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Molecular Characterization of Diffuse Large B-cell Lymphoma and Aspects of Transformation

BY

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Abstract

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Lymphomas are a heterogeneous group of neoplasias originating from B- or T-lymphocytes. In this thesis, we determined the genetic and immunophenotypic characterization of DLBCL and their prognostic impact. Moreover, genomic alterations associated with the transformation to DLBCL from Hodgkin lymphoma (HL) and follicular lymphoma (FL) were elucidated.

In order to outline the impact of cytogenetic as well as immunophenotypic prognostic markers in DLBCL, we firstly studied a series of 54 DLBCL tumors using comparative genomic hybridization (CGH) and we identified several frequently occurring chromosomal imbalances. Loss of 22q was more often found in the diagnostic tumors with a more advanced clinical stage, while gain of 18q21 was more commonly identified in relapses. Secondly, we correlated the expression patterns of CD10, bcl-6, IRF-4 and bcl-2 with clinical parameters in a series of 173 de novo DLBCL patients. Patients with a germinal center (GC) phenotype displayed a better survival than the non-GC group. Expression of bcl-6 and CD10 was correlated with a better survival while bcl-2 expression was associated with a poor prognosis.

In approaching the HL transformation, two novel B-cell lines (U-2932 and U-2940), derived from patients with DLBCL following HL, were characterized. Interestingly, a translocation with materials from 2q and 7q as well as loss of material on 6q was found in both cell lines. For FL transformation, we assessed chromosomal alterations in a panel of 28 DLBCL patients with a previous history of FL. The DLBCL tumors displayed more chromosomal imbalances compared to FL tumors. Loss of 6q16-21 and gain of 7pter-q22 were more commonly found in the DLBCL counterparts, suggesting the chromosomal location of putative genes that may be involved in the transformation process.

Keywords: Diffuse large B-cell lymphoma, Hodgkin lymphoma, follicular lymphoma, transformation, comparative genomic hybridization, prognosis

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To My Family

List of Papers

This thesis is based on the following papers which will be referred to in the text by their Roman numerals.

I Chromosomal imbalances in diffuse large B-cell lymphoma detected by comparative genomic hybridization.
Berglund M, Enblad G, Flordal E, Lui WO, Backlin C, Thunberg U, Sundström C, Roos G, Allander SV, Erlanson M, Rosenquist R, Larsson C and Lagercrantz S. *Modern Pathology 2002;15:807-817.*

II Evaluation of immunophenotype in diffuse large B-cell lymphoma and impact on prognosis.
Berglund M, Thunberg U, Amini RM, Book M, Backlin C, Roos G, Erlanson M, Linderoth J, Dictor M, Jerkeman M, Cavallin-Ståhl E, Sundström C, Rehn-Eriksson S, Hagberg H, Rosenquist R and Enblad G. *Submitted.*

III Molecular cytogenetic characterization of four commonly used cell lines derived from Hodgkin lymphoma.
Berglund M, Flordal E, Gullander J, Lui WO, Larsson C, Lagercrantz S and Enblad G. *Cancer Genetics and Cytogenetics 2003;141:43-48.*

IV A novel B-cell line (U-2932) established from a patient with diffuse large B-cell lymphoma following Hodgkin lymphoma.
Amini RM, **Berglund M**, Heideman A, Sambade C, Lagercranz S, Sundström C and Enblad G. *Leukemia and Lymphoma 2002;43:2179-2189.*

V U-2940: a human B-cell line derived from a diffuse large cell lymphoma sequential to Hodgkin lymphoma.
Sambade C, **Berglund M**, Lagercrantz S, Sällström J, Reis RM, Enblad G, Glimelius B and Sundström C. *Manuscript.*

VI Gain of 7pter-q22 and loss of 6q16-21 and association with transformation of follicular lymphoma to diffuse large B-cell lymphoma.
Berglund M, Enblad G, Amini RM, Thunberg U, Book M, Roos G, Erlanson M, Rosenquist R, Larsson C and Lagercrantz S. *Manuscript*.

Papers on related topics

The following two papers concerns related topics but are not included in the thesis.

- Clonal development of a blastoid mantle cell lymphoma studied with comparative genomic hybridization.
  Flordal E, **Berglund M**, Rosenquist R, Erlanson M, Enblad G, Roos G, Larsson C and Lagercrantz S. *Cancer Genetics Cytogenetics 2002;139:38-43.*

- Spontaneously immortalized human T-lymphocytes develop gain of chromosomal region 2p13-24 as an early and common genetic event.
  Siwicki JK, **Berglund M**, Rygier J, Pienkowska-Grela B, Grygalewicz B, Degerman S, Golovleva I, Chrzanowska K, Lagercrantz S, Blennow E, Roos G and Larsson C. *Genes Chromosomes and Cancer. In press*

Contents

ABBREVIATIONS

ABC	Activated B-cell like
CC	Centrocyte
CB	Centroblast
CGH	Comparative genomic hybridization
cHL	Classical Hodgkin lymphoma
CR	Complete remission
DAPI	4,6-diamidino-2-phenylindole
DLBCL	Diffuse large B-cell lymphoma
DSB	Double strand break
EFS	Event free survival
FDC	Follicular dendritic cell
FISH	Fluorescence *in situ* hybridization
FL	Follicular lymphoma
GC	Germinal center
GCB	Germinal center B-cell like
HL	Hodgkin lymphoma
HR	Homologous recombination
HRS	Hodgkin Reed-Sternberg
IGH	Immunoglobulin heavy chain
IHC	Immunohistochemistry
IPI	International prognostic index
NHL	Non-Hodgkin lymphoma
NLPHL	Nodular lymphocyte predominant Hodgkin lymphoma
OS	Overall survival
PCR	Polymerase chain reaction
REAL	Revised european american lymphoma
SHM	Somatic hypermutation
SKY	Spectral karyotyping
SSCP	Single stranded conformation polymorphism
TSG	Tumor suppressor gene
WHO	World health organization

INTRODUCTION

Cancer is a disease originating from a cell that has acquired a phenotype which enables it to outgrow its neighbors. To become fully cancerous, it is believed that a cell must accumulate mutations in a number of growth-controlling genes, as well as in other genes that enable it to become invasive or to spread throughout the body. This far almost 300 cancer genes have been reported, and of these 90% have shown somatic mutations in tumors, and 20% have exhibited germ line mutations in cancer patients [1]. Commonly the genes were identified when altered by chromosomal translocations creating a chimeric protein or placing the gene under the control of regulatory elements of another gene. Most cancer genes have been identified in leukemias, lymphomas or sarcomas even though these entities only constitute 10% of all human cancers. The known cancer genes are of two major types, i.e. oncogenes and tumor suppressor genes (TSGs).

Oncogenes

Oncogenes are mutated versions of normal genes, so called proto-oncogenes that are involved in a variety of cellular functions. Until date more than 100 proto-oncogenes have been identified and classified into five different categories based on their functional and biochemical properties: *(i)* growth factors, *(ii)* growth factor receptors, *(iii)* signal transducers, *(iv)* transcription factors, and *(v)* regulators of programmed cell death [2]. Activation of a proto-oncogene can be quantitative (increased production of the unaltered product) or qualitative (production of a modified product with altered abilities). The different mechanisms by which a proto-oncogene may be activated

include eg. point mutation, translocation, and amplification. The activation eventually leads to transformation of the normal cell into a neoplastic cell, able to form a tumor.

