PROGNOSTIC IMPORTANCE OF THE TUMOUR MICROENVIRONMENT IN FOLLICULAR LYMPHOMA PATIENTS TREATED WITH IMMUNOCHEMOTHERAPY

Minna Taskinen

Genome-Scale Biology Program
Biomedicum Helsinki
Faculty of Medicine
and
Department of Oncology
Helsinki University Central Hospital

University of Helsinki
Finland

ACADEMIC DISSERTATION

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Supervised by:

Docent Sirpa Leppä
Department of Oncology, Helsinki University Central Hospital
Genome-Scale Biology Program, Biomedicum Helsinki
University of Helsinki
Helsinki, Finland

Reviewed by:

Professor Pirkko-Liisa Kellokumpu-Lehtinen
Department of Oncology, Tampere University Hospital
University of Tampere
Tampere, Finland

Docent Panu Kovanen
Department of Pathology, Helsinki University Central Hospital
University of Helsinki
Helsinki, Finland

Opponent:

Professor Sirpa Jalkanen
Department of Medical Microbiology
MediCity Research Laboratory
University of Turku
Turku, Finland

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TABLE OF CONTENTS

1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I Harjunpää, A.*, **Taskinen, M.***, Nykter, M., Karjalainen-Lindsberg, M.-L., Nyman, H., Monni, O., Hemmer, S., Yli-Harja, O., Hautaniemi, S., Meri, S., and Leppä, S. (2006). Differential gene expression in non-malignant tumour microenvironment is associated with outcome in follicular lymphoma patients treated with rituximab and CHOP. *Br J Haematol.* 135:33-42.

II **Taskinen, M.**, Karjalainen-Lindsberg, M.-L., Nyman, H., Eerola, L-M., and Leppä, S. (2007). A high tumor-associated macrophage content predicts favourable outcome in follicular lymphoma patients treated with rituximab and CHOP. *Clin Cancer Res.* 13:5784-89.

III **Taskinen, M.**, Karjalainen-Lindsberg, M.-L., and Leppä, S. (2008). Prognostic influence of tumor-infiltrating mast cells in patients with follicular lymphoma treated with rituximab and CHOP. *Blood.* 111:4664-67.

IV **Taskinen, M.**, Jantunen, E., Kosma, VM, Bono, P., Karjalainen-Lindsberg, M.-L., and Leppä, S. (2010). Prognostic impact of CD31-positive microvessel density in follicular lymphoma patients treated with immunochemotherapy. *Eur J Cancer.* 46:2506-12.

V **Taskinen, M.**, Valo, E., Karjalainen-Lindsberg, M.-L., Hautaniemi, S., Meri, S., and Leppä, S. (2010). Signal transducers and activators of transcription 5a–dependent cross-talk between follicular lymphoma cells and tumor microenvironment characterizes a group of patients with improved outcome after R-CHOP. *Clin Cancer Res.* 16:2615-23.

*) equal contribution

Publication I is included in the doctoral thesis of Antti Sommarhem, University of Helsinki.

2. ABBREVIATIONS

ABC	activated B-cell-like
aCGH	array comparative genomic hybridization
ADCC	antibody-dependent cellular cytotoxicity
ASCT	autologous stem-cell transplantation
BCL2/6	B-cell leukemia/lymphoma 2/6
BCR	B-cell receptor
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BP-VACOP	bleomycin, cisplatin, etoposide, doxorubicin, cyclophosphamide, vincristine, and prednisone
CDC	complement-dependent cytotoxicity
CHOP	cyclophosphamide, doxorubicin, vincristine, and prednisone
CHVP	cyclophosphamide, adriamycin, etoposide, and prednisolone
CI	confidence interval
CR	complete response
Cru	complete response unconfirmed
CT	computed tomography
CVP	cyclophosphamide, vincristine, and prednisone
DLBCL	diffuse large B-cell lymphoma
DNA	deoxyribonucleic acid
DSMZ	Deutche Sammlung von Mikroorganismen und Zellkulturen
DSS	disease specific survival
EFS	event-free survival
ERK	extracellular signal-regulated kinase
FCM	fludarabine, cyclophosphamide, mitoxantrone
FCS	fetal calf serum
FDC	follicular dendritic cell
FFS	failure-free survival
FLIPI	Follicular Lymphoma International Prognostic Index
FL	follicular lymphoma
FOXP3	forkhead box protein 3
GC	germinal centre
GCB	germinal centre B cell
GEP	gene expression profiling
HPF	high-power field
HS	human serum
IFN	interferon
Ig	immunoglobulin
IHC	immunohistochemical

IL	interleukin
IMDM	Iscove’s modified Dulbecco’s medium
IPI	International Prognostic Index
IRF	interferon regulatory factor
IWC	International Workshop Criteria
JAK	Janus kinase
LAM	lymphoma-associated macrophage
LDH	lactate dehydrogenase
MAC	membrane attack complex
MAPK	mitogen-activated protein kinase
MBR	major break point region
MC	mast cell
MCL	mantle cell lymphoma
MCL-1	myeloid cell leukemia-1
MCP	mitoxantrone, clorambucile, prednisolone
mcr	minor cluster region
miRNA	microRNA
MR	maintenance rituximab
MVD	microvessel density
NA	not applicable
NF-κB	nuclear factor kappa B
NHL	non-Hodgkin lymphoma
NK	natural killer
NO	nitric oxide
OS	overall survival
PCR	polymerase chain reaction
PD	progressive disease
PFS	progression-free survival
PI	proliferation index
PI3K	phosphatidylinositol-3 kinase
PKC	protein kinase C
PMBL	primary mediastinal B-cell lymphoma
PR	partial response
ProMACE-MOPP	prednisone, methotrexate, doxorubicin, cyclophosphamide, etoposide, mechlorethamine, vincristine, procarbazine, and prednisone
R	rituximab
RD	relapsed disease
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RR	relative risk (of death)

RT	radiotherapy
SD	stable disease
SHM	somatic hypermutation
SOCS	suppressor of cytokine signalling
SPD	sum of the product of the greatest diameters
STAT	signal transducer and activator of transcription
TAM	tumour-associated macrophage
TGF	transforming growth factor
TMA	tissue microarray
TNF	tumour necrosis factor
Treg	T-regulatory cell
TTF	time to treatment failure
VEGF	vascular endothelial growth factor
WHO	World Health Organization
YY1	yin-yang 1

3. ABSTRACT

Follicular lymphoma (FL) is the second most common non-Hodgkin lymphoma (NHL). It is an indolent and clinically heterogeneous disease, with median survival expectations of 8–10 years. Although the disease is sensitive to radio- and chemotherapy, most patients relapse several times and the disease is considered incurable. Currently, immunochemotherapy has significantly improved the outcome of FL patients. This is based on the combination of rituximab, a monoclonal anti-CD20 antibody, with chemotherapy, and is used at present as a standard first-line therapy in FL. Thus far, however, patients have been selected for treatment based on clinical risk factors and indices that were developed before the rituximab era. Therefore, there is a growing need to further understand the molecular mechanisms underlying the disease, which would not only provide information to predict survival in the rituximab era, but also enable the design of more targeted therapeutic strategies.

In this study, our aim was to identify genes predicting the outcome in FL patients treated with rituximab in combination with chemotherapy. Therefore, we performed an oligonucleotide-based cDNA microarray. A total of 24 FL patients treated with immunochemotherapy were grouped into long-term or short-term responders, and gene expression differences from diagnostic tumour samples were related to the clinical outcome. We identified novel genes with a prognostic impact on survival, many of them involved in the regulation of cell growth, signal transduction and immune responses. The expression of selected genes was further characterized with quantitative PCR and immunohistochemical staining. Interestingly, the prognostic influence of these genes often seemed to be associated with their expression in non-malignant cells instead of tumour cells.

Encouraged by the observed gene expression patterns, and increasing general knowledge of the importance of the microenvironment in FL, we analyzed the abundance and prognostic value of other non-malignant immune cell types of the FL tissue in patients treated with immunochemotherapy. We observed that a high content of tumour-associated macrophages (TAM) was a marker of a favourable prognosis. In contrast, the accumulation of mast cells (MCs) correlated with an unfavourable outcome and was further associated with tumour vascularity. Increased microvessel density also correlated with an inferior outcome.

In addition to the findings that the genes with a prognostic impact were mainly expressed by non-malignant cells, we identified several genes known to be involved in cell signalling. To identify the signalling pathways or groups of genes capable of separating patients according to outcome, the same data were analyzed using systems biology approach. Among the transcripts, there was a strong representation of genes

associated with signal transducers and activators of the transcription (STAT5a) pathway. When immunohistochemistry was used as validation, STAT5a expression was mostly observed in T-cells and follicular dendritic cells, and expression was found to predict a favourable outcome. In cell cultures, rituximab was observed to induce the expression of STAT5a-associated interleukins in human lymphoma cell lines, which might provide a possible link for the cross-talk between rituximab-induced FL cells and their microenvironment.

In conclusion, we have demonstrated that the microenvironment has a prognostic role in FL patients treated with immunochemotherapy. In addition, the results address the importance of re-evaluating the prognostic markers in the rituximab era of lymphoma therapies.

4. REVIEW OF THE LITERATURE

4.1 Non-Hodgkin lymphomas

Non-Hodgkin lymphomas (NHLs) are a heterogeneous group of lymphoid malignancies with large variation in clinical and biological features. They comprise the sixth most common cancer in Finland (Finnish Cancer Registry). In 2008, 1114 new cases were diagnosed, accounting for approximately 4% of all malignancies. NHLs can be divided into B-cell and T-cell type subgroups, of which B-cell lymphomas encompass nearly 90%. Lymphomas may originate from primitive stem cells (precursor B- or T-cell neoplasms) or from later stages of differentiating cells (mature B- or T-cell neoplasms). The three most common B-cell lymphomas are diffuse large B-cell (DLBCL), follicular (FL), and mantle cell (MCL) lymphomas, comprising 31%, 22%, and 6% of all NHLs, respectively (The Non-Hodgkin's Lymphoma Classification Project 1997). The World Health Organization (WHO) classification divides only mature B-cell neoplasms into 30 different entities based on morphology, immunophenotype, genetic features and clinical presentation (Swerdlow et al. 2008).

4.1.1. Cellular origin of B-cell lymphomas

The vast majority of B-cell lymphomas derive from germinal centre (GC) or post-GC B cells due to disturbed B-cell development. In the early stages, lymphocytes differentiate from immature stem cells in the bone marrow, where they go through recombination of immunoglobulin (Ig) heavy (H) and light (L) chain genes to generate a functional B-cell receptor (BCR) (Figure 1). In a T-cell-dependent immune response, antigen encounter “drives” B cells to undergo clonal expansion in GCs, where the Ig genes are further modified to improve antigen specificity by somatic hypermutation (SHM, the occurrence of many point mutations) and class-switch recombination. These processes eventually generate centrocytes, which are capable of producing high affinity antibodies of different subclasses. Newly generated centrocytes that produce an unfavourable antibody undergo apoptosis. Centrocytes can eventually either differentiate into plasma cells, which can produce large amounts of specific antibodies, or memory B-cells, which are programmed to respond to antigens on repeated exposure, thus mediating specific immune responses (reviewed in MacLennan 1994, Klein and Dalla-Favera 2008).

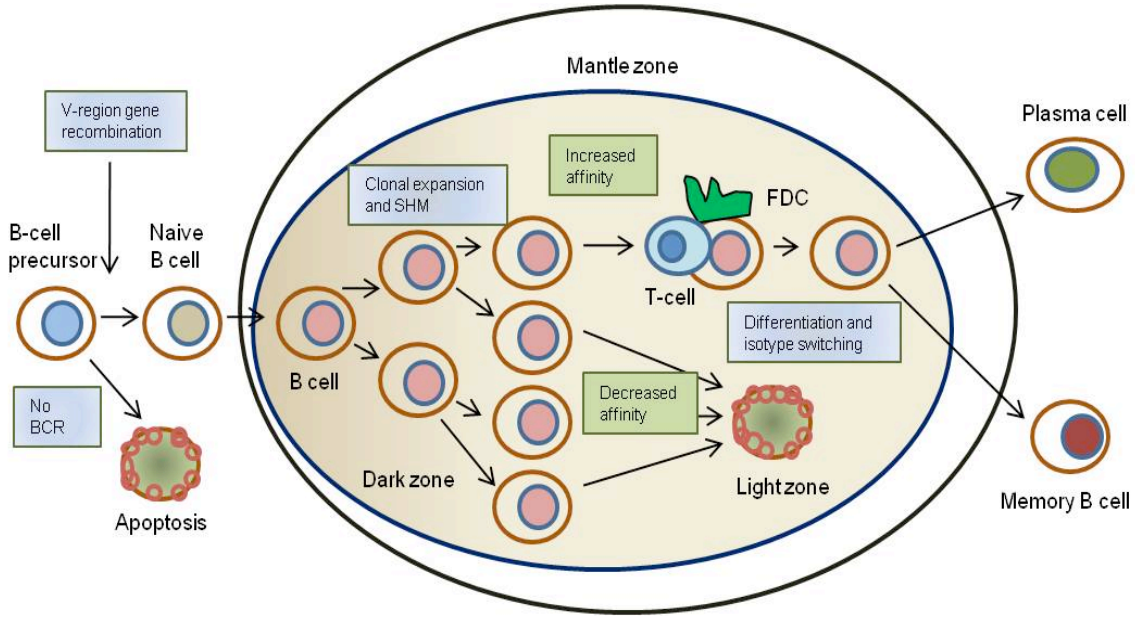

Figure 1. Schematic representation of B-cell differentiation (modified from Küppers 2005).

As distinct stages of B-cell maturation are characterized by a particular structure of BCR or the expression of specific differentiation markers, different lymphoma subtypes have been classified according to these features in such a way that most malignant B-cell lymphomas seem to be “frozen” at a certain stage of B-cell differentiation (Figure 2). B-cell lymphomas often display chromosomal translocations and aberrant SHM. These translocations usually involve a proto-oncogene, which comes under the control of the active Ig locus, causing deregulated expression of the oncogene. For example the B-cell leukemia/lymphoma 2 (*BCL2*)-*IgH* translocation t(14;18) is usually associated with FL (Cleary et al. 1985, Tsujimoto et al. 1985), whereas MCL is characterized by translocation, resulting in aberrant expression of cyclin D1 (Rimokh et al. 1994). However, the same chromosomal translocation may be detected in different lymphoma entities (e.g. *BCL2-IgH* translocation is also detected in roughly 20% of DLBCL) (Huang et al. 2002), and the characteristic cytogenetic alteration is usually only present in part of a given lymphoma subtype.

The cellular origin of B-cell lymphomas has been further elucidated by gene expression profiling (GEP) studies. This method identifies the genetic "signature" of a cancer specimen. For instance, both FL and a subgroup of DLBCL express genes representing the GC B-cell signature. GEP has also had a significant role in identifying previously unidentified lymphoma subtypes (Alizadeh et al. 2000, Rosenwald et al. 2002, Wright et al. 2003). Furthermore, by combining the GEP results with clinical data, statistical predictive models of the clinical behaviour of the disease can be generated (Rosenwald et al. 2002, Rosenwald et al. 2003, Dave et al. 2004).

