeneScan analysis of the CDR2 region, 16 cases yielded results, and eleven cases showed same-sized bands, while different sized bands were detected in five cases. All of the obtained PCR products were subjected to sequencing analysis of the IgVH gene. In our cohort, 78% of B-CLL cases in classical Richter's syndrome showed clonal progression to DLBCL as indicated by same-sized PCR products and identical sequences in both components. In contrast, in 22% of classical Richter's syndrome cases the DLBCL did not demonstrate clonal relationship to the preceding B-CLL and should therefore be considered as a secondary neoplasm (see Figures 6 and 7).
Clinically, there was no statistical difference between clonally related and unrelated Richter's syndromes in terms of survival (Figure 10), but the number of evaluable cases was relatively low.

In conclusion, DLBCL in Richter' syndrome can either develop by clonal evolution from the preceding B-CLL or arise as an independent, secondary neoplasm. Our study comprises the largest collection of Richter's syndrome cases reported to date and suggests that more than 75% of DLBCL cases in Richter's syndrome are clonally related to the pre-existing B-CLL. In view of this finding, a major task in future studies will be the identification of markers in conventional B-CLL that predict likely transformation into DLBCL (Richter's transformation).

4.1.3 Clonal Transformation in Richter's Syndrome Occurs Predominantly in B-CLL with Unmutated IgVH Genes

The majority of clonally identical DLBCL cases (78%) in our cohort derived from B-CLL with unmutated IgVH genes. However, four clonally related cases carried mutated IgVH genes. While one of these cases showed a relatively low rate of mutations (97% homology with the respective germ line IgVH gene), the three remaining cases carried a high load of somatic mutations (89%, 90% and 92% homology, respectively). The finding that IgVH-mutated B-CLL can clonally transform into DLBCL in Richter's syndrome is in contrast to the report of Timar et al. who analyzed the mutational status of the IgVH genes in eight cases of classical Richter's syndrome. In their study, all five clonally identical DLBCL evolved from B-CLL tumors that carried unmutated IgVH genes suggesting that a clonal transformation from B-CLL to DLBCL may exclusively occur in the IgVH-unmutated subgroup of B-CLL (Timar, Fulop et al. 2004). In a very recent study, Smit et al. also reported that Richter's transformation occurs almost exclusively in IgVH-unmutated B-CLL (Smit, van Maldegem et al. 2006). However, the numbers of cases investigated in the previous two cohorts were small (n=8 and n=9, respectively) and, in line with reports of single cases (Cherepakhin, Baird et al. 1993; Nakamura, Kuze et al. 2000), we clearly provide evidence that IgVH-mutated B-CLL can show clonal transformation into DLBCL. After transformation, survival was not statistically different between IgVH-mutated B-CLL and IgVH-unmutated B-CLL cases (see Figure 10). The overall survival after transformation was 7 months in our series which is longer than 3-5 months as reported in the literature. However, when we excluded cases involving the gastrointestinal tract (based on the observation that these cases may be biologically and clinically distinct from other cases of Richter's syndrome), the survival in our cohort was also only 5 months.

The mutational status of the IgVH gene of a neoplastic B-cell is thought to reflect, at least in part, the developmental stage at which the neoplastic transformation occurred. B-cell lymphomas with somatically mutated IgVH genes may therefore develop from germinal center or post germinal center B-cells, whereas tumor cells that carry unmutated IgVH

genes are likely to originate from pre-germinal center B-cells (Kuppers, Klein et al. 1999). Initially, B-CLL was regarded as a homogeneous disease derived from naive B-cells. Based on the finding that B-CLL can carry either mutated or unmutated IgVH genes that showed dramatically different clinical behavior (Fais, Ghiotto et al. 1998; Hamblin, Davis et al. 1999), B-CLL was divided into two variants, one derived from unmutated, pre-germinal center B-cells with a poor clinical course, and one derived from mutated, post-germinal center B-cells with a favorable clinical course. Gene expression profiling studies, however, demonstrated homogeneous gene expression patterns in both IgVH-mutated and IgVH-unmutated B-CLL (Wiestner, Rosenwald et al. 2003) supporting the idea that B-CLL should be considered one single entity. Biased usage of the IgVH genes in the IgVH-unmutated B-CLL subgroup suggests, however, that both B-CLL subgroups may be derived from antigen-experienced B-cells rather than from naïve B-cells in the case of IgVH-unmutated cases (Klein, Tu et al. 2001).