Tumor suppressor genes

Tumorigenesis does not only involve dominant activated oncogenes but also recessive genes, TSGs, which have diverse functions such as DNA repair, cell cycle control and apoptosis. In general both alleles must be inactivated by loss-of-function mutations or epigenetically by methylation, in order to produce a phenotypic effect. TSGs are commonly referred to as being of two major types, gatekeepers and caretakers [3]. Gatekeepers are genes that directly regulate the normal proliferation by inhibiting growth or promoting death such as *TP53* and *RB1*. Inactivation of such genes is rate-limiting for the initiation of a tumor. Caretakers, on the other hand, are DNA repair genes such as *ATM*, *Ku70* and *XRCC4*. When mutated, these genes do not directly promote tumor initiation, but rather gives a genetic instability with accumulation of mutations in gatekeepers or oncogenes, and finally tumor development. However, the underlying mechanism is still unknown. For some TSGs, the type of function is not fully understood, and some TSGs apparently have overlapping properties of both gatekeeper and caretaker such as in the cases of *BRCA1* and *BRCA2*.

Cancer cytogenetics

As mentioned earlier chromosomal aberrations and cancer cytogenetics have played a crucial part in the discovery of genes involved in cancer. It should also be noted that not only the occurrence of a chromosomal imbalance is important but also the chronological order in which they appear. Acquisition of microscopically visible mutations is a multistage process. It is of importance to distinguish between primary and secondary aberrations where pri-

mary aberrations are often found as sole karyotypic abnormalities. This implies that they have a causal role in establishing the neoplasm but is not necessarily the first mutation since a submicroscopic mutation may precede the chromosomal abnormality. Secondary aberrations, as the name implies, almost always occur in neoplasias already carrying a primary aberration. The term cytogenetic noise can be used to describe the non-clonal abnormalities that occur more as a consequence of the genetic instability produced by mutations in genes important for cell homeostasis. Structural chromosomal rearrangements has been associated with distinct tumor subtypes in many different types of human cancer [4]. Since most of these rearrangements has been found in hematological disorders it has been generally believed that the genetic mechanism for tumor initiation is different depending on tissue origin. However, a recent study show that the differences may be just a function of the number of cases with abnormal karyotypes and that the genetic mechanism for tumor initiation may be the same [5].

Hematopoetic neoplasias

Two major categories of cancer have been established including cancer originating from epithelial cells (carcinomas), and from mesenchymal tissues, such as lymphomas, leukemias and myelomas. Lymphomas or leukemias is the fifth most common type of cancer, constituting approximately 7-8 % of all cancer cases in Sweden [6]. Lymphoproliferative tumors are a group of malignant neoplasias derived from lymphocytes in various stages of differentiation (Figure 1). Depending on the stage of differentiation of the originating normal cell, tumor subtypes with unique characteristics are formed.

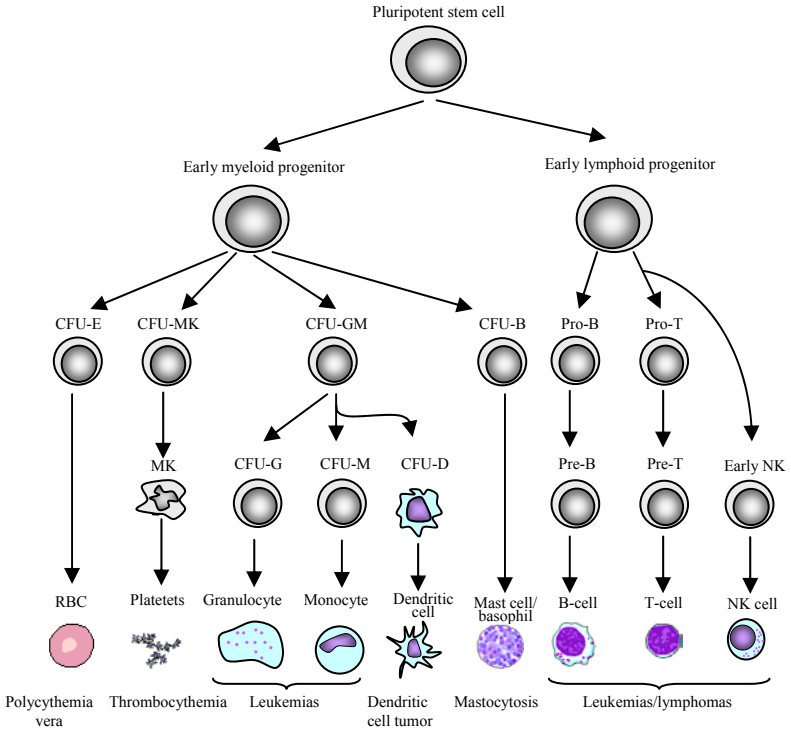

Figure 1. The hematopoesis and neoplasias derived from different cell types.

Historically the diagnosis and treatment strategy has been based on clinical and morphological features but molecular markers are becoming increasingly interesting. Classically lymphomas have been classified as Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL) where lymphomas of both T- and B-cell origin are included in the NHL category. This classification was maintained until recently when the REAL [7] and the WHO classifications were introduced [8] in which morphological, and in addition molecular and clinical features are taken into account. In the most recent classification, from WHO, three major categories of lymphoid neoplasms are recognized, i.e. B-cell neoplasms, T and NK cell neoplasms, and Hodgkin lymphoma (Table 1).

Table 1. Major categories and subtypes of lymphoid neoplasms according to the WHO classification.

Categories	Examples of subtypes
B-cell neoplasms	Precursor B-cell neoplasms Lymphoblastic leukemia/lymphoma Mature B-cell lymphomas Follicular lymphoma Diffuse large B-cell lymphoma Chronic lymphocytic leukemia Mantle cell lymphoma
T/NK-cell neoplasms	Precursor T-cell neoplasms Lymphoblastic leukemia/lymphoma Blastic NK-cell lymphoma Mature T-cell neoplasms Peripheral T-cell lymphoma Anaplastic large cell lymphoma Mycosis fungoides
Hodgkin lymphoma	Nodular lymphocyte predominant Classical Nodular sclerosis Mixed cellularity Lymphocyte rich Lymphocyte depleted

The germinal center

The B-cell development begins with differentiation of a pluripotent stem cell in the bone marrow which continues to differentiate until a functional B-cell receptor is expressed. The second stage starts when the cell migrates from the bone marrow and encounters an antigen through the B-cell receptor which initiates further differentiation in the lymph nodes. In the lymph nodes the B-cells are localized in follicles and the T-cells are distributed in surrounding paracortical areas. Some B-cell follicles also includes germinal centers (GC) where intense proliferation of B-cells takes place after encoun-

tering their specific antigen and T-helper cell (T_H). The GC reaction with a clonal expansion of antigen-activated B-lymphocytes is a hallmark of antibody-mediated immune responses (Figure 2). Patients suffering from defects impairing the GC formation are immunodeficient. B-cells in the GC are also thought to be involved in various types of human B-cell malignancies such as diffuse large B-cell lymphoma, Burkitt lymphoma, follicular lymphoma and Hodgkin lymphoma [9, 10]. In the GC reaction a naïve B-cell is activated by antigen receptor stimulation from a T_H-cell, and transforms into a centroblast that proliferates in the "dark zone" of the GC. Antigen receptor revision is performed through a process called somatic hypermutation (SHM) whereby single basepair changes are randomly introduced (1×10^{-3} bases per generation) into the IgV regions in order to select for immunoglobulin with higher affinity for a specific antigen [11]. The centroblast then develop into centrocytes (CC) which compose the "light zone" of the GC. Here the CC cells are positively or negatively selected depending on their ability to bind the antigen presented by the follicular dendritic cells and T-cells [11]. Most cells have acquired deleterious mutations in the IgV regions and are destined to apoptosis, while those cells with high affinity are selected for further differentiation. To produce antibodies with different effector functions a fraction of the GC cells also switches the expression from IgM and IgD to other Ig classes by isotype switch recombination. The IGH constant (C)-region is then replaced by a downstream C-region gene, i.e. Cγ, Cα and Cε. This allow expression of IgG, IgA and IgE, without altering the specificity, since this recombination occurs in a different part of the Ig-gene than the SHM.