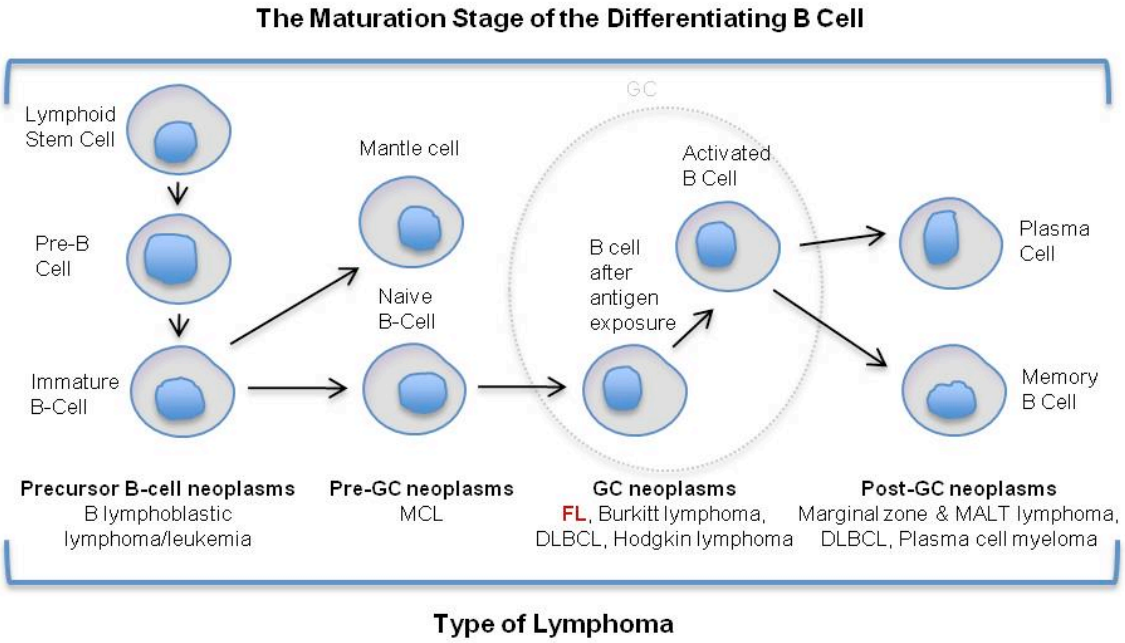

Figure 2. Cellular origin of B-cell lymphomas (modified from Swerdlow et al. 2008).

4.2. Follicular lymphoma

4.2.1. Definition and clinical features

FL is the second most common subtype of NHL after DLBCL (The Non-Hodgkin's Lymphoma Classification Project 1997). The most distinctive morphological feature of the disease is the nodular pattern of growth that attempts to recapitulate the cellular composition of the normal secondary lymphoid follicle (Figure 3A). This is derived from the clonal expansion of GC B-cells, with centrocyte (cleaved cells) and centroblast (large noncleaved cells) morphology in variable ratios (Figure 3B).

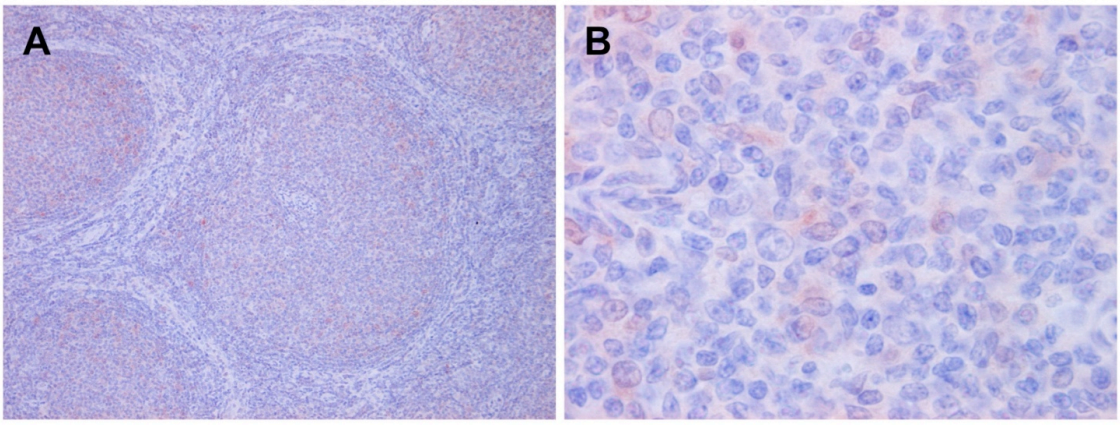

Figure 3. A) The nodular growth pattern of FL. B) The neoplastic follicle contains a mixture of small and large lymphoid cells.

The follicular structures are often uniform in size and shape, have poorly formed or absent mantles, and fail to illustrate a normal distinction between light and dark zones. In some cases, the follicular growth pattern is lost and a diffuse growth pattern predominates. Diffuse areas comprised entirely or mainly of large blastic cells are thought to be equivalent to DLBCL. FLs are graded according to the number of centroblasts present within a standard (0.159 mm^2) high-power field into the grades 1, 2, 3a and 3b (Table 1). However, the newest WHO classification combines the grades 1–2 (low grade with few centroblasts) (Swerdlow et al. 2008). The grade 3b is more closely related to DLBCL (Ott et al. 2002).

Table 1. Grading of FL.

Grading	Definition
Grade 1–2 (low grade)	0–15 centroblasts per hpf
1	0–5 centroblasts per hpf
2	6–15 centroblasts per hpf
Grade 3	>15 centroblasts per hpf
3A	Centrocytes present
3B	Solid sheets of centroblasts

hpf, high-power field of 0.159 mm^2

In addition to tissue morphology, the reliable diagnosis of lymphoma is based on the immunophenotype of the tissue. FL cells usually express surface Ig (IgM +/- IgD, IgG or rarely IgA) and pan-B-cell markers (CD19, CD20, CD22, CD79a). Furthermore, BCL2, BCL6 and CD10 are frequently expressed, whereas most FL cells are negative for CD5 and CD43 (Swerdlow et al. 2008).

Low grade FL is typically diagnosed in an advance stage, with a median age of around 60 years at diagnosis. The disease is characterized by an indolent clinical course with a median survival in the range of 8 to 10 years. Although the response rates to different therapies are high, most of the patients usually do relapse. In addition, the disease is characterized by recurrent progressions, with shorter intervals between relapses (reviewed in Hiddemann et al. 2005a). Moreover, transformation to a more aggressive B-cell lymphoma type (usually DLBCL) is common and affects at least 37% of patients in the 15 years following diagnosis (Montoto et al. 2007).

4.2.2. Cytogenetics

FL is characterized by the chromosomal translocation t(14;18)(q32;q21) (Cleary et al. 1985, Tsujimoto et al. 1985). This translocation is present in 70–90% of FL patients (Bakhshi et al. 1985, Weiss et al. 1987, Yunis et al. 1987). As a consequence, the *BCL2* proto-oncogene comes under the control of the active Ig locus, causing a deregulated and constitutive expression of antiapoptotic Bcl-2 protein (Cleary et al. 1986, Graninger et al. 1987, Ngan et al. 1988, Seto et al. 1988). This provides a survival advantage to B-cells normally destined to undergo apoptosis and allows the accumulation of additional genetic hits to occur (Vaux et al. 1988, Nunez et al. 1989, Küppers 2005). The presence of t(14;18) is not sufficient to cause the transformation of neoplastic B-cells on its own, since *BCL2* transgenic mice develop lymphomas only after a long latency period and the acquisition of secondary chromosomal alterations (McDonnell et al. 1989, McDonnell and Korsmeyer 1991). Furthermore, among 25–45% of healthy persons, t(14;18) translocation can be detected by polymerase chain reaction (PCR) in the peripheral blood with no evidence of clinical disease (Limpens et al. 1995, Summers et al. 2001, Schmitt et al. 2006, Schüler et al. 2009). There are also rare variant translocations t(2;18) and t(18;22) leading to abnormal expression of *BCL2* (Hillion et al. 1991, Bertheas et al. 1992). Overall, only approximately 5% of FL cases have t(14;18) as the sole detectable abnormality, and around the same percentage are negative for that translocation (Johnson et al. 2008a). Moreover, gains in chromosome 18q21 are among the most common genomic alterations, reported to occur in 33% of FL patients (Berglund et al. 2007).

4.2.3. Clinical prognostic parameters

Accurate evaluation of the extent of the disease is essential both at the initial presentation and on follow-up, as this is used to guide management decisions. The modified Ann Arbor staging classification originally developed for Hodgkin lymphomas (Lister et al. 1989) is applied to define the extension of the lymphoma in most NHLs. In only 15–20% of diagnosed FL cases the lymphoma is localized to a single lymph node region (stage I), or to two or more lymph node regions restricted to one side of the diaphragm (stage II). Patients with more widespread stage III disease have affected lymph node regions on both sides of the diaphragm. If one or more extranodal sites are involved, the lymphoma is considered to be stage IV, which occurs in over 40% of FL patients at diagnosis.

The first prognostic classification of FL separated patients into three categories according to small-, mixed-, and large-cell type (The Non-Hodgkin's Lymphoma Pathologic Classification Project 1982). Subsequently, the International Prognostic Index (IPI) has been widely used as a tool to differentiate NHL patients into low and high risk groups (The International Non-Hodgkin Lymphoma Prognostic Factor Project 1993). IPI is defined according to five clinical factors: age, tumour stage, serum lactate dehydrogenase (LDH) concentration, performance status, and the number of extranodal disease sites. Even though IPI was generated to predict the outcome of aggressive NHLs, it has also been shown to be applicable in FL (Lopez-Guillermo et al. 1994). The FL International Prognostic Index (FLIPI) is a clinical index that was particularly developed to predict outcome of patients with FL (Solal-Celigny et al. 2004). It is based on five clinical parameters: age, Ann Arbor stage, number of nodal areas, LDH, and haemoglobin level, which differentiate patients into three separate risk groups (low, intermediate and high) with a ten-year survival of 71%, 51% and 36%, respectively (Table 2) (Solal-Celigny et al. 2004). Although the patient cohort used to build up the FLIPI was treated with conventional chemotherapy, the FLIPI has been demonstrated to also predict survival in patients treated with immunochemotherapy (Buske et al. 2006). Recently, a new FL International Prognostic Index 2 (FLIPI2) has been proposed using partially different variables (β2-microglobulin higher than the upper limit of normal, longest diameter of the largest involved node longer than 6 cm, bone marrow involvement, haemoglobin level lower than 12g/dL, and age older than 60 years) (Federico et al. 2009).

Table 2. Outcome and relative risk of death according to risk group as defined by the Follicular Lymphoma International Prognosis Index (FLIPI).

Risk group	Number of factors	Distribution of patients (%)	5-year OS (%)	10-year OS (%)	RR (95% CI)
Low	0–1	36	90.6	70.7	1 (NA)
Intermediate	2	37	77.6	50.9	2.3 (1.9–2.8)
High	≥ 3	27	52.5	35.5	4.3 (3.5–5.3)

N = 1795. OS: overall survival; CI: confidence interval; RR: relative risk (of death), and NA: not applicable.

One controversial prognostic parameter in FL is the molecular monitoring of minimal residual disease, which is currently tested by analyzing the residual *BCL2/IgH*-positive cells in peripheral blood and bone marrow by PCR. Some studies have documented that the achievement of a sustained molecular response after chemotherapy with or without rituximab is a favourable prognostic factor correlating with prolonged progression free survival (PFS) (Lopez-Guillermo et al. 1998, Rambaldi et al. 2005), whereas others have stated that the prognostic value of residual *BCL2/IgH*-positive cells is not useful for decisions on subsequent therapy for patients with relapsed/resistant FL (Paszkiewicz-Kozik et al. 2009, van Oers et al. 2010a). The molecular monitoring of the disease is complicated by the fact that only approximately 70–90% of FL patients harbour the t(14;18) translocation juxtaposing *BCL2* and *IgH* (Bakhshi et al. 1985, Weiss et al. 1987, Yunis et al. 1987). In addition, up to 50% of patients carrying the translocation have a break point outside the major break point region (MBR) or the minor cluster region (mcr) of the *BCL2* gene (Albinger-Hegyi et al. 2002, Weinberg et al. 2007). The reliable analysis of residual disease with the PCR method may therefore require novel primer sets specific for individual patients.

4.2.4. Transformation

Transformation of FL to a more aggressive disease is often marked by a sudden change in clinical features, e.g. rapidly enlarging lymph nodes, the development of B symptoms including weight loss, fever or night sweats, and a rise in serum LDH levels. It is associated with shortened survival and resistance to therapy. Estimates of the frequency of histological transformation vary significantly, ranging from 10% to 60% (Montoto et al. 2007, Al-Tourah et al. 2008). The transformed disease mostly has the morphology of DLBCL, but the features may occasionally be reminiscent of Burkitt lymphoma or precursor B-lymphoblastic lymphoma (Alsabeh et al. 1997, Parker et al. 2009).

Several studies have investigated changes in genetic and global gene expression associated with transformation (Lossos et al. 2002, de Vos et al. 2003, Elenitoba-Johnson et al. 2003, Martinez-Climent et al. 2003, Glas et al. 2005, Davies et al. 2007). These alterations often lead to changes in the expression of genes reflecting increased proliferation levels or enhanced metabolism. Recently, the role of micro RNAs (miRNAs) as potential diagnostic and prognostic markers in NHLs was examined (Lawrie et al. 2009). Specific miRNA signatures correctly distinguished the lymphoma subtypes between FL and DLBCL in >95% of cases, and differential expression of six miRNAs was found to predict transformation in FL. Some chromosomal aberrations, for example gains in chromosomes 2, 3q and 5, have been linked to higher grade transformations and inferior survival (Eide et al. 2010).

4.2.5. Treatment

The treatment of FL is based on the extent of the disease at initial diagnosis. In most cases, the treatment of FL is not curative, but treatment options that extend the duration of remission and may reflect in improved overall survival (OS) have been developed. FL is detected in the early stages (I or II) in only 15% to 20% of cases. These patients have been conventionally treated with radiation therapy. In a long-term follow-up study on stage I and II low-grade FL patients treated with radiotherapy at Stanford University, 64% and 35% of the patients were alive after 10 and 20 years, respectively, and 44% and 37% of them were relapse-free (Mac Manus et al. 1996). If a patient with advanced disease is asymptomatic, a watchful waiting policy may be a good option, and spontaneous regression sometimes occurs. However, patients with advanced symptomatic disease need treatment, which may consist of a single chemotherapeutic agent, such as orally administered chlorambucil, or polychemotherapy. With the latter, the most commonly used treatment options in Finland are CVP (cyclophosphamide, vincristine, and prednisone) and CHOP (cyclophosphamide, adriamycin, vincristine, and prednisone). Rituximab, an anti-CD20 antibody, has had a large impact on the treatment of FL. Its effectiveness as a single agent (McLaughlin et al. 1998) and in conjunction with chemotherapy (Czuczman et al. 1999) has been validated in several large randomized studies (Table 3) comparing immunochemotherapy with chemotherapy in FL. Subsequent meta-analyses have further evaluated the data obtained from clinical trials, and indicated that the addition of rituximab to chemotherapy has resulted in a higher overall response rate and OS (Gao et al. 2009). Furthermore, rituximab is currently being investigated as a maintenance agent with encouraging results (Table 3) (Vidal et al. 2009). On the grounds of these positive results, immunochemotherapy is considered as a standard of care for patients with FL. The role of autologous stem-cell transplantation (ASCT) in the treatment of FL is controversial.