Almost as a dogma, *de novo* DLBCL carry mutated IgVH genes (Nakamura, Kuze et al. 1999). Based on microarray-generated gene expression profiles, two subtypes of DLBCL can be distinguished. The germinal center type (GCB type) of DLBCL shows similarity in gene expression with non-neoplastic germinal center B-cells, while the activated B-like type (ABC type) shows features of in vitro-activated B-cells. In addition, these two subtypes of DLBCL differ in underlying molecular characteristics (Khan, Miyashita et al.) and show different clinical behaviour (Alizadeh, Eisen et al. 2000). In line with their gene expression profiles, DLBCL appear to be derived from germinal center B-cells or post germinal center B-cells and, accordingly, most, if not all DLBCL cases should carry mutated IgVH genes. The finding that DLBCL in Richter's syndrome patients can also carry unmutated IgVH genes is therefore striking and points to a distinct pathogenesis as compared to *de novo* DLBCL. The frequency of unmutated IgVH genes among *de novo* DLBCL will have to be determined in larger series; if it is confirmed that unmutated IgVH genes occur rarely in *de novo* DLBCL, this molecular feature may be used as a future diagnostic and prognostic marker that points to a DLBCL that has likely arisen from a pre-existing B-CLL.

4.1.4 Biased Usage of IgVH Genes in Richter's Syndrome: A Role for the Involvement of Antigen?

We found that the cases of B-CLL in our study used the IgVH genes VH1, VH3 and VH4 at frequencies of 13.6%, 77.3%, and 9%, respectively (see Figure 8). The distribution of IgVH usage was different from Timar's report (Timar, Fulop et al. 2004), in which 50% of cases carried VH1 genes, while VH3 (37.5%) and VH4 (12%) were less frequently used. Within the VH3 family, VH3-11 was used in two cases. Among nine cases studied by Smit et al., VH1-69 was used twice. The VH1 and VH3 families were most frequently used, while VH4 and VH5 families were less frequently detected (Smit, van Maldegem et al. 2006). Although the major distribution of the IgVH gene usage in our study was similar to previous studies in conventional B-CLL (Fais, Ghiotto et al. 1998; Sakai, Marti et al. 2000; Widhopf, Rassenti et al. 2004), VH3 family usage was more frequent as compared to the IgVH gene usage in 1188 conventional B-CLL cases, whereas VH1 and VH4 families were less commonly detected (Widhopf, Rassenti et al. 2004).

Previous studies have already shown a non-random utilization of IgVH genes in B-CLL with overrepresentation of the VH1-69, VH3-7, VH3-21 and VH4-34 genes (Fais, Ghiotto et al. 1998; Hamblin, Davis et al. 1999; Ghia, Stamatopoulos et al. 2005). Interestingly, the CD5-positive B-cell repertoire in healthy adults is also skewed towards VH3-23, VH3-7 and VH4-34 (Brezinschek, Foster et al. 1997).

In our study, VH3-23, VH3-21 and VH3-9 were the most commonly expressed IgVH genes, and each gene was used in 3 cases of Richter' syndrome. VH3-74 and VH1-2 were found in two cases each (see Figure 9). In cases with a clonal relationship between B-CLL and DLBCL, VH3-9, VH3-23, VH3-74 and VH1-2 were most frequently used (two cases each). Frequent usage of VH3-23 and VH1-2 was similar to previous reports in B-CLL patients (Fais, Ghiotto et al. 1998; Ghia, Stamatopoulos et al. 2005) and VH1-2 was also observed in one case of Richter's syndrome.

VH3-9 and VH3-74 were also overused in our series. VH3-74 was also observed in a B-CLL case with clonal transformation (Smit, van Maldegem et al. 2006) and in a B-CLL case with Richter's transformation in which the DLBCL was clonally unrelated (Timar, Fulop et al. 2004). These two IgVH genes were rarely expressed in previous studies of B-CLL and in the normal adult CD5-positive B-cell repertoire (Fais, Ghiotto et al. 1998; Brezinschek, Foster et al. 1997). Taken together, the usage of IgVH genes in B-CLL cases that show transformation to DLBCL appears to be restricted; this finding points to a possible role of antigen involvement also in the pathogenesis of B-CLL cases that undergo Richter's transformation.