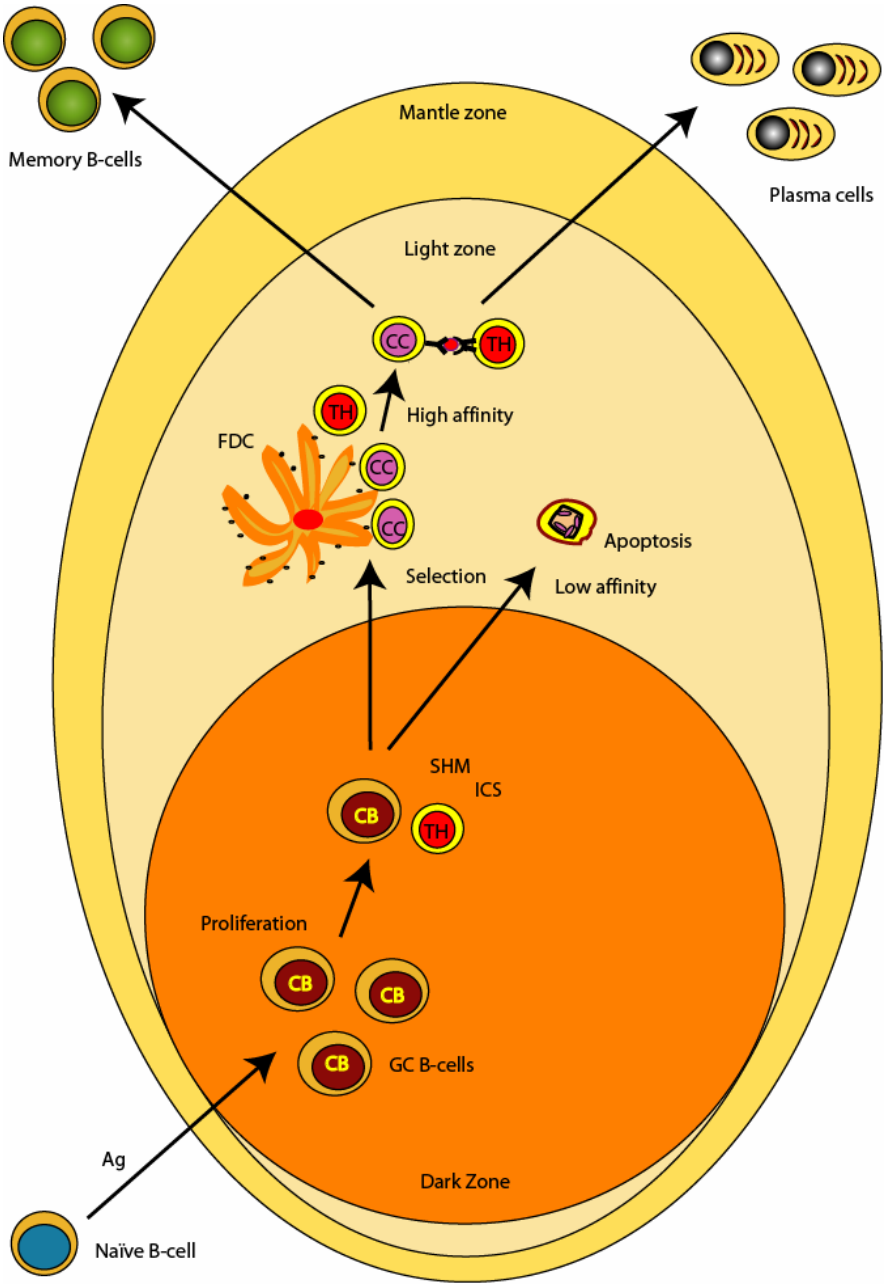

Figure 2. Overview of events during the GC reaction where a naïve B-cell by clonal expansion and selection gives rise to specific antibody producing plasma cells or memory B-cells. CB= centroblast, CC= centrocyte, FDC= follicular dendritic cell, ICS= isotype class switching, SHM= somatic hypermutation, T_H= T-helper cell, Ag= antigen.

Diffuse large B-cell lymphoma (DLBCL)

Pathology

The most common type of lymphoma is diffuse large B-cell lymphoma (DLBCL) which represents 30-40% of all new cases of B-cell lymphomas diagnosed in Western countries each year [12-15]. DLBCL tumor cells typically express B-cell markers such as CD19, CD20 and CD79a. The proliferative fraction as detected by Ki-67 is usually high which may have clinical as well as biological importance [16, 17]. Microscopically the tumor is often homogenous blastic with few infiltrating T-cells and histiocytes. The architecture of the lymph node or normal tissue in extranodal cases is typically replaced in a diffuse pattern. Different morphological variants have been described, i.e centroblastic, immunoblastic, T-cell / histiocyte rich, anaplastic and the more uncommon variants plasmablastic and DLBCL with full-length ALK-expression (Figure 3) [13]. Due to poor intra- and inter-observer reproducibility most pathologists choose to classify these tumors as DLBCL without specification of morphological variants.

Clinical presentation and treatment

DLBCL is considered an aggressive disease where the patients present with rapidly enlarging tumor and may show nodal or extranodal disease, of which the latter is found in up to 40% of patients [13]. The median age at diagnosis is about 65 years but the range is broad and the disease might even be found in young children [18]. Overall men are slightly more commonly affected than women. This far no known cause to the disease exists, but it is well documented that DLBCL can occur *de novo* or by progression of a previous lymphoma such as follicular lymphoma [19].