In patients with relapsed FL, rituximab-based chemotherapy followed by ASCT may provide superior disease control (Witzens-Harig and Dreger 2010).

Table 3. Major randomized studies comparing immunochemotherapy with chemotherapy in FL.

Study	Regimen	Age	Number of FL patients	EFS, PFS or TTF	OS
FL untreated					
Hiddemann et al. 2005b	R-CHOP vs. CHOP	≥18	N = 428	2-year TTF: 82% vs. 63%	2-year OS: 95% vs. 90%
Herold et al. 2007	R-MCP vs. MCP	≥18	N = 358	4-year PFS: 71% vs. 40%	4-year OS: 87% vs. 74%
Salles et al. 2008	R-CHVP-IFN vs. CHVP-IFN	18–75	N = 358	5-year EFS: 53% vs. 37	5-year OS: 84% vs. 79%
Marcus et al. 2005, Marcus et al. 2008	R-CVP vs. CVP	>18	N = 321	Median TTF: 27 vs. 7 months	4-year OS: 83% vs. 77%
FL relapsed					
Forstpointner et al. 2004	R-FCM vs. FCM	≥18	N = 93 (total N = 147)	Median PFS: not reached vs. 21 months	2-year OS: 90% vs. 70%
FL maintenance					
Hochster et al. 2009	CVP + MR vs. CVP	>18	N = 282 (total N = 311)	3-year PFS: 64% vs. 33%	3-year OS: 91% vs. 86%
van Oers et al. 2006	CHOP +/- R + MR vs. CHOP +/- R	≥18	N = 465	Median PFS: 52 vs. 15 months,	3-year OS: 85% vs. 77%,
van Oers et al. 2010b				Median PFS: 3.7 vs. 1.3 years	5-year OS: 74% vs. 64%
Forstpointner et al. 2006	R-FCM + MR vs. R-FCM	≥18	N = 125 (total N = 195)	Median PFS: not reached vs. 26 months	3-year OS: 77% vs. 57% (N=195)

CHOP: cyclophosphamide, doxorubicin, vincristine, prednisone; CHVP: cyclophosphamide, adriamycin, etoposide, prednisolone; CVP: cyclophosphamide, vincristine, prednisone; EFS: event free survival; FCM: fludarabine, cyclophosphamide, mitoxantrone; IFN: interferon-α2a; MCP: mitoxantrone, clorambucile, prednisolone; MR: maintenance rituximab; OS: overall survival; PFS: progression free survival; R: rituximab; TTF: time to treatment failure.

4.2.5.1. Rituximab

Rituximab is a chimeric IgG monoclonal antibody directed against the CD20 surface antigen expressed on most normal and neoplastic B-lymphocytes (Figure 4) (McLaughlin et al. 1998). It is the first monoclonal antibody approved for the treatment of cancer. The *in vivo* mechanisms of rituximab-mediated antitumour effects include antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), the induction of apoptosis, and direct growth arrest (Figure 5).

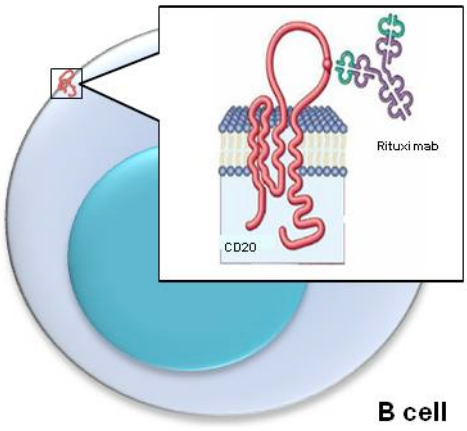

Figure 4. Rituximab targets B-cells by binding specifically to CD20 on the cell surface (modified from www.niaid.nih.gov).

CD20 is a tetra-membrane spanning 33–37 kDa phosphoprotein (Einfeld et al. 1988, Tedder et al. 1988). It is expressed on over 90% of all B-cell NHLs (Nadler et al. 1981, Anderson et al. 1984). Importantly, CD20 is not expressed on early pre-B cells, or on antibody secreting plasma cells (Loken et al. 1987). The precise function of CD20 or its natural ligand is not currently known, and CD20 knockout mice show normal B-cell development and function (O'Keefe et al. 1998). However, CD20 appears to play a role in Ca^{2+} conductance (Bubien et al. 1993, Daniels et al. 2008, Polyak et al. 2008), and it is also involved in cell-cycle progression through interaction with Src family kinases (Popoff et al. 1998). In some lymphoma patients, surface and cytosolic expression of CD20 is lost after rituximab therapy (Haidar et al. 2003), which may partly be regulated by some epigenetic mechanisms (Hiraga et al. 2009).

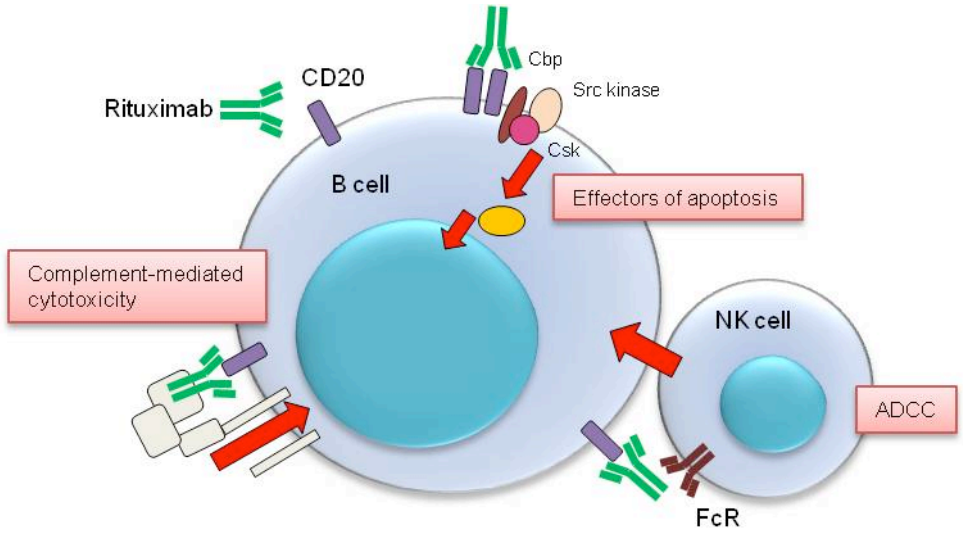

Figure 5. Effector mechanisms of rituximab (modified from Olszewski and Grossbard 2004).

Antibody-dependent cellular cytotoxicity (ADCC). One of the effector mechanisms of rituximab is ADCC. The human IgG1 Fc domain of rituximab interacts with FcγRs, which are the activating receptors of immune effector cells, such as monocytes/macrophages, granulocytes/neutrophils, and natural killer (NK) cells (Reff et al. 1994, Flieger et al. 2000, Golay et al. 2003). This binding initiates a series of signalling pathways, leading to the release of inflammatory and/or cytotoxic immune modulators, and eventually to the phagocytosis or elimination of targeted CD20-positive cells. Macrophage-mediated ADCC against B-cell lymphoma cells targeted with rituximab has been shown to be regulated by the phosphatidylinositol-3-kinase (PI3K) /Akt pathway (Joshi et al. 2009). The importance of ADCC as an effector mechanism in the immunotherapy of lymphoid malignancies has recently been demonstrated to be dependent on Fc gamma-chain signalling (de Haij et al. 2010).

Complement-dependent cytotoxicity (CDC). Together with ADCC, CDC also has a role in the antitumour activity of rituximab (Harjunpää et al. 2000, Golay et al. 2000). Now the Fc domain of rituximab is responsible for activating the classical complement pathway, leading to target cell lysis via the formation of the membrane attack complex (MAC) (Zhou et al. 2008).

Apoptosis. Crosslinking of CD20 receptors on human B cells has been shown to inhibit cell proliferation, trigger DNA fragmentation, and induce apoptosis (Shan et al. 1998). Binding of rituximab to CD20 leads to the aggregation and rapid translocation of CD20 into specialized plasma membrane domains, known as lipid rafts (Janas et al. 2005). Within these signalling platforms, CD20 is associated with Src family tyrosine kinases Lyn, Fyn, and Lck (Deans et al. 1995), which leads to the activation of phospholipase

Cγ (Deans et al. 1993, Shan et al. 2000). These signalling cascades employed by CD20 are strikingly similar to those utilized by the BCR, and CD20 has been shown to directly associate with the BCR (Polyak et al. 2008, Walshe et al. 2008). In addition, cytolic Ca^{2+} flux induced by CD20 is dependent on BCR expression and signalling (Walshe et al. 2008). The resultant Ca^{2+} flux has been implicated in CD20-mediated apoptosis by the observation that intra- or extracellular calcium chelation inhibited apoptosis induced by rituximab in a Burkitt lymphoma cell line (Hofmeister et al. 2000). Recent studies have also demonstrated alterations in BCR signalling pathways in FL and DLBCL (Irish et al. 2006, Chen et al. 2008). Inhibition of tonic BCR signalling may therefore be a rational treatment target in these lymphomas, and rituximab has been shown to directly alter the expression, dynamics and signalling of BCR (Kheirallah et al. 2010). Only recently, therapeutic doses of monomeric rituximab were shown to trigger prolonged intracellular calcium release distinct from lipid raft-dependent and Src kinase-related pathways, independently of CD20 and Fcγ receptors (Unruh et al. 2010). This may indicate that unique regions within IgG that do not define antigen specificity or mediate FcγR binding contribute to the direct effects of rituximab. Furthermore, rituximab-mediated cell apoptosis and sensitivity to chemotherapeutic regimens has been reported to be independent of its Fc functions (Vega et al. 2009).

Rituximab-induced apoptosis has been shown to involve the death receptor pathway and proceed in a caspase-8-dependent manner (Stel et al. 2007). Other possible mechanisms of action include downmodulation of p38 mitogen-activated protein kinase (MAPK), nuclear factor-κB (NFκB), extracellular signal-regulated kinase (ERK) 1/2, and PI3K / Akt survival pathways. This may contribute to the downregulation of anti-apoptotic Bcl-2/Bcl-X_L or transcriptional repressor yin-yang 1 (YY1) and chemosensitization of drug-resistant B-NHL cell lines (Jazirehi et al. 2004, Vega et al. 2004, Jazirehi et al. 2005, Vega et al. 2005, Suzuki et al. 2007).

Particularly in FL cells, one possible mechanism of the rituximab-mediated antitumour effect is the protein kinase C zeta (PKCζ)-mTOR pathway. Treatment of FL cells with rituximab resulted in the disruption of the PKCζ-MAPK module and subsequent inhibition of mTOR (Leseux et al. 2008), which may have an essential function in FL cell survival (Leseux et al. 2006).

Rituximab resistance of lymphoma cells has been studied by developing rituximab-resistant cell clones (Jazirehi et al. 2007). These resistant clones have diminished surface CD20 molecule expression, and they exhibit constitutive hyperactivation of NFκB– and ERK 1/2 pathways, leading to overexpression of Bcl-2, Bcl-XL and Mcl-1. Pharmacological inhibitors of the survival pathways or Bcl-2 family members reduced the activity of these pathways, diminished anti-apoptotic protein expression and chemosensitized the rituximab-resistant cells (Jazirehi et al. 2007, Stolz et al. 2008).

These observations suggest that pharmacological modulation of Bcl-2 family proteins is a promising strategy to overcome rituximab resistance.

4.2.5.2. Response evaluation

Standardized response definitions are essential to ensure reliable analysis of comparable patient groups among various studies and the acquisition of similar data. The International Workshop Criteria (IWC) have become the widely accepted standard for assessment of the disease response in NHL (Cheson et al. 1999). The response in NHL patients is most often defined on the basis of a regression in the size of enlarged lymph nodes or confluent lymph node masses.

The IWC is based primarily on computed tomography (CT), but bone-marrow biopsy as well as clinical and biochemical information are also considered. A complete response (CR) is defined as the complete disappearance of all detectable disease by imaging, with nodes previously > 1.5 cm regressing to < 1.5 cm and nodes of 1.0–1.5 cm to < 1.0 cm. In addition, there is resolution of disease-related symptoms, normalization of biochemical abnormalities and normal bone-marrow biopsy. Complete response unconfirmed (Cru) is classified as CR, but with a residual mass > 1.5 cm in diameter that has regressed by > 75%. In partial response (PR) there is at least 50% reduction in the sum of the product of the greatest diameters (SPD) of the six largest nodes. There should be neither an increase in the size of other nodes nor any new sites of disease. Hepatic and splenic lesions should also reduce > 50% in size in the SPD. The response is defined as stable disease (SD), when it is less than a PR but there is no progressive disease. Instead, when there is more than a 50% increase in the product of the diameters in any previously abnormal node or the development of new disease sites either during or at therapy, the response is termed progressive disease (PD). Relapsed disease (RD) occurs when there is an appearance of any new disease or an increase in size of over 50% of residual lesions in patients who had previously achieved CR or Cru.

4.3. Gene-expression studies on FL

Microarray technology has enabled a quantitative approach to study cancer-related genes with a huge amount of information. In a DNA microarray, arrayed series of tens of thousands of DNA oligonucleotides consisting of a specific DNA sequence are attached to a solid surface, like a chip. This is hybridized with a fluorochrome – or chemiluminescence-labelled cDNA or cRNA from the research sample under high-

stringency conditions. Resulting hybridization pairs are visualized and quantified to detect the relative abundance of specific nucleotide sequences in the sample. Gene expression arrays rely on the fact that in each cell, only part of the DNA is transcribed to messenger RNA depending on the cell lineage, differentiation state and activation status of the cell. Thus, it is possible to investigate the underlying biology by measuring thousands of expressed genes in parallel that constitute the gene expression profile (GEP) of the normal or malignant cells studied. In malignant lymphomas, GEP has revealed that existing diagnostic categories are comprised of several molecularly and clinically distinct diseases. For example, DLBCL is comprised of three different subgroups, termed germinal centre B cell (GCB)-like, activated B cell (ABC)-like, and primary mediastinal B cell lymphoma (PMBL) (Alizadeth et al. 2000, Rosenwald et al. 2002, Rosenwald et al. 2003, Savage et al. 2003, Wright et al. 2003). These subgroups differ in their cell of origin (Figure 2), utilize distinct oncogenic mechanisms, and respond differentially to chemotherapy. Among lymphomas, GEP has also been used to create gene expression-based survival predictors, which have identified characteristic biological features of the tumours to influence their clinical behaviour.