The VH3-21 gene was detected in 3 B-CLL cases in our series. In two cases, the gene was somatically mutated and in one case it was unmutated. Interestingly, recent reports in B-CLL indicate that VH3-21 usage confers a poor clinical prognosis despite the fact that the gene is frequently mutated (Lin, Manocha et al. 2003; Tobin, Thunberg et al. 2003; Thorselius, Krober et al. 2005). In addition, Falt and colleagues described a distinctive gene expression pattern among VH3-21 utilizing B-CLL cases as compared to IgVH-unmutated and IgVH-mutated B-CLL utilizing other VH genes (Falt, Merup et al. 2005). Among differentially expressed genes, cell cycle associated genes such as E2F4 that is involved in the control of transcription (Yoshida and Inoue 2004) were upregulated in the VH3-21 group, while HDAC (histone deacetylase) was downregulated. It is tempting to speculate that B-CLL cases with the usage of VH3-21 may be more prone to undergo Richter's transformation which may explain in part their inferior clinical course. Whether indeed VH3-21 B-CLL cases develop Richter's transformation more frequently as compared to other B-CLL cases, will have to be determined in future studies.

In contrast to the observation by Timar et al. (Timar, Fulop et al. 2004) the VH3-11 gene was only detected once in our series. Likewise, VH1-69 which constitutes the most commonly used IgVH gene in B-CLL in several series and is strongly associated with the IgVH-unmutated B-CLL subtype (Fais, Ghiotto et al. 1998; Hamblin, Davis et al. 1999; Ghia, Stamatopoulos et al. 2005), was observed only once in our study.

In summary, in the largest study performed to date we demonstrate a biased usage of IgVH genes in B-CLL and DLCBL that occur in Richter's syndrome. The VH3-23, VH3-74, VH1-2 and VH3-9 genes were used in over half of the B-CLL cases and, at the same time, the former three IgVH genes were present in almost half of the DLBCL components in clonally related Richter's syndrome. While VH3-23 and VH1-2 had been described to be overused in B-CLL in previous studies, VH3-9 and VH3-74 have not been previously associated with Richter's syndrome. Although the number of cases studied is still limited, our data may indicate that a subset of B-CLL cases that is undergoing Richter's transformation may be biologically distinct from other B-CLL cases. Given a biased usage of IgVH genes it is likely that antigen stimulation may also play a pathogenetic role in B-CLL with Richter's transformation.

4.2 B-CLL and Hodgkin's Lymphoma (HL) as a Variant of Richter's Syndrome

4.2.1 Clonal Relationship between B-CLL and HL in Richter's Syndrome and Association with EBV Infection

Although this variant of Richter's syndrome occurs rarely, we were able to study 6 cases of the HL variant of Richter's syndrome and 8 cases of B-CLL that showed scattered CD30-positive, HRS-like cells. We applied a very strict definition of the HL variant of Richter's syndrome in our study, and only B-CLL cases that showed features of classical Hodgkin's lymphoma (either concomitantly or in a subsequent biopsy) were considered the HL variant of Richter's syndrome. In particular, the large neoplastic HRS cells had to be present in the characteristic lympho-histiocytic background making the morphological picture indistinguishable from classical HL (Figure 11A-B). In contrast, cases with CD30-positive HRS-like cells that were scattered within the background of B-CLL cells without evidence of a polymorphous reactive background were regarded as B-CLL cases with HRS-like cells (Figure 11C-D) and excluded from the category of the HL variant of Richter's syndrome. This is in contrast to some previous reports who included these cases

among the HL variant of Richter's syndrome (Ohno, Smir et al. 1998; de Leval, Vivario et al. 2004).

Due to the low number of tumor cells in HL, studies on the clonal relationship between B-CLL and HL in Richter's syndrome are extremely scarce. A few previous reports suggested that HRS-like cells that are scattered within a background of B-CLL were clonally related to the B-CLL (Rubin, Hudnall et al. 1994). We here demonstrate that CD30-positive HRS-like cells in a background of B-CLL may also represent a different clone. Whether these cases may constitute precursor forms of classical Hodgkin's Lymphoma remains to be seen.