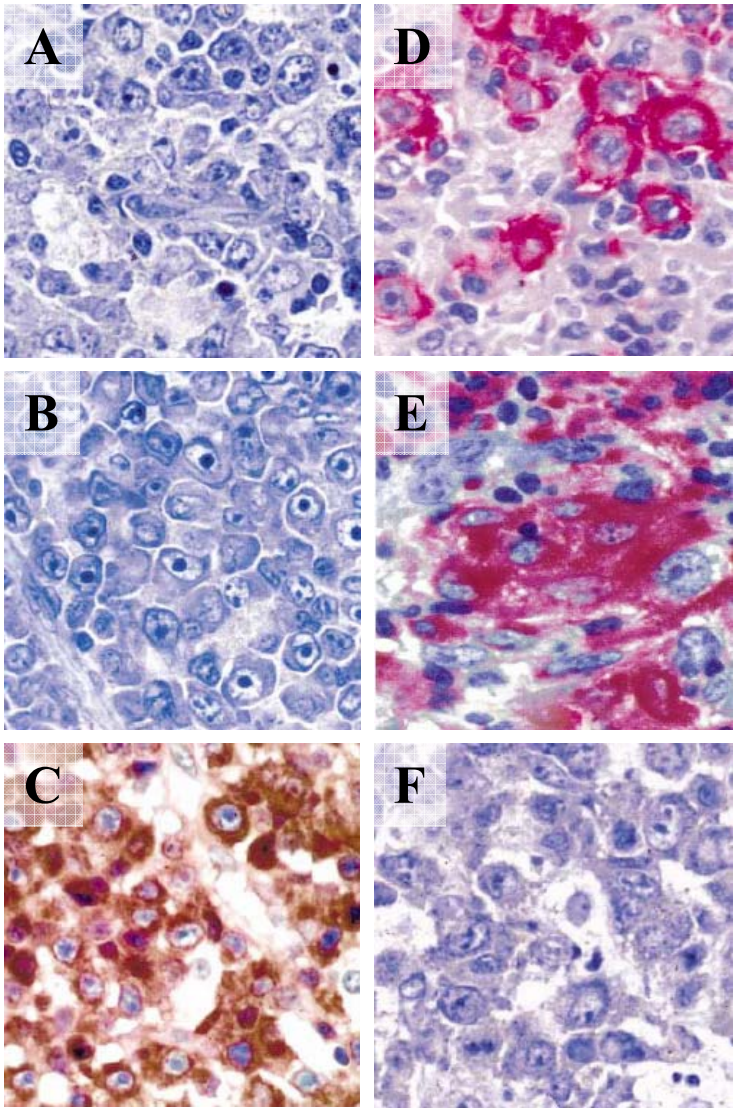

Figure 3. Different morphological variants of diffuse large B-cell lymphoma (DLBCL). (**A**) centroblastic variant (Giemsa); (**B**) immunoblastic variant (Giemsa); (**C**) plasmablastic; (**D**) T-cell rich variant: CD20+ large neoplastic cells are surrounded by small CD20- T-lymphocytes; (**E**) histiocyte-rich variant: neoplastic cells are associated with some CD68+ histocytes; (**F**) anaplastic variant (Giemsa).

Patients with DLBCL are generally treated with standard anthracyclin-based chemotherapy regimens, but high-dose chemotherapy with autologous stem cell transplantation is also used. DLBCL could be considered as a partly curable disease where almost 50% of the patients achieve a longterm complete remission (CR). Recent progress has been made using a chimeric anti-CD20 monoclonal antibody, rituximab, in combination with standard CHOP treatment, which has been shown to significantly increase the CR rate and survival in DLBCL patients [20, 21]. By determining the genetic and expression characteristics of DLBCL its molecular background may be better understood, and possibly serve as a basis for development of new therapeutic strategies.

Tumor biology, genetics and prognostic factors

The difficulties in diagnosis and prognosis prediction by morphology alone have led to the development of other strategies for evaluation and choice of treatment strategy. The International Prognostic Index (IPI) uses a risk score based on the clinical factors age, serum lactodehydrogenase (s-LDH) level, performance status, clinical stage and involvement of extranodal sites [22]. The use of scoring systems like the IPI has been found useful for DLBCL and is today the only reliable prognostic tool used in the clinical practice. Nevertheless significant progress has been made due to the introduction of molecular markers. The clinical heterogeneity of DLBCL makes the possibility of subclassification with molecular markers extremely important. Numerous chromosomal aberrations and other mutations such as point mutations and deletions have been described in DLBCL suggesting a heterogeneous genetic background (Table 2).

Table 2. Examples of candidate genes previously reported in DLBCL.

Gene	Alteration
BCL2	Translocation, amplification
BCL6	Translocation, amplification, mutation
BCL8	Translocation
RhoH/TTF	Translocation, mutation
H4	Translocation
PIM1	Translocation, mutation
IKAROS	Translocation
BOB/OBF1	Translocation
TFRR	Translocation
PAX5	Translocation, mutation
MYC	Translocation, mutation
ATM	Mutation
REL	Amplification
MGMT	Hypermethylation

The translocation t(14;18)(q32;q21.3) involving *BCL2* is cytogenetically found in 20-30% of the cases [23]. However, over-expression of the bcl-2 protein is found in 30-60% of cases and more frequently in nodal than in extranodal tumors [24-27], suggesting that other mechanisms for bcl-2 over-expression are also involved such as gene amplification [28]. Translocations involving the locus for *BCL6* in 3q27 are found in almost one third of the cases [29]. Furthermore, mutations in the 5' regulatory unit of *BCL6* are also seen [30], implying that *BCL6* may be involved in the majority of DLBCL. Almost all DLBCL are likely to be of GC derivation or of post-GC origin, since they show highly mutated Ig genes indicating SHM. The microarray technique used for global gene expression profiling is a powerful tool in cancer research but has so far not been applied in clinical practice. In recent studies, DLBCL were categorized based on their global gene expression profiles into three subgroups: germinal center B-like (GCB), activated B-cell-like (ABC), or type 3 [31-33]. The tumors were found to retain most of the expression pattern of their normal counterparts [31]. In addition the ABC group was associated with shorter survival. It has also been shown that two groups, GC and a non-GC, can be determined using immunohistochemi-

cal markers such as CD10, bcl-6, IRF-4 and that these corresponds to the previous findings using microarray profiling [34]. Furthermore the GC-group show a better survival than the non-GC group similar to the GCB vs. ABC group mentioned above [31].

In clinical practice molecular characterization of DLBCL using a few well known markers studied by established methods in tumor diagnostics such as immunohistochemistry might be the best approach. Protein expression of p53, bcl-2, bcl-6, CD10 and IRF-4/MUM1 alone or in combination with others, have been suggested for prognosis prediction [12, 35-39]. Expression of CD23 and CD40 has also been suggested as prognostically favorable in DLBCL [40]. A small group of DLBCL, about 5% of all cases, is positive for CD5 and show poor prognosis. These cases also show other characteristics such as chromosomal aberrations that are not common in CD5 negative DLBCL, suggesting them as a distinct subtype [41].

Bcl-6

The *BCL6* gene was first characterized due to its involvement in 3q27 translocations and its association with DLBCL [42-45]. The gene encodes a POZ/zinc finger protein that acts as a transcription repressor and is required for GC formation and affinity maturation [46-49]. In addition, bcl-6 controls cell-cycle arrest, inflammation and apoptosis [50, 51]. Recently it has been shown that bcl-6 also regulates terminal plasma cell differentiation by repressing blimp1 (B-lymphocyte-induced maturation protein 1), and that blimp1 expression represses bcl-6 and pax-5 expression to finally drive the cells toward plasma cell differentiation [52]. By understanding the function of the protein we might be able to understand how it is involved in tumorigenesis and progression of DLBCL .The *BCL6* gene product can regulate its own expression by directly binding to the promoter region, which blocks the transcription [30]. Therefore immunohistochemical detection is the best

method to detect all DLBCL with involvement of bcl-6. Expression of bcl-6 in DLBCL has been reported to be associated with better overall survival (OS) [29, 34, 53, 54], however, in a recent study these findings were not confirmed [55].

Bcl-2

The antiapoptotic molecule bcl-2 was originally identified as involved in the t(14;18)(q32;q21.3) that is characteristic for FL. Over-expression of bcl-2 might lead to a survival advantage for the tumor cells by blocking apoptosis and confering resistance to chemotherapy [56, 57]. The prognostic importance of bcl-2 expression is still controversial. While some studies have found no difference in OS [12, 58, 59], most studies have suggested that bcl-2 expression is associated with shorter survival and recurrent disease [55, 56, 60-63].