4.3.1. Clinical outcome prediction

In FL, it was demonstrated and subsequently validated that non-malignant immune cell expression signatures predict the clinical outcome of the patients (Dave et al. 2004). A molecular predictor was first build with supervised methods in a training set of 95 FL patients, and further validated in another set of 96 FL patients. All patients were treated with chemotherapy. Two outcome-related signatures were created, named immune response 1 (IR1) and immune response 2 (IR2). IR1 was composed of genes mostly expressed by T cells (*CD7, CD8B1, ITK, LEF1* and *STAT4*) and macrophages (*ACTN1* and *TNSF13B*), and was found to be associated with an improved clinical outcome. Instead, the IR2 signature was enriched for genes expressed by macrophages and/or follicular dendritic cells (TLR5, FCGR1A, SEPT10, LGMN and C3AR1), and was associated with inferior survival. These two signatures were used to construct a survival predictor, which allowed patients in the test set to be divided into four quartiles with widely disparate median lengths of survival of 13.6, 11.1, 10.8, and 3.9 years, independently of clinical prognostic variables.

In another study, Bohen et al. (2003) used a microarray to identify genes that predict the response to rituximab treatment in a small cohort of 24 FL patients. The association of the genes with survival was not analyzed. Interestingly, however, they noted that gene expression pattern of tumours not responding to treatment appeared to be more similar to those of normal lymphoid tissues than did the responding patients. Many of the genes

expressed at significantly higher levels in nonresponders were involved in the cellular immune response and inflammation. This was suggested to indicate that these tumours are more capable of inducing a growth-stimulating microenvironment. Another important observation was that gene expression phenotype of FL can change over time.

LeBrun et al. (2008) performed a gene expression analysis with 41 FL patients, and identified gene pairs that predicted a poor outcome, measured as death within 5 years of diagnosis. The best gene pairs (*LOXL3 + TSN*, and *NOTCH2 + RIPK5*) displayed >1000-fold discriminative power when compared to single genes or FLIPI, and exceeded 85% outcome prediction accuracy. Representative gene pairs could also divide patients into prognostic groups in Kaplan Meyer survival analysis. This study was suggested to provide a predictive model with a minimum number of genes, which could be further developed to be used in clinical settings.

In a study searching for genes predictive for successful CHOP chemotherapy in FL, Björk et al. (2005) utilized a high-density oligonucleotide array with 57 FL tumour samples and found 14 genes that were highly expressed in patients responding well to therapy. Of these genes, 11 were involved in cell cycle, mitosis, or DNA modulation. However, only the expression of *CCNB1* coding for cyclin-B1 had a positive prognostic value independent of the FLIPI.

4.3.2. Comparison of FL cells with their normal counterparts or other lymphoma subtypes

To specifically identify the differences in gene expression between normal GC B cells and FL cells, Husson et al. (2002) used immunomagnetic beads to purify normal GC B cells of hyperplastic tonsils and malignant B cells, and compared their gene expression. They found a number of genes to be overexpressed (e.g. *BCL2*, *MADH1*, *CDKN1A*, *YY1*, *PAX5*, *TNF*, *IL2RG*, and *IL4R*) or underexpressed (e.g. *TNFRSF5*, *ITGAL*) in FL cells compared to their normal counterparts, and some of these genes were localized to sites of known genetic abnormalities observed in FL. Notably, all the FL patients analyzed in the study had relapsed, and some of the gene expression changes may reflect prior chemotherapies.

Measurement of prognostic gene signatures in routine practice is difficult. To test the use of some indicator genes as a diagnostic tool in FL and DLBCL, Sakhinia et al. (2007) developed a sensitive RT-PCR method and validated previous microarray studies with 36 candidate indicator genes. Altogether, 106 archived frozen lymph node samples were available for the study, including 63 of FL, 25 of DLBCL, 10 reactive lymph nodes and 4 paired samples of FL and subsequent DLBCL. Nine genes out of 36

showed statistically significant differential expression between FL and DLBCL (*CCNB1, COL3A1, NPM3, H731, PRKCB1, OVGL, ZFPC150, HLA-DQ-a,* and *XPB*), and six genes had significantly higher expression in neoplastic nodes compared with reactive nodes *(PRKCB1, BCL6, EAR2, ZFX, CCNB1, YY1*). In FL, expression of *XPB, TNF* and *YY1* showed a statistically significant association with survival, and high levels of *YY1* were especially associated with shorter OS interval. This was of particular interest, since *YY1* was found to be overexpressed in FL cells (Husson et al. 2002) and it was reported to have a role in producing resistance to rituximab treatment in a cell culture model (Vega et al. 2005). The same group also constructed a RT-PCR model in FL with 35 candidate indicator genes selected on the basis of previous microarray studies together with genes associated with B-cell, T-cell and macrophage differentiation (Byers et al. 2008). RT-PCR was applied to 60 FL samples, in parallel with immunohistochemical analysis for tumour infiltrating host immune cell markers. Of these genes, only high levels of CCR1, a marker of monocyte activation, were significantly associated with shorter survival, and high levels of CD3 with better survival.

4.3.3. Genes involved in histological transformation

There have been several gene expression studies addressing the question of how FL patients differ from their transformed counterparts with DLBCL at the molecular level (Lossos et al. 2002, de Vos et al. 2003, Elenitoba-Johnson et al. 2003, Martinez-Climent et al. 2003, Glas et al. 2005, Davies et al. 2007). The transformation is a heterogeneous process, and uniform gene expression changes are not therefore expected. There have, however, been similarities between different studies, e.g. increased *MYC* (Lossos et al. 2002, Martinez-Climent et al. 2003, Glas et al. 2005, Davies et al. 2007, Gentles et al. 2009), and increased *NEK2* (de Vos et al. 2003, Glas et al. 2005, Andreasson et al. 2009) expression in transformed DLBCL. Using supervised classification with a paired samples series of 12 FL patients whose disease later transformed to aggressive disease, Glas et al. (2005) established an FL stratification profile with 81 genes that could distinguish low-grade from high-grade disease with approximately 94% accuracy in an independent validation set. Gentles et al. (2009) used module network analysis with previously published data and found inappropriate activation of pluripotency-related genes, including genes expressed in embryonic stem cells, such as *MYC* and its target genes, to be related to histological transformation in FL. Conversely, high expression of transforming growth factor-β (TGF-β) signalling and stromal genes was associated with an improved prognosis.

Using highly purified tumour cells derived from FL and transformed DLBCL patients, Andreasson et al. (2009) screened a human genome array to define molecular determinants of transformation. Among the 79 upregulated transcripts, 13 genes were selected for validation with immunohistochemistry (IHC). Two of these proteins, galectin-3 and NEK2, could identify a subgroup of transformed DLBCL when compared with FL.

4.3.4. Gene expression changes related to chromosomal aberrations

When the gene expression profiles of FL patients with and without t(14;18) translocation were compared, Leich et al. (2009) reported an enrichment of germinal centre B-cell-associated signatures in patients with translocation, whereas activated B-cell-like, NFκB, proliferation and bystander cell signatures were enriched in t(14;18)-negative patients. When the results were validated with IHC in an independent patient cohort, decreased expression of CD10 and an increased Ki67 proliferation rate was observed in the translocation-negative patient group. Array comparative genomic hybridization (aCGH) demonstrated frequent chromosomal gains in 1q, 2p, 7, 8q, 12q, 18q, and X, whereas losses occurred in 6q, 10q, and 13q. Only amplification in 18q21 correlated with an inferior outcome, but was also present in only 4 of 127 patients.

4.4. Biological prognostic factors in FL

Clinical prognostic parameters, such as the FLIPI, have been developed to risk-stratify FL patients. However, the discriminative power of these parameters is limited due to the complexity of the clinical behaviour of FL, and new markers related to the biology of the disease are needed. The FL tissue mimics the architecture of normal secondary lymphoid follicles, and the FL cells interact with various immune cells, such as follicular helper T-cells, suppressor regulatory T-cells (Tregs), follicular dendritic cells (FDCs) and macrophages, which define the tumour microenvironment. The immune system may either promote or restrict tumour cell development, depending on the relative distribution and activation status of various subpopulations of cells. Although gene expression and IHC studies have highlighted the importance of the tumour microenvironment in FL, some prognostic markers are also defined by the features of the malignant cells.

4.4.1. Characteristics of neoplastic cells

4.4.1.1. Proliferation index

The proliferation index (PI) is measured by Ki67 monoclonal antibody, which recognizes a nuclear antigen present in proliferating cells (Gerdes et al. 1983). In FL, the PI generally correlates with the histological grade (Martin et al. 1995, Koster et al. 2007), but its prognostic influence on the survival of FL patients is more controversial. In a study with 62 chemotherapy-treated FL patients, at a median follow-up of 71 months, patients with a PI below the median had a significantly prolonged PFS (median not reached vs. 15 months for those with a PI above the median, $p = 0.0006$) and improved OS (median not reached vs. 42 months, $p = 0.002$) (Koster et al. 2007). Saito et al. (2004) reported a significant correlation between an inferior response to treatment and high Ki67 expression in 18 FL patients after immunochemotherapy. However, other studies have failed to show a significant prognostic impact of PI (Cibull et al. 1989, Llanos et al. 2001, Farinha et al. 2005, de Jong et al. 2009). Another observation related to subject was described by Leich et al. (2009), that t(14;18)-negative FLs have a higher PI than t(14;18)-positive lymphomas.

4.4.1.2. Bcl-2 family proteins

Bcl-2-family proteins, including both pro- and anti-apoptotic members, play a crucial role in apoptosis by regulating mitochondrial integrity (Tait and Green 2010). Only some of the family members have been investigated in relation to the prognosis in FL. In immunohistochemical analyses, anti-apoptotic myeloid cell leukemia-1 (Mcl-1) protein was shown to correlate positively with an increasing grade of FL (Cho-Vega et al. 2004). In another study, high Mcl-1 expression mainly localized to centroblasts was related to a poor outcome (median OS 40.3 vs. 98.6 months for those with low Mcl-1 expression) (Michels et al. 2006). Using a protein microarray with microdissected follicles from 20 cases of FL and 15 cases of benign follicular hyperplasia, Gulmann et al. (2005) identified high Bcl-2/Bak and Bcl-2/Bax levels to be associated with early death from FL (median OS 4.1 vs. 11.4 years and 7.6 vs. 11.4 years, between FL patients with OS < 10 years vs. OS ≥ 10 years, respectively). However, overexpression of Bcl-X_L gene was demonstrated to correlate with the number of apoptotic lymphoma cells and linked to short OS ($p = 0.0129$) (Zhao et al. 2004). However, this has not been verified at the protein level (Farinha et al. 2005).

4.4.1.3. GC–related markers

Bcl-6 is a transcriptional repressor and master regulator of GC-stage B-cell development (Ye et al. 1997). CD10 is an integral membrane glycoprotein expressed on pro-B cells, and later during antigen-dependent GC maturation (McIntosh et al. 1999). Both of these follicular centre B-cell markers are expressed in a great majority of FLs (Dogan et al. 2000). Bilalovic et al. (2004) reported that the expression levels of Bcl-6 and CD10 varied significantly between 73 FL patients, and high expression of both markers was associated with a favourable OS, disease specific survival (DSS), and time to treatment failure (TTF).The same group also studied the clinical relevance of other GC-associated proteins, and found high PU.1 protein expression to correlate with improved OS and PFS in univariate analysis ($p = 0.003$ and $p = 0.001$) (Torlakovic et al. 2006). While Bcl-6 retained its predictive value in this study, the expression of CD10 lost its significance when FLIPI was included in the analysis. MUM-1/IRF4 is a member of the interferon regulatory factor (IRF) family of transcription factors, expressed in plasma cells and in a small percentage of GC B-cells (Falini et al. 2000). Higher MUM-1/IRF4 expression has been associated with high-grade disease in FL (Natkunam et al. 2001, Naresh et al. 2007). In a study investigating prognostic biomarkers before and after the introduction of monoclonal antibody therapy in FL, the presence of MUM-1/IRF4 was associated with a nearly two-fold increase in the risk of death (relative risk, RR = 1.75, 95% CI 0.95–3.22, $p = 0.07$) in patients who received immunochemotherapy (Sweetenham et al. 2010). Instead, in patients treated with chemotherapy only, MUM1/IRF4 expression was not associated with survival.

4.4.1.4. Cell cycle regulators

The p53 protein is a tumour suppressor involved in many cellular stress pathways (Junttila and Evan 2009). Mutations in the *p53* gene and resulting overexpression of the protein have been associated with the progression and histological transformation of FL (Lo Coco et al. 1993, Sander et al. 1993, Llanos et al. 2001). Within 185 FL patients at diagnosis, heterozygous *p53* mutation was detected in 6% of analyzed cases and it correlated with a shorter PFS (log-rank, $p < 0.001$) and OS (log-rank, $p < 0.001$) (O'Shea et al. 2008). Cyclins are cell cycle regulators often deregulated in human malignancies. In a gene expression study of 57 FL patients treated with CHOP chemotherapy, high cyclin B1 mRNA expression was associated with a favourable prognosis (Björk et al. 2005). However, despite the good correlation between cyclin B1 mRNA and protein levels, a cut-off level for cyclin-B1 protein expression with prognostic significance could not defined.

4.4.1.5. Others

The suppressor of cytokine signalling 3 (SOCS3) is a member of a family of cytokine suppressors that inhibit cytokine-mediated signalling via negative feedback inhibition of the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) signalling pathway and are rapidly upregulated in response to a wide range of cytokines and hormones (Starr et al. 1997, Alexander and Hilton 2004). When searching for genes co-operating with Bcl-2 in neoplastic transformation, Vanasse et al. (2004) identified SOCS3 overexpression in B-cells isolated from Eμ-Bcl-2 transgenic mice when compared to wild-type littermates. In addition, immunohistochemical analysis in a cohort of patients with FL revealed marked overexpression of the SOCS3 protein, which mostly co-localized with Bcl-2 expression in neoplastic FL cells. Later, the prognostic significance of SOCS3 protein overexpression was determined in 82 FL patients, and found to predict a decreased OS (median OS 10 years vs. 22 years for SOCS3-negative patients, $p = 0.001$) (Krishnadasan et al. 2006).). Yin-yang 1 (YY1) is a zinc finger protein reported to positively regulate *IL4* gene expression in lymphocytes (Guo et al. 2001) and to have a role in resistance to Fas-induced apoptosis through binding to the silencer region of the Fas promoter (Garban and Bonavida 2001). In addition, rituximab has shown to up-regulate Fas expression and sensitize B-NHL cell lines to Fas-induced apoptosis via inhibition of YY1 (Vega et al. 2005). High levels of YY1 gene expression have been associated with a shorter survival interval in FL (Sakhinia et al. 2007).