Since the number of CD30-positive HRS or HRS-like cells was low in the cases studied, microdissection had to be applied and careful precautions had to be taken in order to avoid contamination. A two-step procedure was performed to reduce the possibility of contamination: First, small cells surrounding the HRS or HRS-like cells were removed by using the P.A.L.M laser microbeam microdissection system. Subsequently, HRS and HRS-like cells were picked up by the Pixcell laser capture microdissection system as a second step (Figure 13 A-C). From isolated cells, DNA was extracted and a semi-nested PCR strategy was employed to amplify the CDR3 region of IgVH gene. Using this tedious approach we were able to successfully amplify the CDR3 region in two HL samples and in two cases of B-CLL with HRS-like cells. Despite many attempts, DNA could not be successfully amplified in several other cases. Two reasons may account for this failure: First, the quantity and quality of the DNA was poor in paraffin-embedded specimens and after the microdissection procedure and, secondly, the binding of primers may have been affected by a high load of somatic mutations present in the IgVH genes of HRS cells.

Taken together, we found that HRS cells in two samples and HRS-like cells in one sample were clonally distinct to the preceding B-CLL clone, whereas in one sample the HRS-like cells were clonally related to the surrounding B-CLL cells (Table 8). In the literature, only a handful of such cases have been molecularly analyzed so far due to their

rare occurrence and the requirement of single cell PCR strategies (Ohno, Smir et al. 1998; Kanzler, Kuppers et al. 2000; de Leval, Vivario et al. 2004; Fong, Kaiser et al. 2005). Among a total of nine cases, five cases (three cases with HL and two cases with HRS-like cells) were clonally related to the B-CLL, whereas four cases (one case with HL and three cases HRS-like cells) were clonally distinct from the B-CLL clone. Our data as well as the published literature therefore suggest that HRS and HRS-like cells can be clonally related or arise as an independent, secondary clone in Richter's transformation. Interestingly, in five cases in which HRS or HRS-like cells were clonally related to the B-CLL clone, CD30-positive cells did not show evidence of an association with an EBV infection. In contrast, in four cases in which the HRS/HRS-like cells were clonally independent, an association with EBV infection was evident. It was therefore suggested that clonally related cases might be restricted to EBV-negative cases, whereas EBV-positive HL may develop as an independent secondary lymphoma (de Leval, Vivario et al. 2004). However, Fong and colleagues reported recently that three of four cases of the HL variant of Richter's syndrome were clonally related with the corresponding B-CLL and two of them were EBV-positive. In our series, the three clonally distinct cases with HRS or HRS-like cells expressed the EBV-associated protein LMP1, whereas in the clonally related case with HRS-like cells LMP1 was not expressed (Table 7). Taken together, an association with EBV infection appears to be more frequent in cases with HRS/HRS-like cells that develop as an independent, secondary clone. In this scenario, an underlying immunodeficiency that is frequently associated with B-CLL may increase the risk of an EBV infection which in turn could lead to an increased risk of the development of secondary neoplasms (de Leval, Vivario et al. 2004).

EBV infection is widespread in most human populations, and EBV persists in the vast majority of individuals as a lifelong, asymptomatic infection in the B-lymphocyte pool. It has the potential to transform resting B-cells into permanent, latently infected lymphoblastoid cell lines in vitro (Young and Rickinson 2004). LMP1, as an oncogene, plays a central part in the process of this transformation (Kaye, Izumi et al. 1995). However, in healthy EBV carriers, infected B-cells are rare (1-50 per 10^6 B cells) (Khan, Miyashita et al. 1996) and usually in a resting state (G0 phase). In the case of EBV

reactivation, EBV-positive B-cells can be quickly eliminated by cytotoxic CD8+ T-lymphocytes (Rickinson and Moss 1997). In B-CLL patients, a dysfunction of CD4+ and CD8+ T-cells has been described (Tinhofer, Marschitz et al. 1998; Scrivener, Goddard et al. 2003) leading to a reactivation of EBV-infected B-cells. These cells may escape the immunosurveillance and acquire additional genetic hits that allow for uncontrolled proliferation. Immunosuppression in B-CLL may also be influenced by chemotherapy, in particular by purine analogs such as fludarabine which may cause marked depletion of T-cells(Cheson 1995; Tsimberidou, Younes et al. 2005). Rubin and colleagues even suggested a possible role of fludarabine therapy in EBV-positive Hodgkin's lymphoma in Richter's transformation (Rubin, Hudnall et al. 1994), and two recently reported B-CLL cases with subsequent EBV-positive HL had also undergone prior treatment with purine analogs (de Leval, Vivario et al. 2004).