P53

TP53 is probably the most frequently mutated gene in human cancer and has also been named as the guardian of the genome. The *TP53* gene encodes a 393 amino acid phosphoprotein and regulates the transcription of multiple other genes [64]. It is considered to protect the integrity of the genome by responding to external and internal signals to stress, such as DNA damage, by inducing cell cycle arrest or apoptosis [65]. Mutations in *TP53* have been found in a wide range of lymphomas such as mantle cell lymphoma, Burkitt lymphoma, chronic lymphocytic leukemia [66, 67] and DLBCL [23, 68, 69]. Interestingly, DLBCLs with *TP53* mutations have been suggested to be more resistant to chemotherapy and hence to have a worse prognosis [70].

CD10

CD10 is a membrane metalloproteinase that normally is found in a variety of lymphoid cells as well as in stromal and epithelial cells [71, 72]. It has also been used as a marker for GC in reactive lymphoid tissue as well as in lym-

phomas [71-75]. The prognostic significance of CD10 expression has been evaluated in many studies with controversial results. In some studies a longer survival was found for patients with tumors expressing CD10 [34, 35]. However, other studies reported opposite findings or no association at all [37, 55, 58].

IRF-4

IRF-4 is a transcriptional regulator thought to be involved in activation of T-cells, lineage commitment in lymphocytes, as well as in Fas-dependent apoptosis [76]. It has also been shown that a small proportion of cells in the apical light zone of GC show a bcl-6^-/IRF-4^+ immunophenotype. IRF-4 is expressed in the ABC group of DLBCL and is associated with a worse clinical outcome [31, 34]. In a recent study no association with outcome was found, but with immunoblastic morphology and primary nodal presentation [55].

Hodgkin lymphoma (HL)

Pathology

Hodgkin lymphoma (HL) is a specific group of lymphomas where the neoplastic cells, the Hodgkin and Reed Sternberg (HRS) cells, represent only a minority of cells in involved lymph nodes. HL is further divided into classical HL (cHL) and nodular lymphocyte predominant HL (NLPHL). In cHL, the HRS cells reside in infiltrates containing lymphocytes, neutrophils, eosinophils, histiocytes, fibroblasts and plasma cells. The cHL is further divided into four subtypes, i.e. lymphocyte rich, nodular sclerosis, mixed cellularity and lymphocyte depleted (Table 1) [7, 13].

Clinical presentation and treatment

Hodgkin lymphomas belong to the most curable of all lymphomas with a general OS of about 80%. The typical HL patient is a young person presenting with enlarged lymph nodes in the neck, chest, or mediastinum. Primary extranodal involvement is rare. Patients in early stages show a very good prognosis with a long-term survival of about 90%. Treatment of these patients today involves a combination of short course chemotherapy and involved-field radiotherapy [77]. Patients with advanced stages are treated with full course chemotherapy, often in combination with involved-field radiotherapy. The outcome has been worse than for earlier stages [77] but with new intensive treatment (e.g. escalated BEACOPP) the survival is approaching that for lower stage [78].

Tumor biology, genetics and prognostic factors

It has been shown that the neoplastic cells in HL tumors are of B-cell origin except for a few percent that are derived from T-cells. In cHL the HRS tumor cells are not capable of producing functional immunoglobulin molecules, since they lack expression of the transcription factors BOB1/OBF1 and Oct2 [79]. The absence of BOB1/OBF1 and Oct2 leads to impaired activation of the immunoglobulin promotor and hence no immunoglobulin expression [79]. Interestingly IRF-4 has been shown to be over-expressed in cHL. Since expression of IRF-4 is found in all normal and neoplastic plasma cells but only in a small proportion of GC cells, it is possible that the mechanisms to switch off the B-cell program is different between HRS cells and plasma cells. Common features of non-GC DLBCL are high IRF-4 expression and constitutive NF-κB activation, suggesting that the HRS cells and non-GC DLBCL tumor cells might have the same origin. Another feature of the HRS cells is genetic and chromosomal instability, which results in a multitude of chromosomal aberrations [80, 81]. Recurrent chromosomal altera-

tions found in classical HL include gains in 2p, 9p, 12q and 17q [82, 83] and high level amplification of 4q16, 4q23-24 and 9p23-24[83]. An amplification of the *REL* locus at 2p has also been described and could be one explanation for the constitutive NF-κB activation in HRS cells [84]. Chromosomal losses are less common and a loss on 16q11-21 has been identified in previous studies where E-cadherin was suggested as a candidate gene [81, 85]. Translocations are frequent events in HL and non-random breakpoints such as 14q32 including the *IGH* gene locus has been identified [80].

The nodular lymphocyte predominant subtype of HL (NLPHL) is now considered as a distinct lymphoma entity and represents about 5% of all HL [8]. The neoplastic cells in NLPHL, the lymphocytic and histiocytic cells (L & H cells), only constitute a minority of the tumor. NLPHL is suggested to be derived from the centroblastic stage of GC in lymphocyte differentiation and the *IGH* is heavily mutated with frequent signs of ongoing somatic hypermutation [86].

Historically a strong association between histological subtype and prognosis was observed for HL, but which is no longer applicable following the introduction of modern therapy. Today the clinical stage and presence or absence of systemic symptoms are much more important [8]. An international prognostic score (IPS) has been developed for advanced stage HL [87]. The IPS includes several binary adverse prognostic factors and can be used for patients between 15 and 65 years of age. Even though 80% of cHL patients are cured today, the search for new prognostic factors has to go on, in order to further improve treatment and diminish the risk for late complications. Biological markers reported to be of prognostic importance are infiltration of mast cells [88], increased serum levels of interleukin 9 [89] and infiltration of eosinophils [90]. Age and the presence of bulky disease [91, 92] are other

parameters that has been suggested to influence survival and treatment outcome.

Table 3. Morphological and immunohistochemical characteristics of GC-DLBCL, non-GC DLBCL, cHL and FL.

Tumor Characteristics	DLBCL		cHL	FL
	GC	non-GC		
Growth pattern	diffuse	diffuse	diffuse or interfollicular	follicular
Cell of origin	GC B-cell	Post-GC B-cell	GC B-cell	GC B-cell
Cytogenetic abnormalities				
t(14;18)	20-30%	20-30%	-	80%
Loss of 17p	<10%	<10%	<10%	<10%
Gain of 12q	10-25%	10-25%	10-30%*	15%
Gain of 7	20%	20%	<10%	20%
Loss of 6q	20-35%	20-35%	5-25%	10-30%
Gain of 2p15-16	<10%	<10%	25-50%	10%
Immunophenotype				
CD20	+	+	-/+	+
CD30	-/(+)	-/(+)	+	-
CD10	+/-	-	-	+
CD79a	+	+	+/-	+
CD19	+	+	+/-	+
Bcl-6	+/-	-/+	+/-	+
Bcl-2	+/-	+/-	+/-	+
IRF-4	-/+	+/-	+/-	-
SHM/Ongoing SHM				
Present	Yes	Yes	Yes	Yes
Ongoing	Yes	No	No	Yes

*Not the same region as in the other lymphoma subtypes,
SHM= somatic hypermutation.