4.4.2. Role of the tumour microenvironment

Cancer cells are in continuous interplay with their microenvironment, and model it to support their own growth (Whiteside 2008). Although an immune infiltrate is a feature of most cancers, there is a failure in the host immune defence (Galon et al. 2006, Mihm et al. 1996). Especially in FL, the role of the microenvironment has been demonstrated in many studies. However, the published data on the prognostic significance of different subpopulations of cells, including macrophages, T-cells, and dendritic cells, is conflicting. This may be a consequence of differences in patient selection, experimental design, different techniques and statistical analysis (Table 4).

Table 4. List of published studies addressing microenvironmental prognostic factors in FL.

Study	Number of patients	End point	Treatment	Median age (years)	(FL)IPI range (%)	Biomarkers studied
Farinha et al. 2005	99	OS, PFS	BP-VACOP +RT	44	59/40/1	Bcl-2, Bcl-XL, Bcl-6, CD3, CD4, CD7, CD8, CD10, CD20, Ki67, CD21, CD57, CD68, TIA-1
Koster et al. 2005	46	OS, PFS	CVP + IFN-α2b	53	56/44	CD34, VEGF
Alvaro et al. 2006a	211	OS, PFS	Various, 44% CHOP, 15% CVP	+/- 56	39/38/23	CD68, STAT1
Alvaro et al. 2006b	211	OS, PFS	Various, 44% CHOP, 15% CVP	+/- 56	39/38/23	CD4, CD8, CD57, FoxP3, TIA-1, CD68, CD123
Carreras et al. 2006	97	OS	Various, 58% CHOP, 14% fludarabine	55	37/27/36	CD3, CD4, FoxP3
Lee et al. 2006	59	OS	Various indolent regimens	61/46	NA	CD4, CD7, CD8, CD25, CD68, FoxP3, Granzyme-B, TIA-1
Glas et al. 2007	58	transformation	Various indolent regimens, mostly CVP	55/46	50/27/23 vs. 97/3/0	Ki67, CD20, CD3, CD4, CD8, T-bet, CD57, FoxP3, CD21, CD23, CD68, CD69
Jorgensen et al. 2007	107	OS, EFS, transformation	Various	54	54/40/6	CD34, VEGF, Ki67
Kelley et al. 2007	94	OS	Various, 47% R	58	36/28/36	CD68, FoxP3, Bcl-2, CD10, MUM-1
Wahlin et al. 2007	139	OS, DSS	Highly various	60	32/32/35	CD19, CD3, CD4, CD8
Canioni et al. 2008	194	EFS	CHVP-I or R+CHVP-I	NA	NA	CD68
Tzankov et al. 2008	86	OS, DSS, FFS	Various	+/-57	7/46/47	FoxP3
Carreras et al. 2009	100	OS, PFS	Various, 62% CHOP	54	39/26/35	PD-1

Study	Number of patients	End point	Treatment	Median age (years)	(FL)IPI range (%)	Biomarkers studied
de Jong et al. 2009	31/30	PFS	Fludarabine / CVP	56/56	26/42/32 vs. 27/47/27	Ki67, CD20, CD3, CD4, CD8, FoxP3, CD21, CD23, CD68, CD69
Farinha et al. 2010a	105	OS, PFS, transformation	BP-VACOP + RT	45	61/38/1	CD4, CD8, CD20, CD21, CD25, FoxP3
Sweetenham et al. 2010	103/30/47	OS	ProMACE-MOPP / R-CHOP / CHOP + tositumomab or iodine I 131 tositumomab	47/55/50	59/33/8 vs. 50/37/13 vs. 58/40/2	CD68, MUM-1, FoxP3
Wahlin et al. 2010	37/33	OS	Various	NA	54/27/19 vs. 6/27/67	CD3, CD4, CD7, CD8, FoxP3, PD-1, GranzymeB, TIA-1, Perforin, CD57, CD56, CD68, Tryptase

BP-VACOP: bleomycin, cisplatin, etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone; CHOP: cyclophosphamide, doxorubicin, vincristine, prednisone; CHVP-I: cyclophosphamide, doxorubicin, etoposide, prednisolone, interferon; CVP: cyclophosphamide, vincristine, prednisone; DSS: disease-specific survival; EFS: event-free survival; FFS: failure-free survival; IFN: interferon; NA: not applicable; PFS: progression-free survival; ProMACE-MOPP: prednisone, methotrexate, doxorubicin, cyclophosphamide, etoposide, mechlorethamine, vincristine, procarbazine, and prednisone; OS: overall survival; R: rituximab; RT: radiotherapy

4.4.2.1. Macrophages

Macrophages are critical mediators of inflammation during the immune response to foreign pathogens, but can also display anti-inflammatory properties. They can be broadly divided into two categories, based on their surface phenotype and functions (reviewed in Gordon and Taylor 2005, Martinez et al. 2009). Type I (M1) macrophages are classically activated, usually proinflammatory effectors, expressing interleukin 12 (IL-12), tumour necrosis factor (TNF)-α, CC chemokines and nitric oxide (NO) synthase. However, type II (M2) or alternatively activated macrophages are considered to have anti-inflammatory properties, and to secrete IL-10. M2-type macrophages also express higher levels of scavenger receptors and proangiogenic factors. Tumour-associated macrophages (TAMs) have generally been shown to display an M2-like

phenotype. They have been associated with many cancers, usually with an increased abundance linked to more progressive disease. In a very recent study, B cells were shown to drive the polarization of macrophages to a unique, but M2-biased phenotype (Wong et al. 2010). The prognostic impact of macrophages in FL has recently been studied by several investigators in different patient cohorts (Table 5). Farinha and coworkers (2005) first reported the adverse prognostic value of an increased lymphoma-associated macrophage (LAM) content, as recognized by CD68 expression, in a homogeneous series of chemotherapy-treated patients. In another study, Alvaro et al. (2006a) observed a STAT1-positive subset of LAMs to be associated with an adverse outcome. Even though CD68 itself had no significant predictive value, considerably more CD68-positive LAMs were detected in patients without bone marrow involvement and with a low Ann Arbor stage (Alvaro et al. 2006a&b). Kelley et al. (2007) found a high extrafollicular LAM content to be associated with shorter survival in an initially treated FL patient cohort. Interestingly, in subgroup analysis, CD68 positivity only remained a statistically significant predictor in rituximab-naïve patients. Subsequently, other studies have also pointed out the ability of rituximab to circumvent the unfavourable outcome associated with a high macrophage count (Canioni et al. 2008). This is suggested to be related to the essential role of macrophages in rituximab-mediated ADCC (Uchida et al. 2004). The predictive value of prognostic markers was also shown to be dependent on specific treatment protocols by de Jong et al. (2009), who reported a dense and interfollicular infiltrate of CD68-positive LAMs to be associated with a longer time to progression in CVP-treated patients, while being a poor prognostic sign in fludarabine-treated patients.

Table 5. Prognostic macrophage markers in FL.

Biomarker	Proposed outcome	References
CD68	Good	Alvaro et al. 2006b, de Jong et al. 2009 (CVP)
	Poor	Farinha et al. 2005, Kelley et al. 2007 (R-naive), Canioni et al. 2008 (CHVP-I), de Jong et al. 2009 (Fludarabine), Wahlin et al. 2010
	No correlation	Lee et al. 2006, Glas et al. 2007, Canioni et al. 2008 (R+CHVP-I), Sweetenham et al. 2010
STAT1	Poor	Alvaro et al. 2006a

The association of microvessels with macrophages was earlier shown among B-cell non-Hodgkin lymphomas in a very heterogeneous patient population, including different lymphoma subtypes (Vacca et al. 1999). In other studies, the LAM content has not been found to be associated with the outcome (Lee et al. 2006, Glas et al. 2007, Sweetenham et al. 2010).

4.4.2.2. T cells

Another important cell type in the non-malignant infiltrate of FL is different T-cell subsets. Markers for T-cell subtypes include pan-T-cell marker CD3, CD4 for follicular and helper T cells, CD8 for cytotoxic T cells, FOXP3 for regulatory T cells (Tregs), and PD-1 for follicular GC T cells.

Several groups have examined the influence of different T cells in the progression of FL (Table 6). In most studies, the total number of T cells has not had an impact on the prognosis of the disease (Carreras et al. 2006, Glas et al. 2007, de Jong et al. 2009). However, the influence of different T-cell subtypes is less clear. Thus, the number of CD4+ cells was not related to survival in some studies (Farinha et al. 2005, Alvaro et al 2006b, Carreras et al. 2006), but a high number of these cells in the intrafollicular compartment was associated with rapid transformation to DLBCL (Glas et al. 2007) and a poor outcome (Wahlin et al. 2010). In contrast, high numbers of CD4+ cells, especially in perifollicular areas, were more common in patients with a long survival (82% of patients with >15 years OS vs. 53% of patients with <5 years OS, $p \leq 0.05$) (Lee et al. 2006). A high number of CD8+ cells was found to be a marker of a favourable prognosis as analyzed with IHC (Alvaro et al. 2006b, Wahlin et al. 2010) and flow cytometry (Wahlin et al. 2007), but it was not related to the transformation to DLBCL (Glas et al. 2007). The frequency and distribution between CD4+ and CD8+ cells have also been under study. A high CD4/CD8 ratio was shown to correlate with poor OS (Wahlin et al. 2010), and a follicular predominance of CD4+ versus CD8+ cells was associated with PFS (RR = 0.54, 95% CI=0.29–1.0, p = 0.05) (Farinha et al. 2010a).

The regulatory T cells (Tregs) have a central role in regulating immunotolerance, predominantly via the suppression of effector T-cell proliferation and cytokine production (Piccirillo and Shevach 2001, Yang et al. 2006, Oberle et al. 2007). The number and topographic distribution of Tregs, and their prognostic value in FL are variable. Interestingly, in contrast to most studies on solid tumours (Banham et al. 2006), an increased number of forkhead box P3 (FOXP3)-positive Tregs was associated with prolonged OS (5-year OS 80%, 74%, and 50% for Treg content of >10%, 5–10%, and <5%) (Carreras et al. 2006). The same study also reported that the number of Tregs was markedly reduced when transformation into DLBCL occurred. Concordant with these observations, a dense infiltrate (Alvaro et al. 2006b, Tzankov et al. 2008), a perifollicular infiltrate (Lee et al. 2006, de Jong et al. 2009) or a follicular infiltrate (Wahlin et al. 2010) of Tregs was shown to be a good prognostic sign. Similarly to observations with tumour-infiltrating macrophages, de Jong et al. (2009) found a high content of FOXP3+ Tregs to be associated with a favourable time to progression in CVP-treated patients, while being a marker of poor prognosis in fludarabine-treated patients. Some other studies have also reported a follicular Treg content to be associated

with an unfavourable prognosis (Kelley et al. 2007, Farinha et al. 2010a). Sweetenham et al. (2010), who investigated the prognostic value of different immune cells in FL patients obtained from three sequential Southwest Oncology Group trials, could not find any correlation between the Treg composition of lymph nodes and survival in patients treated either with or without therapy including rituximab.

An additional T-cell marker is PD-1, which is preferentially expressed in follicular GC T-cells (Keir et al. 2008). In a cohort of 100 FL patients with various treatments, a higher number of PD-1+ T cells was associated with improved survival (5-year OS of 50%, 77%, and 95% for patients with PD-1 ≤ 5%, 6–33%, and > 33%, $p = 0.004$) and a lower risk of transformation (5-year risk of transformation of 7% vs. 29% for patients with PD-1 ≤ 5%, $p = 0.05$) (Carreras et al. 2009). Another study also confirmed the positive prognostic importance of PD-1+ cells (Wahlin et al. 2010). CD57 is also a marker for GC follicular helper T cells, and more than 90% of CD4+/CD57+ cells were shown to coexpress PD-1 (Carreras et al. 2009). However, most studies have not found a correlation between the CD57+ cell content and outcome (Farinha et al. 2005, Glas et al. 2007, Wahlin et al. 2010), although Alvaro et al. (2006b) observed a lower infiltration of CD57+ cells more persistently in FL patients with fewer than four nodal sites and without bone marrow involvement.

Table 6. Prognostic T-cell markers in FL.

Biomarker		Proposed outcome	References
CD4	dense	Good	Lee et al. 2006
		No correlation	Farinha et al. 2005, Alvaro et al. 2006b, Carreras et al. 2006
	follicular	Poor	Glas et al. 2007, Wahlin et al. 2010
CD8	dense	Good	Alvaro et al. 2006b, Wahlin et al. 2007
		No correlation	Glas et al. 2007
	perifollicular	Good	Wahlin et al. 2010
FoxP3	dense	Good	Alvaro et al. 2006b, Carreras et al. 2006, Tzankov et al. 2008, de Jong et al. 2009 (CVP)
		Poor	deJong et al. 2009 (Fludarabine)
		No correlation	Glas et al. 2007, Sweetenham et al. 2010
	follicular	Good	Wahlin et al. 2010
		Poor	Kelley et al. 2007, Farinha et al. 2010a
	perifollicular	Good	Lee et al. 2006, de Jong et al. 2009
PD-1	dense	Good	Carreras et al. 2009, Wahlin et al. 2010
CD57	dense	Poor	Alvaro et al. 2006b
		No correlation	Farinha et al. 2005, Glas et al. 2007, Wahlin et al. 2010

In FL tissue, the malignant B cells reside in close contact with different T cells. In an *in vitro* model, large CD25+ primary human B cells have been shown to costimulate or downregulate T-cell responses, depending on the environmental conditions and activation status (Tretter et al. 2008). In particular, FL B cells have been shown to induce conventional T cells to express FoxP3 and to acquire a regulatory function, whereas normal B cells were not able to induce Treg conversion (Ai et al. 2009). In addition, FL B cells have been suggested to induce T-cell dysfunction by impaired immunological synapse formation (Ramsay et al. 2009). These regulatory functions induced by FL tumour cells may act as an immunosuppressive mechanism enabling cancer progression.

4.4.2.3. Microvessel density (MVD)

Angiogenesis, the formation of new blood vessels, is essential for the growth of both solid and haematologic malignancies. Linked to the number of infiltrating macrophages and mast cells, increased angiogenesis has been reported to correlate with progressive disease in B-cell NHL (Vacca et al. 1999, Ribatti et al. 2000). However, the study populations were very heterogeneous, consisting of various lymphoma subtypes and treatment regimens. Koster et al. (2005) investigated the significance of vascularization in a uniformly treated (CVP induction chemotherapy combined with IFN-α2b) FL patient cohort. In this study, increased vascularization was associated with improved survival (median OS > 94 months compared with 59 months for patients with lower MVD, $p = 0.03$). The authors also investigated mRNA expression of vascular endothelial growth factor (VEGF) in lymphoma specimens with *in situ* hybridization, but could not find any correlation between VEGF expression and vessel density due to a lack of VEGF expression.

In contrast, Jorgensen et al. (2007) studied MVD from 107 pretreatment FL samples and found an increased interfollicular MVD to be associated with progressive disease and inferior EFS and OS. They also noted a difference in patient survival according to the VEGF expression pattern: those with diffuse VEGF expression in the lymphoma cells had poorer OS than those with a focal expression pattern. Subsequently, other groups have similarly reported a negative association of increased MVD with survival in FL (Clear et al. 2010, Farinha et al. 2010b).