4.2.2 The HL Variant of Richter's Syndrome is Mainly Associated with IgVH-Mutated B-CLL

As described above, we were able to investigate the clonal relationship between the B-CLL clone and HRS/HRS-like cells in four cases. In addition, however, we performed sequence analysis of the IgVH gene in eight additional B-CLL cases with either the HL variant of Richter's syndrome or the presence of HRS-like cells. Surprisingly, 83.3% of the B-CLL patients with the HL variant of Richter's syndrome or HRS-like cells carried mutated IgVH genes, which is a much higher frequency than observed in classical Richter's syndrome or in conventional B-CLL (approximately 50%). This indicates that different types of Richter's syndrome may be associated with different subsets of B-CLL. In a previous report, a B-CLL case with clonally related Hodgkin's lymphoma and anaplastic large cell lymphoma (ALCL) carried mutated IgVH genes (van den Berg, Maggio et al. 2002) and Kanzler and colleagues (Kanzler, Kuppers et al. 2000) reported on three IgVH-mutated B-CLL cases with HRS-like cells. Our data in altogether 12 cases now strongly associates the presence of HRS or HRS-like cells with the IgVH-mutated type of B-CLL. The reason why the HL variant of Richter's syndrome is frequently

associated with IgVH-mutated B-CLL is unclear and cannot be explained at the present time.

As observed in classical Richter's syndrome, we found a biased IgVH family usage among the B-CLL cases with the HL variant of Richter's syndrome. In particular, the VH4 family (50%) and especially the VH4-34 gene (33.3%) as well as the VH3-48 (33.3%) gene were overused in IgVH-mutated B-CLL cases (Figure 12). The VH4 gene family is used in approximately 20% of conventional B-CLL (Widhopf, Rassenti et al. 2004) and, interestingly, the VH4-34 gene, which was found to be strongly associated with cold agglutinin disease (Potter 2000) and autoimmune disorders (Pascual and Capra 1992), has been demonstrated to be overused in conventional B-CLL as well (Fais, Ghiotto et al. 1998; Hamblin, Davis et al. 1999; Ghia, Stamatopoulos et al. 2005). In contrast, the VH3-48 gene that was detected in two out of six cases with the HL variant of Richter's transformation has not been frequently reported in conventional B-CLL or in the repertoire of CD5-positive B-cell in healthy individuals. The biased usage of certain IgVH gene families in the HL variant of Richter's syndrome again suggests a possible role for antigen involvement in tumorigenesis.

The survival times of B-CLL patients with the HL variant of Richter's syndrome or with the presence of HRS-like cells appears to be superior to that of patients with classical Richter's syndrome. In our series, patients with the HL variant of Richter's syndrome had a median survival of 21 months and patients with B-CLL and the occurrence of CD30-positive HRS-like cells had a median survival of 33 months, which is markedly superior to patients with classical Richter's syndrome (7 months).

Chapter 5

Conclusions

We conclude from the work performed in this study:

1. In Richter's syndrome, diffuse large B-cell lymphoma (DLBCL) can evolve by clonal transformation of the pre-existing B-CLL clone or occur as an independent, clonally unrelated neoplasm. In the majority of cases (78% in our series), B-CLL and DLBCL are clonally identical.

2. In Richter's syndrome, clonal transformation into DLBCL occurs predominantly in B-CLL patients with unmutated IgVH genes. This finding points to important pathogenetic differences between DLBCL cases derived from a pre-existing B-CLL as compared to *de novo* DLBCL cases, since *de novo* DLBCL is characterized by mutated IgVH genes.

3. The Hodgkin's lymphoma (HL) variant of Richter's syndrome and the presence of CD30-positive, HRS-like cells are mainly associated with B-CLL patients carrying mutated IgVH genes. EBV-association in the HL variant of Richter's syndrome occurs more frequently in clonally unrelated secondary malignancies, possibly as a consequence of an underlying immunodeficiency.

4. Considering all cases of Richter's syndrome, there is a relationship between ZAP-70 expression and IgVH-unmutated B-CLL cases as described for conventional B-CLL.