Transformation from HL to DLBCL

Patients treated for HL have a higher risk of developing a non-Hodgkin lymphoma (NHL) [93-95], and similarly the risk for HL is elevated in NHL patients [96]. The frequency of NHL after treatment for HL is about 1-6% [97, 98], and if the time interval is long between the diagnoses it is classically considered as therapy induced lymphoma [99]. However, the question if the secondary lymphoma is therapy induced or a clonal progression is contro-

versial. Recent studies are in favor of clonal progression and transformation[100-102]. Firstly, clonal relationship has been proven in most cases where data is available from both the HL and NHL tumor [103]. Composite lymphomas where both HL and DLBCL are found in the same patients at the time of diagnosis have shown to be clonally related in most cases [101-103]. Secondly, a recent study showed a significant correlation between the primary and secondary lymphoma regarding expression of LMP and p53 indicating a clonal relationship [100]. Thirdly, in contrast to secondary leukemias, lymphomas are commonly seen after treatment of another lymphoma but almost never after treatment of any other malignancy. Furthermore, the frequency of secondary NHL after HL was reduced when chemotherapy intensity was increased in the study by the German Hodgkin study group [97]. Chromosomal aberrations have been found in peripheral blood lymphocytes in both treated and untreated HL patients [104, 105]. This indicates an underlying chromosomal instability, suggesting that these patients may have an increased risk for treatment induced secondary lymphomas. Therefore it is more likely that the secondary lymphoma is a progression of the first, or that they share a common ancestral cell. In HL patients the secondary lymphoma occurring after transformation is most commonly a DLBCL, but cases with FL and high grade T-cell lymphomas have been reported as well [99].

Both DLBCL and cHL are suggested to be derived from B-cells within GC or B-cells which have passed GC (Table 3) [101, 106, 107]. DLBCL tumor cells may be of GC or post GC origin, while cHL is suggested to be derived only from the GC stage of differentiation [108]. This far no single chromosomal aberration has been specifically associated with HL or DLBCL, although both frequently show translocations involving the *IGH* locus at 14q32 [109]. Translocations or other chromosomal events involving the *IGH*-locus is a common feature of most mature B-cell lymphomas [110].

Very frequent mutations in the variable region of the Ig heavy chain genes, so called somatic hypermutation (SHM), is found in both diseases [108, 111]. Activation of the V(D)J recombination machinery has been suggested to contribute to lymphomagenesis by enhancing the genomic instability and increasing the risk of new mutations leading to transformation [103].

Follicular lymphoma (FL)

FL and DLBCL are the most common types of lymphoma [7, 15, 112]. It is mainly characterized by the activation of *BCL2* through the t(14;18) translocation, however, other variants do occur [113-115]. Over-expression of bcl-2 is not regarded as sufficient to confer the malignant phenotype in FL. Additional necessary events have been suggested to include alterations of chromosomes 7, 12 and X [116, 117].

Clinical presentation and treatment

At diagnosis most FL patients present with a widespread disease but are usually asymptomatic except for the lymph node enlargement. The bone marrow is involved in about 40% of the patients [8]. If the patient has no symptoms, the treatment usually starts with a watch and wait approach, followed by chemotherapy and if necessary radiotherapy. The CD20 antibody rituximab alone or in combination with chemotherapy has improved the survival for some patient groups such as patients with low tumor burden and those who are not able to tolerate chemotherapy [118].

Transformation from FL to DLBCL

Transformation of FL to a lymphoma of higher histopathological grade occur in about 10-70% of the cases with a following progressive lymphoma where most patients succumb to the disease [7, 19, 119-121]. Since the trans-

formation event is almost impossible to predict, the finding of molecular markers indicating a higher risk for transformation would be very important for the clinical handling of these patients. A few mechanisms involved in the transformation have been proposed, such as c-myc deregulation [122-125], *TP53* mutation and/or over expression [126-128] and inactivation of *CDKN2A* and *CDKN2B* [129-131]. Global gene expression profiling have revealed not only differences between the original FL tumor and the DLBCL but also that when compared to *de novo* DLBCL the transformed cases resemble the original FL tumor [125]. In FL the tumor cells show a close resemblance to normal GC cells both morphologically and immunophenotypically, therefore FL is considered as a GC derived lymphoma.

The SHM of immunoglobulin genes occur in the GC and introduce mutations for affinity maturation in the B-cells. This has been postulated as a key mechanism for chromosomal translocation, and indeed cytogenetically, clonal evolution has been found in FL suggesting that late relapses normally show a more aberrant karyotype than preceding tumors [117]. Therefore it is possible that the transformation involves a series of events, a cytogenetic evolution of the tumor, which in most cases ends with a histologically detectable transformation to DLBCL. Several putative candidate genes have been proposed to work synergistically to produce the more aggressive tumor. In general the post-transformation DLBCL tumors are resistant to chemotherapy. This underlines the importance of exactly defining the molecular events that are responsible for transformation, and to subsequently use this knowledge for targeted therapy.

AIMS OF THE STUDY

The overall aim of the study was to molecularly characterize diffuse large B-cell lymphoma (DLBCL) and to investigate aspects of transformation from Hodgkin lymphoma (HL) and follicular lymphoma (FL) to DLBCL. More specifically the aims were to:

- Identify chromosomal imbalances and to correlate the findings with prognosis, as well as to elucidate the cytogenetic evolution during the tumor progression of DLBCL.

- Evaluate immunophenotypes and its prognostic impact in DLBCL.

- Identify recurrent cytogenetic alterations in HL derived cell lines.

- Characterize two novel B-cell lines derived from DLBCL patients with a previous history of HL.

- Elucidate the chromosomal imbalances involved in the transformation of FL to DLBCL.

MATERIALS AND METHODS

Patients and tumor specimens

A total of 226 patients diagnosed with DLBCL and collected from the files of the Departments of Pathology at the Uppsala University Hospital, Umeå University Hospital and Lund University Hospital, Sweden, were included in this thesis. In paper I, 54 DLBCL specimens were obtained from 40 patients, 28 men and 12 women, diagnosed between 1985 and 1998. The clinical history revealed that 8 of the patients had a history of low grade malignant lymphoma prior to the DLBCL diagnosis. In paper II, 173 *de novo* DLBCL patients (86 men and 87 women), diagnosed between 1984 and 2002 with a median age of 66 years (range 16-91) were included in the study. From paper I the IHC results for bcl-2 and bcl-6 from 15 patients with *de novo* DLBCL were included. In paper VI, 28 patients showed a previous history of FL. The CGH results from 7 DLBCL patients with a previous history of FL in paper I were incorporated in study VI. All DLBCL tumors were confirmed according to WHO classification [8] by a histopathologically re-evaluation.

The clinical history was obtained from the patient records. The International Prognostic Index (IPI) [22] was retrospectively evaluated including the following risk factors: age (>60years), stage (III or IV), elevated S-LDH (>nl), and ECOG performance status ($\geq$2) and extranodal site (>1 site). The patients were mainly treated with CHOP or CHOP-like regimens.

Four commonly used HL derived cell lines were studied (DEV, HDLM-2, L-540 and CO). Two of these were established from patients with nodular sclerosis (NS) subtype (HDLM-2 and L-540), and one was from a patient with nodular lymphocyte predominance (NLPHL) subtype (DEV) (III). The fourth cell line (CO) was found to be identical with a T-ALL cell line. Furthermore, two cell lines (U-2932, U-2940) were established from two female patients suffering from DLBCL who previously had been treated for HL with multiple chemotherapy regimens and radiotherapy (IV and V).

DNA preparation

Molecular studies on the DNA level constitute an important part of modern cancer research. The analyses include both normally occurring DNA variants and tumor related mutations on the chromosomal and single gene level. DNA can be isolated from all types of cells, including fresh frozen, cultured and archival material.