Other studies have compared the expression pattern of VEGF or other angiogenic factors in different entities of NHL, and observed little difference in the staining patterns between low- and high-grade lymphomas (Steward et al. 2002), or higher expression of VEGF in DLBCL (Tzankov et al. 2007, Giatromanolaki et al. 2008).

Paydas et al. (2009) reported OS to be significantly shorter in aggressive lymphomas expressing VEGF-A and VEGF-C, but not in indolent lymphomas.

Besides IHC studies in tumour tissue, serum angiogenic factors have been analyzed in heterogeneous groups of NHL patients. The higher pretreatment levels of serum endostatin, VEGF and basic fibroblast growth factor (bFGF) have been linked to poor survival (Salven et al. 1997, Salven et al. 2000, Bono et al. 2003). In a recent study including only FL patients, high serum levels of VEGF were associated with poor PFS (Labidi et al. 2009). Nevertheless, no valid biomarkers are currently available to predict the outcome in response to immunochemotherapy.

5. AIMS OF THE STUDY

The aim of the study reported in this thesis was to search for novel molecular insights into the clinical heterogeneity of FL. The main questions addressed in the study were:

1) What are the genes that predict the outcome of FL patients treated with rituximab in combination with chemotherapy?
2) Can microarray-based data be validated at the protein level, and more specifically, what are the expression patterns of prognostic genes in tumour tissues of FL patients treated with immunochemotherapy ?
3) What is the prognostic significance of the tumour microenvironment in the rituximab era of lymphoma therapies?
4) What are the functions of the identified biomarkers in lymphoma cells?

6. PATIENTS AND METHODS

6.1. Patients and treatments

Detailed descriptions of patient cohorts in different studies are provided in the original publications (I-V). Patients were selected on the basis of the availability of clinical information and histological material. As our aim was to specifically study the biologically relevant prognostic factors in FL patients in response to immunochemotherapy, the study populations consisted of FL patients treated with a combination of rituximab (R) and anthracycline-containing chemotherapy (CHOP: cyclophosphamide, vincristine, doxorubicin, and prednisone). Patients received rituximab as a front-line therapy or at relapse. Studies II and III included a control group of patients treated with first line chemo- or radiotherapy without rituximab. All samples were taken before treatment.

6.2. Cell lines

All human lymphoma cell lines were cultured in a humidified 5% CO_2 atmosphere at 37 °C in a medium described in Table 7, supplemented with 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml of streptomycin.

Table 7. Cell lines used in the study.

Cell line	Description	Culture medium	Study
HF-1	human FL (described in Eray et al. 1994)	RPMI + 10% FCS	V
Granta-519	human MCL (DSMZ ACC342)	D-MEM + 10% FCS	V
SuDHL4	human DLBCL, GC type (gift from J. Martinez-Climent, University of Navarra, Spain)	RPMI + 10% FCS	V
OCILy-3	human DLBCL, activated B-cell type (gift from J. Martinez-Climent, University of Navarra, Spain)	IMDM + 20% HS + 55μM β-mercaptoethanol	V

DSMZ: Deutche Sammlung von Mikroorganismen und Zellkulturen; FCS: fetal calf serum; HS: human serum; IMDM: Iscove's modified Dulbecco's medium; RPMI: Roswell Park Memorial Institute medium

6.3. Antibodies and other reagents

Table 8. Antibodies and cytokines used in the study.

Antigen	Description	Dilution or concentration	Source	Study
Epha1	polyclonal rabbit	1:200	Santa Cruz	I
Smad1	monoclonal mouse	1:100	Santa Cruz	I
CD31	monoclonal mouse	1:200	Novocastra Laboratories	I, IV
CD34	monoclonal mouse	1:100	Dako Cytomation	IV
CD3	monoclonal mouse	1:100	Novocastra Laboratories	II, V
CD4	monoclonal mouse	1:150	Novocastra Laboratories	V
CD68	monoclonal mouse	1:2000	Dako Cytomation	II, III
Mast cell tryptase	monoclonal mouse	1:2000	Dako Cytomation	III, IV
STAT5a	monoclonal mouse	1:500	Zymed Laboratories	V
FoxP3	monoclonal mouse	1:150	Serotec	V
Rituximab	chimeric	10 μg/ml	Mabthera, Roche	V
Cytokine	**Description**	**Concentration**	**Source**	**Study**
IL-2	recombinant human IL-2	10 ng/ml	Promokine	V
IL-4	recombinant human IL-4	10 ng/ml	Promokine	V
IL-6	recombinant human IL-6	10 ng/ml	Promokine	V
IL-7	recombinant human IL-7	10 ng/ml	Promokine	V

6.4. Gene expression analyses

6.4.1. Microarray with FL patient samples

Initially, 24 FL patients for the microarray study were selected on the basis of the treatment regimen including rituximab and the availability of fresh frozen lymph node tissue, containing enough material for mRNA analyses. All samples were taken before rituximab treatment, and all patients received rituximab for the first time. Of these patients, 17 had primary and 7 had relapsed disease. Details of the RNA extraction and the microarray and statistical methods are presented in studies I and V, and Appendix S1. Briefly, total RNA was isolated and analyzed for gene expression with the Agilent Human 1A Oligo Microarray (Agilent Technologies, USA). In study I, patient samples

were sorted into long-term responders (TTF >35months, n = 11) and short-term responders (TTF <23 months, n = 13), or into patients with a response (complete or partial response, n = 21) and no response (n = 3). In study V, patients were classified into groups of favourable (continuous remission, n = 11) or adverse (relapsed disease, n = 13) outcomes.

6.4.2. Microarray with cell line samples

To identify rituximab-induced genes, human B-lymphoma cell lines were treated with rituximab (10 µg/ml, Mabthera, Roche) for 3 h. The cells were pelleted and mRNA was extracted with a Nucleospin RNA II Kit (Macherey-Nagel GmbH) according to the manufacturer's instructions. The gene expression profiles of control- and rituximab-stimulated cells were compared with the Agilent Human 44K oligonucleotide microarray (Agilent Technologies). The gene expression analyses are described in detail in study V.

6.5. qRT-PCR

Total RNA was available from 22 patients used in the microarray. The expression of CUL4B, EPHA1, MARCO1, MXI1, RRAD, and SMAD1 mRNAs was measured by quantitative real-time PCR (qRT-PCR) with the TaqMan methodology (Inventoried TaqMan Gene Expression assays) using a Gene Amp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). GAPDH and TBP served as an endogenous control. A detailed description of the method is available in Appendix S1 of study I.

6.6. Immunohistochemistry

IHC staining was performed with formalin-fixed paraffin-embedded tissue sections, either as a part of a tissue array or on individual slides. At first, the sections were incubated at 56 °C for 30 min. After deparaffinization, heat-induced epitope retrieval was performed in 0.01 mol/l sodium citrate buffer (pH 6) at 121 °C for 3 minutes. The slides were usually incubated with antibodies overnight at 4 °C, and the staining was completed with Vectastain ABC kit reagents (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. The immunoreactions were visualized with 3-amino-9-ethylcarbazole, and counterstaining was performed with haematoxylin.

Antibodies used in IHC are listed in Table 8. A representative example of positive expression of each antibody is presented in Figure 6. A detailed description of the evaluation of the staining is provided in the original publications (I-V). All scorings were performed blinded.

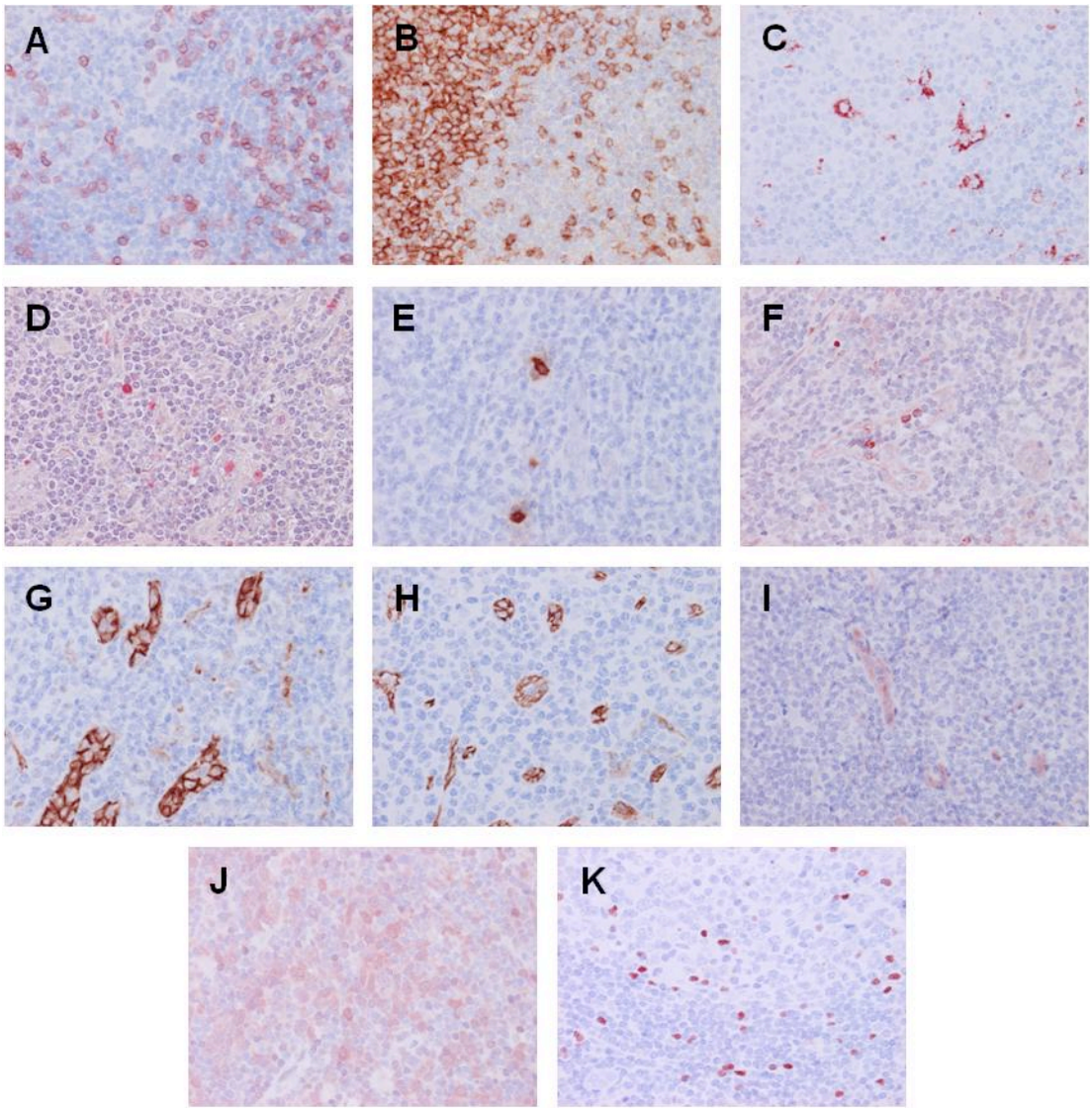

Figure 6. Positive expression in IHC staining for A) CD3, B) CD4, C) CD68, D) Leder stain, E) MCT, F) EphA1, G) CD31, H) CD34, I) Smad1, J) STAT5a and K) FoxP3.

6.7. Proliferation assays

HF-1 cells were plated at a density of $2x10^5$ cells/ml in 96-well plates, and exposed to rituximab (10 μg/ml) or rHu ILs (10 ng/ml) for indicated periods of time (V). Cell proliferation was measured with WST-1 reagent (Roche Diagnostics GmbH, Mannheim, Germany).

6.8. Statistical methods

The statistical analyses were carried out with SPSS 17.0 for Windows (SPSS, Chicago, IL, USA). The chi-squared test and Mann-Whitney U-test were used to compare the baseline characteristics and expression of mRNA and the protein level, or to evaluate the differences in the frequency for the prognostic factors. Survival rates were estimated using the Kaplan-Meier method, and the differences between the subgroups were compared with the log-rank test. PFS and OS were calculated from the time of the first day of therapy to the time of relapse or death from any cause or the date of the last follow-up. Both univariate and multivariate analyses were performed according to the Cox proportional hazards regression model. All P-values were two-tailed, and a significant level of probability was considered as <0.05.

7. RESULTS AND DISCUSSION

Many gene expression profiling and immunohistochemical studies have shown that the outcome of FL in response to chemotherapy is mainly related to the activity of non-malignant immune cells in the microenvironment of lymphoma tissue. The combination of rituximab with chemotherapy has significantly improved the outcome of FL patients, and immunochemotherapy is currently used as a standard first line therapy. However, most of the prognostic factors for FL have been identified during pre-rituximab era.

The current research has focused on identifying prognostic factors in FL in the rituximab era, exploiting both gene expression and immunohistochemical methods.

7.1. Identifying differentially expressed genes associated with the outcome in FL in response to immunochemotherapy (I)

7.1.1. Gene expression microarray

In study I our aim was to identify biomarkers, whose expression in lymphoma tissue was associated with the clinical outcome of immunochemotherapy treated FL patients. The screening cohort consisted of 24 FL patients treated with R-CHOP. The work was performed with an oligonucleotide-based cDNA microarray, with reference RNA from a panel of cell lines.

To obtain an overview of the gene expression data, we performed hierarchical clustering. In line with previous studies with chemotherapy-treated patients (Glas et al. 2005), the expression patterns between patients were relatively homogeneous, and the arising clusters did not correlate with clinical parameters. Therefore, we conducted a supervised learning classification, and ranked the genes according to their capability to separate the patients into favourable versus unfavourable groups.

Based on different clinical courses, the patients were first divided into those with a response (complete or partial response, n = 21) and with no response (n = 3). However, since the groups were unbalanced in size and the PFS varied significantly in the responding group, the patients were further divided into long-term responders (TTF >35months, n = 11) and short-term responders (TTF <23 months, n = 13), which included the non-responders. The groups were well balanced in age, grade, disease status and FLIPI. The genes whose corresponding mRNA levels varied most

significantly between differently responding patients with both distinctions were selected for further analyses. Interestingly, many of these genes were involved in the regulation of cell proliferation, apoptosis, signal transduction or immune responses.

Bohen et al. (2003) also analyzed the gene-expression patterns of 24 FL patients in response to rituximab. In their study, however, all patients had received at least one course of chemotherapy or anti-idiotype antibody (one patient) before receiving rituximab. Gene expression was only analyzed by comparing differentially expressed genes between rituximab responders and non-responders, and a list of genes expressed at significantly higher levels in either of the patient groups was presented. In addition to differences in patient cohorts, comparison of these gene lists with ours was complicated by the use of different array platforms with diverse probe sets and nomenclature.