5. Biased usage of IgVH genes in the two subtypes of Richter's syndrome suggests a possible role for antigen involvement in tumorigenesis also in B-CLL cases that undergo Richter's transformation during the course of the disease.

List of Abbreviations

ABC	Activated B-cell
ABVD	Doxorubicin, bleomycin, vinblastine, and dacarbazine
ALCL	Anaplastic large cell lymphoma
APS	Ammonium Persulfate
ATM	Ataxia telangiectasia mutated
B-CLL	B-cell chronic lymphocytic leukemia
Bi	Biclonal
BM	Bone marrow
HP	Helicobacter pylori
CB	Centroblastic variant
CDRs	Complementarity-determining regions
CGH	Comparative genomic hybridisation
CNT	Central nervous system
CTL	Cytotoxic T lymphocyte
DAB	3-3’-diaminobenzidine
DMF	N,N-dimethylformamide
DNA	Deoxyribonucleic acid
DLBCL	Diffuse large B-cell lymphoma
ddNTP	Dideoxyribonucleoside triphosphates
dNTP	Deoxynucleotide
DW	Distilled water
EB	Ethidium Bromide
EBV	Epstein-Barr virus
EBNAs	EBV nuclear antigens
EDTA	Ethylenediamine tetraacetic acid
FISH	Fluorescence in situ hybridisation
FL	Follicular Lymphoma
FR	Framework region
GCB	Germinal-centre
GI	Gastrointestinal
HDAC	Histone deacetylase
HL	Hodgkin’s Lymphoma
HRS cells	Hodgkin/Reed-Sternberg cells

IgVH	Immunoglobulin heavy chain variable region
IB	Immunoblastic variant
IHC	Immunohistochemistry
IRF-4	Interferon regulatory factor-4
IP	Incomplete product
LCM	Laser capture microdissection
LMP1	Latent membrane protein-1
LN	Lymph nodes
LCLs	Lymphoblastoid cell lines
LPC	Laser pressure catapulting
M	Monoclonal
MALT	Mucose-associated lymphoid tissue
MCL	Mantle cell lymphoma
MOPP	Mechlorethamine, vincristine, procarbazine, and prednisone
MPC	Monoclonal with polyclonal background
MSI	Microsatellite instability
NHL	Non Hodgkin's Lymphoma
NS	No signal
Olig.	Oligoclonal
RB1	Retinoblastoma gene
PCR	Polymerase Chain Reaction
PLL	Prolymphocytic leukemia
RER+	Replication error positive phenotype
PBS	Phosphate-buffered saline
Poly.	Polyclonal
SLL.	Small lymphocytic lymphoma
SDS	Sodium dodecyl sulfate
TBE	Tris-borate EDTA electrophoresis buffer
TEMAD	Tris-(hydroxymethyl)-aminomethane
TNFR	Tumor necrosis factor receptor
Tris	N,N,N´,N´-Tetramethylethylenediamine
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
ZAP70	Zeta associated protein 70

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Figure 7:Clonal Relationship between B-CLL and DLBCL Based on IgVH gene Sequences in Classical Richter's Syndrome

Figure 8: IgVH Gene Family in B-CLL and DLBCL in Classical Richter's Syndrome

Figure 9: IgVH Gene Usage in B-CLL and DLBCL in Classical Richter's Syndrome

Figure 10: Survival Analysis of Patients with Classical Richter's Syndrome

Figure 11: Morphology of Richter's Syndrome with the HL Variant (**A-B**) and B-CLL with CD30-Positive HRS-like Cells (**C-F**)

Figure 12: IgVH Gene Usage of B-CLL Cells with the HL Variant (**A**) or with CD30-Positive HRS-like Variant (**B**)

Figure 13: Immuno-laser Capture Microdissection (LCM)

Figure 14: Survival Analysis of all Patients with Richter's Syndrome

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ERKLÄRUNG

Hiermit erkläre ich, die vorliegende Dissertation selbst angefertigt und nur die angegebenen Quellen und Hilfsmittel verwendet zu haben.

Weiterhin erkläre ich, dass die vorliegende Dissertation weder in gleicher noch ähnlicher Form einem anderen Prüfungsverfahren vorgelegt wurde.

Hiermit bewerbe ich mich erstmals um den Doktorgrad der Naturwissenschaften der Bayerischen Julius-Maximilians-Universität Würzburg.

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