In this thesis DNA was prepared from fresh frozen tumor tissue and cell cultures and used for comparative genomic hybridization and analyses of clonal relatedness. A standard protocol was applied including proteinase K digestion, phenol-chloroform separation and ethanol precipitation. Using this methodology good quantity of high quality DNA is obtained that is suitable for most types of DNA analyses. In addition, DNA extraction was performed in some cases using QIAamp® DNA Mini Kit. The quality and concentrations of the samples were determined by spectrophotometry and verified by agarose gel electrophoresis.

Establishment of cell lines and culturing

For U-2932 the cell line was established from ascites from the DLBCL in a patient who earlier had been diagnosed for cHL. Mononuclear cells were separated using a Ficoll-Isopaque gradient centrifugation. The cell line U-2940 was established from a pleural effusion. All cells were cultured in RPMI 1640 containing 10% (U-2932, U-2940) or 20% (DEV, L-540, HDLM-2, CO) fetal calf serum, glutamine and antibiotics (100 IU/ml of penicillin and 50μg/ml of streptomycin) at 37 ^{o}C, in a 5% CO_2 humidified environment. For all cell lines growth medium was changed twice per week.

Immunohistochemistry (IHC)

Immunohistochemistry is generally used to determine the presence, absence and localization of proteins in cells or tissue sections. A simplified outline of IHC is illustrated in Figure 7. In this thesis, paraffin-embedded sections (4 μm) from DLBCL tumors were stained with monoclonal antibodies targeted against bcl-2, bcl-6, CD10 and Ku70 antigens. A polyclonal antibody was used to stain for IRF4/MUM1 expression. The proportion of positively stained cells was estimated on sections from each tissue biopsy. The proportion of positively stained tumor cells was visually estimated under the microscope. For each case the core with the highest percentage of tumor cells was used for analysis.

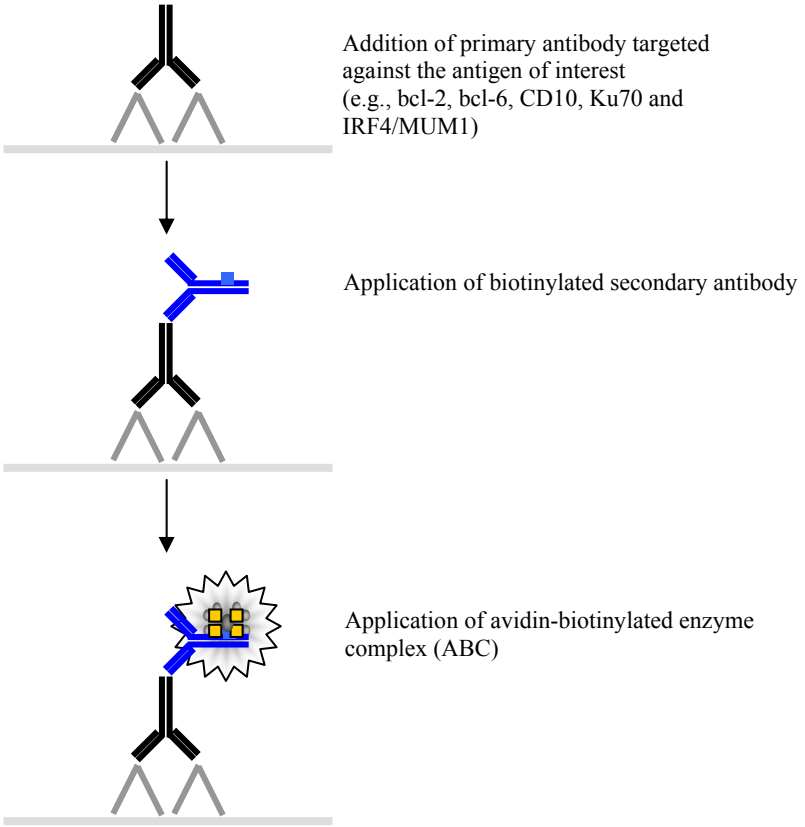

Figure 7. Immunohistochemical detection of protein expression in tumor tissue sections using the ABC technique.

Fluorescence *in situ* hybridization (FISH)

The fluorescence *in situ* hybridization (FISH) techniques are all based on the same principle, the ability of single stranded DNA to bind to its complementary DNA strand (target) in a specific manner. The use of fluorescent dye makes it possible to take live images and visualize the target gene/s, another advantage is that there is no need to use radioactive isotopes. The work performed in this thesis is based on metaphase slides with chromosomes fixed in methanol:acetic acid according to standard procedures.

Comparative genomic hybridization (CGH)

A major problem in cancer cytogenetics is the need of culturing cells in order to obtain metaphases, this means that it is only possible when viable cells with a relatively high rate of mitosis (a high mitotic index) are available. The CGH method does not depend upon metaphase preparation from tumor cells since the hybridization is performed of metaphase spreads from normal cells. This means that tumors with low mitotic index as well as archived material such as fresh frozen tumors can be used. With CGH the entire tumor genome is screened for chromosomal gains and losses (Figure 4), however, alterations that do not lead to a net alteration of the DNA content of a particular sequence are not detected by this technique. The sensitivity is within the range of conventional cytogenetics and detects low copy gains and losses at 10 Mb resolution and high copy amplification at about 2Mb. For balanced alterations such as translocations and insertions a method based on chromosomal karyotyping is preferred. Similarly, alterations leading to imbalances between the chromosome homologues should be approached by methods identifying loss of heterozygosity in the tumor. For a higher sensitivity, recent techniques such as array CGH can detect chromosomal aberrations as small as 100 kb [132].

CGH was first described by Kallioniemi et al (1992) [133] and is based on the method dual color FISH. Test and reference DNA are labeled with green and red fluorochromes, respectively, and cohybridized to normal metaphase chromosomes. The difference in fluorescence intensities is measured along the length of each chromosome and reflects the relative copy number. In the microscope the chromosome areas of gain are displayed in green while losses are visualized in red. At least 10 metaphases are captured and analyzed by digital image analysis. In our study at least 12 ratio profiles were averaged for each chromosome except the Y chromosome. Green-to-red

ratios exceeding 1.2 were considered as gains while ratios below 0.8 were scored as losses. The cut-off level for high-level amplifications was set to 1.5. Heterochromatic regions as well as the p-arms of the acrocentric chromosomes and the Y-chromosome were not included in the evaluation.