In 2004, Dave et al. presented a survival model for FL with two survival-associated gene expression signatures, termed IR1 and IR2 for a good and bad prognosis, respectively. The IR1 signature included a number of genes encoding T cell-restricted proteins, whereas the IR2 signature reflected an immune infiltrate dominated by macrophages and/or follicular dendritic cells. Thus, the nature of the infiltrating immune cells was the predominant feature of the tumour that predicted the length of survival. GEP was performed on 191 patients, but none of them was treated with rituximab. Nevertheless, as a quality control for our microarray, we collected the genes representing IR1 and IR2 signatures, and constructed predictor scores with 31/43 and 18/24 genes corresponding to IR1 and IR2 signatures found from the array. In our patient cohort, only the IR1 signature had a prognostic impact on PFS in the univariate analyses ($p = 0.034$). The reason that IR2 was not associated with survival may be related to the role of macrophages in the mechanism of action of rituximab. This association will be discussed further in the following section.

7.1.2. Verification of differentially expressed genes with qRT-PCR (I)

The differences in mRNA expression between the long- and short-term responders were verified with qRT-PRC from 22 patients whose mRNA was still available for further analyses. From the group of distinctive genes with prognostic value according to univariate analysis, we randomly selected six genes with interesting biological functions and available primers. From these, *SMAD1*, *EPHA1*, *MARCO*, *RRAD*, and *MXI1* correlated between microarray and qRT-PCR, whereas the differential expression of *CUL4B* mRNA could not be validated with this method.

When the qRT-PCR results were correlated with patient outcomes, low *SMAD1* and *MARCO* mRNA levels predicted a significantly better PFS (median PFS 58 vs. 11

months, p = 0.019 for *SMAD1* and median PFS 58 vs. 15 months, p = 0.018 for *MARCO*). In addition, the patients with high *EPHA1* mRNA levels maintained a trend towards a better PFS (median PFS 58 vs. 21 months, p = 0.077).

The number of the genes validated by qRT-PCR was relatively small. However, expression levels from five out of six genes were consistent with microarray results, yielding a validation rate of 83%. In survival analysis, only two genes according to qRT-PCR results were significantly associated with survival. Obviously, our study population was small and the results require additional validation in an independent cohort of patients. Overall, comparisons between different studies are difficult (Johnson and Gascoyne 2008b). Byers et al. (2008) measured the expression levels of 35 candidate indicator genes selected from previous microarray studies using qRT-PCR, and found only two of these genes to be significantly associated with survival in their cohort of 60 FL patients.

7.1.3. Analysis of protein expression and localization with IHC (I)

To extend our results on gene expression to the protein level, and to examine the localization of protein expression, we performed immunohistochemical (IHC) analysis of paraffin-embedded lymphoma tissue obtained from 22 patients used in the microarray. Since EphA1 and Smad1 had previously been demonstrated to have an active role in lymphocyte signalling (Husson et al. 2002, Munoz et al. 2004, Aasheim et al. 2005), and commercial antibodies suitable for IHC were available, these molecules were selected.

The expression of EphA1 in FL tissue varied from total absence to strong intensity. However, the observed immunoreactivity was not localized to malignant cells, but instead to the surrounding microenvironment. Especially the vascular endothelium and granulocytes near to high endothelial venules showed the most intense staining. Interestingly, Smad1 staining also resulted in a similar finding. Although the expression pattern of Smad1 was quite diffuse, and tumour cells were also faintly positive, the most intense immunoreactivity was found in vascular structures.

Because of the diffuse staining pattern of Smad1, we first graded the stainings semiquantitatively. This approach, however, did not show a consistent correlation with either the mRNA results or patient outcome. Instead, if we counted the number of strongly EphA1-positive granulocytes from the whole tissue microarray (TMA) sections, a good correlation between mRNA and protein expression was achieved. Furthermore, long-term responders had higher numbers of EphA1-positive granulocytes than short-term responders (mean 14 vs. 8 per TMA core, p = 0.039). When we

subsequently validated these results in a separate group of 40 R-CHOP treated FL patients, we observed a trend towards a better outcome among patients with high EphA1 expression. This association of EphA1 expression with PFS in the validation group verified the reproducibility of the primary data.

Of the further verified genes, *SMAD1* expression was associated with a poor prognosis in our microarray results. Smad1 is a transcription factor mediating the signalling of bone morphogenetic protein (BMP) and transforming growth factor-β (TGFβ). It has been shown to be the most differentially overexpressed gene in FL cells when compared with normal GC cells (Husson et al. 2002). A role of Smad1 in TGFβ-mediated inhibition of cell proliferation in FL cells has also been suggested (Munoz et al. 2004). In our stainings, the immunoreactivity of Smad1 in lymphoma tissue was most prominent in vascular structures, although also observed in malignant lymphocytes. Likewise, Munoz et al. detected phosphorylated forms of Smad1 in FL cells, whereas no staining was observed in normal GC B-cells. However, normal lymphoid tissue frequently contained phospho-Smad1-positive endothelial and epithelial cells (Munoz et al. 2004).

EphA1 is a receptor tyrosine kinase, which has been shown to be involved in trans-endothelial migration (Aasheim et al. 2005) and to regulate T cell interactions (Sharfe et al. 2008). Patients with reduced EphA1 expression have been reported to comprise poor survival among patients with colorectal carcinomas (Dong et al. 2009). Eph and ephrin molecules control the angiogenic remodelling of blood vessels, and recent evidence suggests this to also happen in tumour neovascularization (reviewed in Kuijper et al. 2007). We observed EphA1 activity in the vasculature of FL tissue, but most intensively in granulocytes located close to high endothelial venules. Supported by a study indicating that granulocytes contribute to the biological activity of rituximab (Hernandez-Ilizaliturri et al. 2003), we can speculate that Epha1-expressing granulocytes might migrate to lymph nodes and enhance the ADCC effect of rituximab.

The genes associated with distinction in the outcome also included *MARCO*, which is a scavenger receptor expressed in a subpopulation of macrophages (Elomaa et al. 1995). It has a role in immune responses by mediating binding and phagocytosis of bacteria (van deer Laan et al. 1999), but also in tumour antigen-stimulated phagocytosis by dendritic cells (Grolleau et al. 2003). In a recent study, targeting of MARCO expression was shown to enhance both the trafficking and anti-tumour efficacy of tumour lysate-pulsed dendritic cells (Matsushita et al. 2010). This supports our notion that high *MARCO* expression is a marker of inferior survival. Unfortunately, we could not validate these findings with immunohistochemistry, because at the time of these analyses no commercial antibody was available for MARCO.

7.2. The role of innate immune cells (II, III)

The finding that the molecular signature in the surrounding cells of the microenvironment was associated with the outcome of FL patients treated with R-CHOP encouraged us to investigate the predictive value of other bystander cell types in these lymphoma tissues. Malignant B cells are surrounded by many types of innate immune cells, including macrophages, mast cells, T-cells, and follicular dendritic cells. The presence of these cells has been examined in FL patients treated with chemotherapy, but not revalidated in the rituximab era. Together with the fact that effector cells are required for the ADCC capacity of rituximab (Flieger et al. 2000, de Haij et al. 2010), we were interested in evaluating the role of tumour-infiltrating immune cells as a response to R-CHOP treatment.

7.2.1. High tumour-associated macrophage (TAM) content predicts a favourable outcome in FL patients treated with R-CHOP (II)

In order to investigate the prognostic value of macrophages in FL tissues, we performed anti-CD68 staining on paraffin-embedded tissues from 96 FL patients treated with R-CHOP either first line (n = 71) or at relapse (n = 25). As a control group we had 45 patients treated with chemotherapy or radiotherapy. The number of CD68+ cells was counted as absolute cell numbers in areas with the highest abundance of positive cells, mainly in interfollicular areas. Similarly to previously published results (Farinha et al. 2005), a high TAM content was associated with worse PFS in the control group (median PFS 17 months vs. 41 months for patients with low TAM score, $p = 0.026$). Surprisingly, however, in R-CHOP-treated patients a high TAM number was associated with a significantly better prognosis (median PFS not reached vs. 45 months with low TAM score, $p = 0.006$). As a continuous variable in Cox univariate analysis, the TAM score was also a predictor for OS ($p = 0.030$). This effect was specific, regardless of the disease stage before treatment, since PFS rates tended to be better with an increasing TAM number in both small subpopulations of patients treated upfront or later at relapse.

Subsequently to our findings, other groups have also observed the reversing effect of the addition of rituximab to chemotherapy on TAM-associated survival (Canioni et al. 2008). These contradictory observations on the prognostic role of TAM content depending on the treatment with or without rituximab are likely to be related to the critical role of macrophages in B-cell depletion after CD20 antibody-based therapy through their functions in ADCC (Uchida et al. 2004). In a mouse model deficient in the Fc common γ-chain, B cells were not depleted after CD20 antibody treatment. In another study, depletion of macrophages in a mouse lymphoma model eliminated the

anti-tumour effect of CD20 antibody (Minard-Colin 2008). The correlation between FcγR polymorphism and the outcome was proven to be specific to immunochemotherapy, and not to result from the chemotherapy response or underlying clinical features of the disease (Weng et al. 2009). Taken together, the prognostic role of TAMs appears to be treatment-dependent. In the context of immunochemotherapy, a high TAM content is suggested to be critical for the efficacy of rituximab, and thus to be associated with a favourable outcome.

Leidi et al. (2009) investigated the factors modulating the tumour cell killing potential of macrophages, and observed that cytokines secreted by lymphoma cells can favour the alternative activation of TAMs with a phagocytic capacity towards rituximab-opsonized target cells. In addition, they reported that M2-type macrophages had 2- to 3-fold greater phagocytic activity compared with M1-type. The "classical" macrophage marker CD68 has been shown to cross-react with fibroblasts and endothelial cells (Kunisch et al. 2004, Gottfried et al. 2008). To precisely define the contribution of TAMs to lymphoma growth, in-depth characterization of TAMs would be warranted. CD163 is a scavenger receptor restricted to the monocyte/macrophage lineage, and suggested to be a useful marker of anti-inflammatory or alternatively activated (M2) macrophages (Buechler et al. 2000). In a study by Clear et al. (2010), increasing numbers of CD163+ TAMs were shown to correlate with angiogenic sprouts in a poor prognosis group in FL. Therefore, it would also be of interest to further characterize the prognostic TAMs in our rituximab-treated FL patients.

7.2.2. High content of tumour-infiltrating mast cells is associated with a poor prognosis in FL patients treated with R-CHOP (III)

Mast cells (MCs) were another interesting inflammatory cell type not previously investigated in FL. In addition, as our immunohistochemical results with Epha1 localized into neutrophils, we also wanted to more generally examine the abundance of neutrophils in FL tissue. These cell populations are possible to detect with enzymatic Leder staining (Leder 1964). To confirm the results from MC staining, a subset of samples was also evaluated for MC tryptase immunoreactivity.

The patient population in this study consisted of 98 FL patients, from which 70 received R-CHOP front line and 28 at first relapse. Whereas the neutrophil count had no significant influence on the outcome, the MC content was negatively associated with survival. In Kaplan-Meyer analysis with the median as the cut-off level, PFS after the R-CHOP regimen was significantly worse among the patients with high numbers of MCs compared to those with low numbers (4-year PFS 34% vs. 74%, $p = 0.002$).

When the patients receiving R-CHOP either as a front- or second-line therapy were analyzed separately, a high MC content remained as a negative prognostic factor in both groups. In contrast, if the patients treated with rituximab at relapse were analyzed in relation to their first treatment option, the MC count lost its prognostic significance, and instead showed an opposite trend towards an improved outcome.

To further assess the prognostic value of tumour-infiltrating MCs relative to other cell types, and especially TAMs, we investigated TAM-related PFS separately in patients with a low and high MC content. In line with our previous results with TAM, patients with low TAM scores (≤ upper tertile) had a worse PFS than those with high TAM scores (> upper tertile) (4-year PFS 60% vs. 100%, $p = 0.006$). On the contrary, among the patients with a high MC score, no effect of TAM content on survival was observed (median PFS 43 months vs. 35 months, $p = 0.98$).

Over the past few years, many studies have highlighted the correlation between the presence of tumour-infiltrating MCs and the degree of tumour aggressiveness in B-cell neoplasms, including Hodgkin lymphoma and DLBCL (Molin et al. 2002, Canioni et al. 2009, Hedström et al. 2007). MCs are mainly located in tissues that interface with the external environment, where they secrete various biologically active mediators, including growth factors, cytokines and proteases, that might be beneficial to the tumour (Theoharides and Conti 2004). In a pancreatic islet tumour model, activation of the pleiotropic transcription factor Myc triggered a rapid recruitment of mast cells to the tumour site, leading to angiogenesis and tumour expansion (Soucek et al. 2007). In another study, tumour-infiltrating MCs were demonstrated to remodel the tumour microenvironment and promote tumour growth by intensifying inflammation and immunosuppression through augmenting tumour cell NF-kappaB and AP-1 activities, and by enhancing the suppression of T cells and NK cells (Huang et al. 2008). Recruitment and activation of MCs was mainly mediated by the tumour-derived stem cell factor (SCF). MCs are also suggested to directly influence B cells. In a coculture assay with mouse cells, MCs were shown to promote both the survival and activation of naive B cells as well as to induce the proliferation and further plasma cell differentiation of activated B cells (Merluzzi et al. 2010).

The finding that a high MC content predicts inferior PFS in the same patient cohort only after combined rituximab and chemotherapy, and not after chemotherapy alone, suggests that rituximab has a significant influence on the prognostic strength of MCs in FL. In addition, the presence of many MCs disturbed the prognostic impact of TAMs. This may imply that tumour-infiltrating MCs negatively regulate ADCC either by controlling the activation of macrophages or by the engagement of rituximab via their own Fcγ receptor (Siberil et al. 2007).

7.3. Increased CD31-positive microvessel density correlates negatively with survival in FL patients after R-CHOP therapy (I, IV)

7.3.1. Analysis of CD31-positive microvessel density (MVD)(I, IV)

Considering that both Epha1 and Smad1 expression were partly localized to vascular structures, we evaluated more precisely the vasculature with CD31 staining in our microarray patient cohort. However, we could not observe a significant difference in microvessel density (MVD) between long- and short-term responders (mean number of CD31-positive vessels 13.9 vs. 13.2 per TMA core, $p = 0.74$). Interestingly, when the mRNA values were compared between EPHA1, SMAD1 and CD31, we detected a correlation between SMAD1 and CD31 expression ($r_s = 0.588$, $p = 0.003$). In addition, low mRNA levels of CD31 were associated with better PFS (median PFS not reached vs. 21 months, $p = 0.036$).

Based on our findings at the gene-expression level, and knowing the capability of tumour-infiltrating MCs in remodelling the tumour microenvironment and growth by potentiating angiogenesis, we investigated whether the tumour vasculature is associated with the MC content in a larger cohort of 95 FL patients. Immunohistochemistry was performed with CD31 staining as before, but CD34 staining was also applied ($n = 55$).

In immunohistochemical staining, a typical pattern of vessel distribution was observed, showing prominent vascularization in the interfollicular areas, and only few vessels within the follicles. MCs co-localized with the vessels, and a statistically significant correlation between the MC count and CD31+ MVD was found ($r_s = 0.260$, $p = 0.023$). According to Cox univariate analyses, CD31+ MVD had a prognostic impact on PFS as a continuous variable ($p < 0.001$). Furthermore, according to Kaplan-Meyer analyses, the patients in the high CD31+ MVD group (> lowest tertile) had a significantly worse outcome than those in the low MVD group (5-year PFS, 43% vs. 81%, $p = 0.002$, 5-year OS, 82% vs. 97%, $p = 0.048$). When grouped together with the MC content, those patients having both high CD31+ MVD and a high MC content had a very poor outcome. In contrast, those patients having low values in both parameters had an exceptionally good prognosis (5-year PFS 35% vs. 90%, $p = 0.007$). Although we observed a borderline correlation between CD34+ and CD31+ MVDs ($r_s = 0.341$, $p = 0.056$, $n = 32$), CD34+ MVD was not associated with survival.

Our finding of the negative impact of MVD on the prognosis in FL is opposite to the results reported by Koster and co-workers (Koster et al. 2005). This might reflect their treatment regimen including IFN-α2b, which is known to have antiangiogenic effects (Minischetti et al. 2000). Therefore, the patients with a high MVD might be more susceptible to the antiangiogenic effects of IFN-α2b therapy than those with low vascularity, and thus the reverse prognostic role of MVD would be treatment dependent.

Other studies have mainly shown results similar to ours, although these have included only chemotherapy-treated patients (Jorgensen et al. 2007, Farinha et al. 2010b). Considering that CD31 expression can also be detected in macrophages and dendritic cells lining the sinuses of the lymph nodes (McKenney et al. 2001, Hattori et al. 2003), we performed additional CD34 staining, which is specific for vascular endothelial cells (Podgrabinska et al. 2002). However, we observed no correlation between CD34+ MVD and survival. Clear et al. (2010) investigated the smallest neovascular angiogenic sprouts in diagnostic biopsies of FL patients at the extremes of survival and found higher angiogenic activity in the poor prognostic group. In accordance with our results, they observed that only CD31 staining was significantly prognostic in contrast to CD34. This difference was suggested to result from CD31 expressing vascular channels, because analysis with lymphatic markers showed no difference between prognostic groups. They also reported an association between increased sprouting and elevated numbers of tumour-infiltrating CD163+ macrophages.

So far, our group is the only one that has investigated the prognostic impact of MVD on the survival of FL patients uniformly treated with R-CHOP. In this regard, the addition of rituximab to chemotherapy appears not to counteract the predictive role of vascularity.

7.3.2. Prognostic impact of other angiogenic markers (IV)

In order to obtain support for our immunohistochemical results, we analyzed the prognostic significance of genes involved in angiogenesis using our previous microarray data set with updated follow-up information (median follow up 73 months). Consistent with the data from CD31 staining, high PECAM1 mRNA levels in the FL tissue (>median) associated with an adverse outcome (5-year PFS 8% vs. 82%, $p = 0.001$). The patients with high CD34 mRNA levels also had a non-significant trend towards poorer PFS (5-year PFS 64% vs. 25%, $p = 0.097$). When we studied the expression of other angiogenic factors in relation to survival, we identified high VEGF mRNA levels (>highest tertile) to be associated with a favourable outcome (5-year PFS 86% vs. 25% in low VEGF group, $p = 0.015$).

Consistent with our microarray results, Lenz et al. (2008) reported a gene signature enriched with genes attributed to endothelial cell characterization and function (e.g. CD31) to be associated with a poor patient outcome in DLBCL. Studies concerning the role of VEGF in lymphomas have mainly focused on comparisons between different histological lymphoma subtypes (Jorgensen et al. 2007, Paydas et al. 2009). Lymphoma growth effected by VEGF or its receptors seems to be potentiated by at least two different mechanisms: the autocrine stimulation of tumour cells via the expression of

VEGF and VEGF receptors on lymphoma cells, and the paracrine effect of the tumour microenvironment. These distinct angiogenic mechanisms appear to be potential therapeutic targets in lymphomas. Preliminary clinical studies on antiangiogenic therapies have reported some promising results, and various antiangiogenic drugs are under development (Levine et al. 2006, Stopeck et al. 2009).

7.4. Identifying signalling pathways having clinical importance in FL (V)

7.4.1. Analysis of gene expression data (V)

In addition to the findings that the genes with a prognostic impact were mainly expressed on non-malignant cells, we also identified several genes known to be involved in cell signalling. We used the same microarray data with a systems biology approach to identify signalling pathways or groups of genes capable of separating patients into groups with favourable (continuous remission, n = 11) or adverse (relapsed disease, n = 13) outcomes. The Gene Ontology analysis of 404 differentially expressed mRNAs between patient groups demonstrated a statistically significant overrepresentation of genes involved in the biological processes of lymphocytes, such as activators of lymphocyte differentiation, and regulators of the I-κB kinase / NF-κB cascade and B-cell activation. Consistent with Onto Tool pathway analysis, PI3K signalling and Janus kinase (JAK) – signal transducer and activator of transcription (STAT) pathways separated the groups with favourable and adverse outcomes. Based on the literature concerning the importance of cytokine and STAT signalling in lymphoma biology (Guiter et al. 2004, Abraham et al. 2005, Alvaro et al. 2006a, Lam et al. 2008), we were motivated to further analyze whether the expression levels of STAT pathway-related transcripts differed between the patients who were in remission or had relapsed. The only STAT differentially expressed between the groups was *STAT5a*. However, many regulators of STAT activity correlated with *STAT5a* expression and were also differentially expressed between the patient groups. These included *SOCS1*, *PIM1*, *IL2RA*, *IL4R* and *IL7*.

In univariate analyses, *STAT5a*, *SOCS1*, *PIM1*, *IL2RA*, *IL4R* and *IL7* had a prognostic effect on PFS, as also did a signature score constructed from them. This STAT score was built by summing the mRNA expression values of the genes positively correlating with PFS (*STAT5a* and *PIM1*), and dividing it by the sum of the mRNA expression values of the genes negatively associated with the outcome (*SOCS1*, *IL2RA*, *IL4R* and *IL7)*.

STATs are a family of proteins mediating cytokine and growth factor-induced signals. STAT signalling has been implicated in the control of multiple cellular responses, including proliferation, differentiation and apoptosis (reviewed in Levy and Darnell 2002). STAT5a and STAT5b are encoded by two closely linked genes and share 96% homology (Liu et al. 1995). Mice deficient in Stat5a/b have severely impaired lymphoid development and differentiation (Yao et al. 2006). Supressors of cytokine signalling (SOCS) proteins are important negative regulators of STATs. However, the serine-treonine kinase Pim-1 is a target gene for STAT5, but it has also been found to co-operate with SOCS proteins in inhibiting STAT5 activity, suggesting the existence of a negative feedback mechanism (reviewed in Paukku and Silvennoinen 2004). Somatic hypermutation of *SOCS1* has been reported in lymphocyte-predominant Hodgkin lymphoma (Mottok et al. 2007), whereas *PIM1* mutations are found in DLBCL (Pasqualucci et al. 2001). IL-4 regulates proliferation, differentiation and apoptosis in B cells and mediates immunoglobulin class-switching to IgE and IgG (Avery et al. 2008). In addition, IL-4 is reported to be a potent stimulator of FL cells in *vitro* (Schmitter et al. 1997), and the FL microenvironment is observed to contain increased levels of IL-4 (Calvo et al. 2008). IL-7 is an essential, nonreduntant growth factor for early B- and T-cell precursors (Sudo et al. 1993) that also exerts effects on mature peripheral T cells (Schluns et al. 2000). In addition, IL-7 has been identified to have a role in the development and maintenance of dendritic cells (Vogt et al. 2009). The IL-7-STAT5 signalling pathway has been indicated to have a role in early B-cell lymphopoiesis, and this may contribute to leukemogenesis in humans (Malin et al. 2010).

Taken together, the association of several STAT pathway components with survival suggests that this pathway may have some previously unidentified functions in FL biology.

7.4.2. STAT5a protein levels and their association with outcome (V)

To validate the gene-expression data and to confine the localization of STAT5a expression, we performed IHC staining for STAT5a on the lymphoma tissue of 81 FL patients uniformly treated with first-line R-CHOP. Overall, faint STAT5a reactivity was seen throughout the lymphoma tissue. STAT5a expression localized to both perifollicular and follicular areas, but was generally low in neoplastic follicles. STAT5a positivity often seemed to colocalize with CD4-positive lymphocytes, which was equivalent to a positive correlation in the mRNA level ($r_s = 0.537$, $p = 0.007$). However, no correlation with common T-cell marker CD3 or regulatory T-cell (Treg) indicator FoxP3 was observed. Instead, based on morphological features, a subpopulation of follicular dendritic cells appeared to show prominent STAT5a reactivity.

We next investigated the prognostic value of STAT5a expression in the lymphoma tissue. In accordance with the findings on the mRNA level, high STAT5a expression (>median) was associated with improved PFS (median PFS not reached vs. 46 months, $p = 0.001$).

The finding that the expression of STAT5a did not correlate with FoxP3 was rather surprising, since STAT5a is a known activator of FoxP3-positive Tregs (Yao et al. 2007, Passerini et al. 2008). Neither did we observe any prognostic significance of FoxP3-positive Tregs, although several reports have illustrated the predictive value of tumour infiltrating Tregs in FL (Carreras et al. 2006, Lee et al. 2006, de Jong et al. 2009, Wahlin et al. 2010). Instead, we found a common T cell marker CD3 to correlate with FoxP3+ cells ($r_s = 0.468$, $p = 0.001$, $n = 49$). Clearly, our sequential stainings with STAT5a and CD4 were too few ($n = 10$) to draw any definite conclusions and will require additional validation. The specific characterization of the STAT5a-expressing cell population in FL tissue will be of further interest, and may be accomplished with double staining in IHC or flow cytometry.

In this regard, a subpopulation of GC B-cells characterized by phosphorylated STAT5 expression with an activated centrocyte phenotype has recently been identified (Scheeren et al. 2005). Activated STAT5a was shown to modulate Bcl-6 transcription and thereby regulate memory B cell differentiation. In lymphomas, however, the STAT5 responsive region of the *BCL6* gene has been reported to be frequently mutated, which might contribute to the pathogenesis of the disease (Walker et al. 2007).

7.5. Assessment of functional significance with cellular studies (V)

To further understand the signalling between non-malignant and FL cells, we performed experiments with CD20-expressing HF-1, SuDHL-4, Granta-519, and OciLy-3 B-cell lymphoma cell lines. Of these cells, HF-1 represents the FL cell type, whereas SuDHL-4 and OciLy-3 resemble DLBCL (germinal centre derived and activated B-cell type) and Granta-519 MCL. The cells were exposed to rituximab for various times and their growth monitored. In agreement with previous studies, the growth of HF-1 and SuDHL-4 cell lines was significantly reduced after rituximab treatment (Czuczman et al. 2008, Mattila and Meri 2008). However, in Granta-519 the growth-inhibitory effect of rituximab was less obvious, and OciLy-3 cells did not seem to respond to rituximab at all. In a growth assay with various interleukins (ILs; IL-2, IL-4, IL-6, and IL-7), we could not observe any further enhancement of the growth-inhibitory effect of rituximab. Neither did any of the ILs have a direct effect on the growth of the tested cells.

To identify the genes regulated by rituximab, we performed a gene expression microarray with lymphoma cell lines, comparing control and rituximab-stimulated samples. This screening identified differentially expressed ILs especially in germinal centre derived and rituximab sensitive cell lines, HF-1 and SuDHL-4. In HF-1 cells, we detected 4.1-, 3.4-, and 2.7-fold induction of IL15, IL4 and IL23A genes in response to rituximab. In SuDHL-4 cells IL15 expression was 4.4 -fold induced.

Of these cytokines, IL-15 has been identified as an activator of regulatory and effector T-cells (Passerini et al. 2008). It has also suggested to stimulate NK cells and to enhance rituximab-mediated ADCC against B-cell lymphoma (Moga et al. 2008). IL-7 and IL-15 have together been reported to stimulate the DNA binding activity of STATs in cutaneous T-cell lymphoma (Qin et al. 2001). IL-4 is mainly reported to signal via STAT4/6, and it has been shown to be highly expressed in FL compared to follicular hyperplasia (Calvo et al. 2008). IL-23A has been observed to provide effective protection against malignant diseases. This feature was shown to be dependent on CD8-positive T-cells (Lo et al. 2003).

The finding that rituximab only induced IL expression in rituximab-sensitive cell lines suggests a central role of IL-mediated signalling in these cells. In addition, ILs are known regulators of STAT5a. Since high STAT5a expression was a favourable prognostic factor in R-CHOP-treated FL patients, we might speculate that rituximab-induced secretion of ILs in FL cells could activate STAT5a in T-cells. Consecutively, STAT5a levels might contribute to the efficacy of rituximab. Irish et al. (2010) used multiplex phosphoflow cytometry to profile single cells within human FL tumours. Stimulation of cells with IL-2, IL-7 and IL-15 led to specific phosphorylation of STAT5 in tumour-infiltrating T cells. In addition, lower IL-7 signalling in tumour-infiltrating T cells was observed in tumours with impaired BCR signalling, which was negatively correlated with overall patient survival. In accordance with our results, Meier et al. (2009) found that the expression of tyrosine-phosphorylated STAT5 was associated with a better prognosis in FL. In addition to tyrosine phosphorylation, the activity of several STAT proteins was shown to be modulated by serine phosphorylation (reviewed in Decker and Kovarik 2000). For example, in a mouse transplantation model, serine phosphorylation was reported to play a crucial role in STAT5-driven leukemogenesis (Friedbichler et al. 2010). Therefore, although we observed a correlation between the basal expression level of STAT5a and the prognosis in FL, both at the mRNA and protein level, analysis of activated forms of STAT5a would be also warranted.

In the future, further characterization will be needed to determine how rituximab modifies STAT5a activity. Besides, it would be of interest to specifically define the interacting cell populations and signalling networks.

8. CONCLUSIONS

During recent years we have witnessed a significant improvement in the life expectancy of FL patients as a consequence of the combination of rituximab with chemotherapy and rituximab maintenance therapy. On the other hand, GEP studies and immunohistochemical analyses have demonstrated the importance of the microenvironment in FL. The precise nature of the different cell populations and the molecular mechanisms involved in the reciprocal interactions between malignant and non-malignant cells are still unclear. In addition, the published data concerning the prognostic impact of diverse cell populations are conflicting and were mainly produced in the pre-rituximab era.

In this study, we have identified genes that may help to identify FL patients who benefit from the current therapy. Our results suggest that the characteristic properties of the tumour microenvironment in FL determine the responses to immunochemotherapy, and that these characteristics may even have opposite effects on the outcome depending on the specific treatment. We have also identified the JAK–STAT pathway to be associated with the prognosis in FL patients, and to possibly be involved in mediating cross-talk between FL cells and tumour inflammatory cells. Therefore, signals from FL cells to the surrounding microenvironment can also have a biological effect.

Taken together, prognostic biomarkers need to be re-evaluated in the current rituximab or antibody era of lymphoma therapies. In addition, when evaluating these biomarkers, the balance of the entire microenvironment should be considered instead of individual cell subsets. A more complete understanding of the molecular mechanisms involved will provide the basis for the optimal use of existing therapies and the development of novel treatments for FL.

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