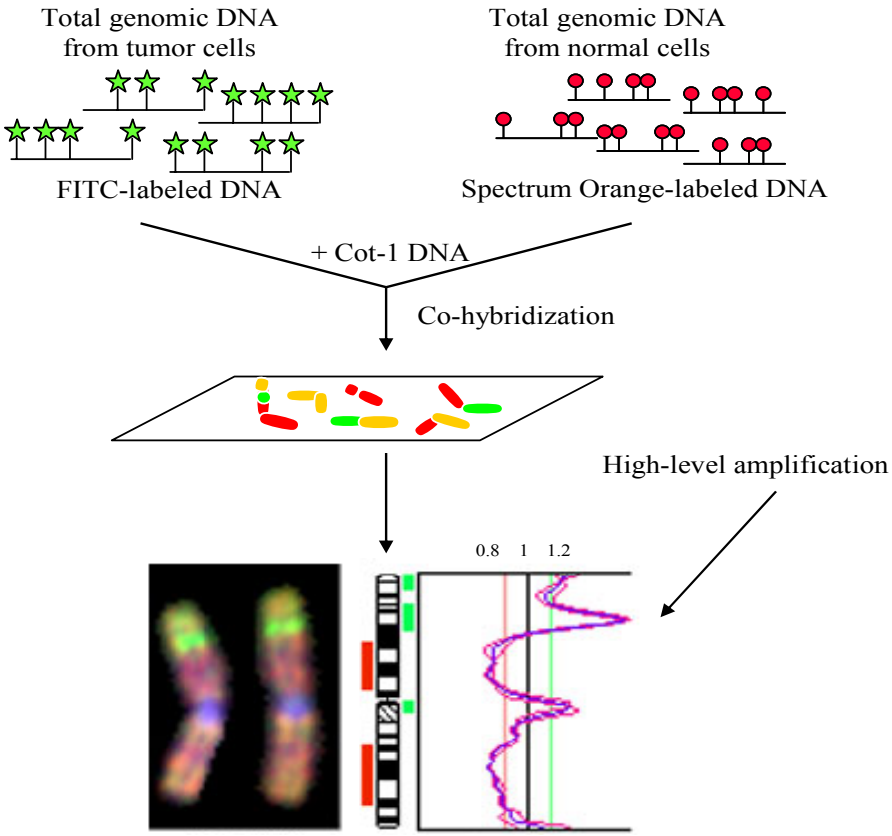

Figure 4. Detection of net DNA sequence copy alterations by CGH. Differentially labeled DNAs are cohybridized onto chromosome spreads from a normal donor, whereby gains and losses are detected by deviations from a green-to-red ratio of 1.0. In this figure a high-level amplification in 1p is illustrated.

Spectral karyotyping (SKY)

With the development of multiplex FISH (m-FISH) [134] and Spectral karyotyping (SKY) [135] in 1996 it became possible to simultaneously stain all chromosomes in different colors and thereby visualize complex rearrangements not solved by classic cytogenetics. Still metaphase spreads are needed, however, chromosome rearrangements may be resolved even if the chromosome quality is inferior. The SKY method combines Fourier spectroscopy (interferometry), charge coupled device (CCD) imaging and optical microscopy to measure the emission spectrum in the visible and near infrared spectral range in all measure points. This makes it possible to discriminate multiple and spectrally overlapping fluorochromes. Each chromosome is labeled with one to four different fluorochromes and in total 5 different fluorochromes are used. With introduction of the SKY technique the resolution is highly increased compared to traditional banding techniques, and previously unidentified or misinterpreted chromosomal changes have been frequently characterized. The principles of the method are outlined in Figure 5. For optimal resolution of complex karyotypes, SKY is used to identify the chromosomal composition. The subchromosomal regions involved are then determined from some type of regular banding supplemented by CGH. The combination of SKY and CGH techniques result in a good description of the chromosomal alterations in a tumor since it combines the karyotype obtained by SKY on a single cell level with the net gain and losses detected by CGH [136, 137] within the tumor.

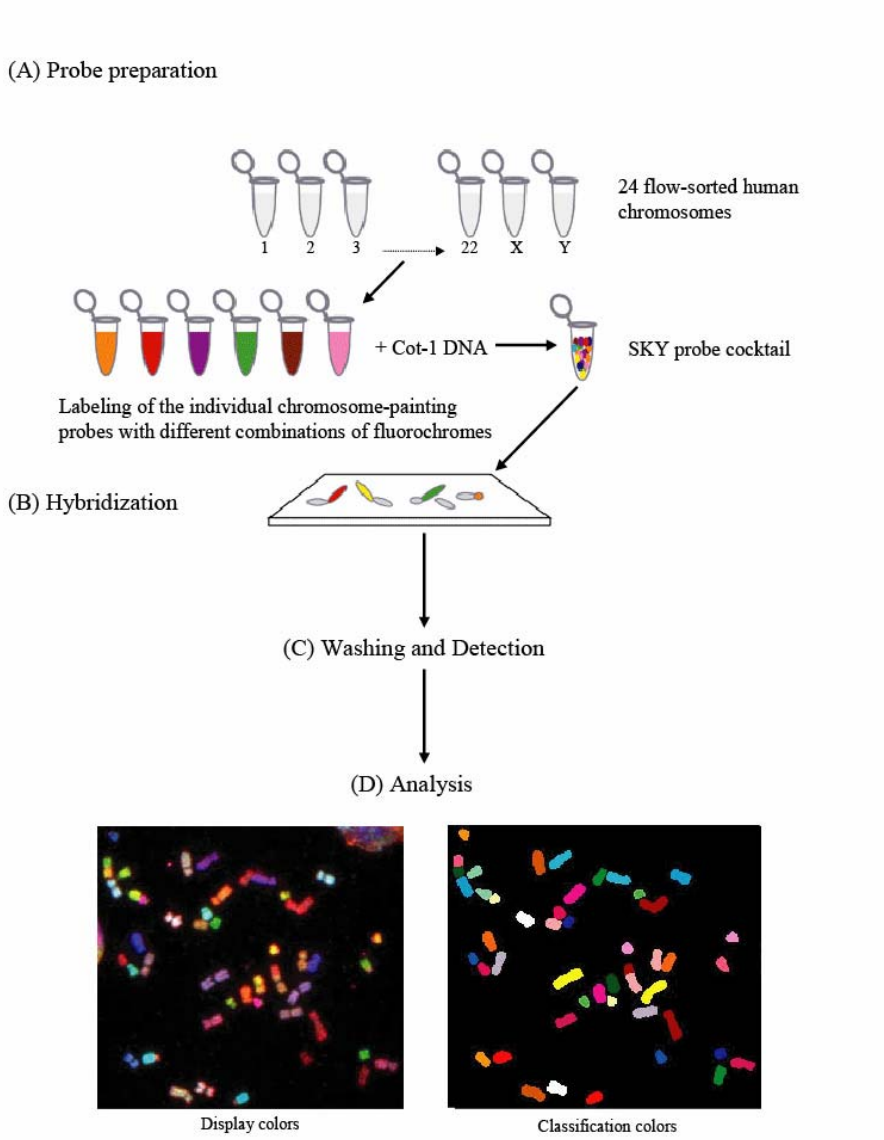

Figure 5. Multicolor FISH analysis by spectral karyotyping (SKY). In this example a typical metaphase from the cell line U-2940 (paper V) is shown in display and classification colors.

Analyses of clonal relatedness

Lymphocytes have highly variable *IGH*-sequences. This is due to rearrangements of the *IGH* locus, and to normally occurring somatic hypermutation (SHM). By comparing the *IGH*-sequences of two tumors from an individual it can be determined if they represent multiple primary tumors or originated from the same primary tumor. In this study *IGH*-rearrangements were first amplified by PCR [138, 139], and subsequently monoclonal PCR-products were distinguished from polyclonal using single-strand conformation polymorphism (SSCP) analysis [140].

SSCP analysis utilizes the fact that single DNA strands that carries a mutation migrate at a different rate through a non-denaturing polyacrylamide gel. This difference in mobility depends on an alteration in the three dimensional structure of the single-stranded PCR products resulting from the mutation (Figure 6). In one case for which a clonal PCR product was lacking in the primary tumor, further analyses were carried out using Southern blotting and hybridization with a J_H probe to determine clonality of the *IGH* gene as previously detailed [141]. To confirm the SSCP-finding that some tumors from the same patient were not clonally related, the PCR products from these samples were sequenced and aligned with *IGH* sequences retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/igblast/).

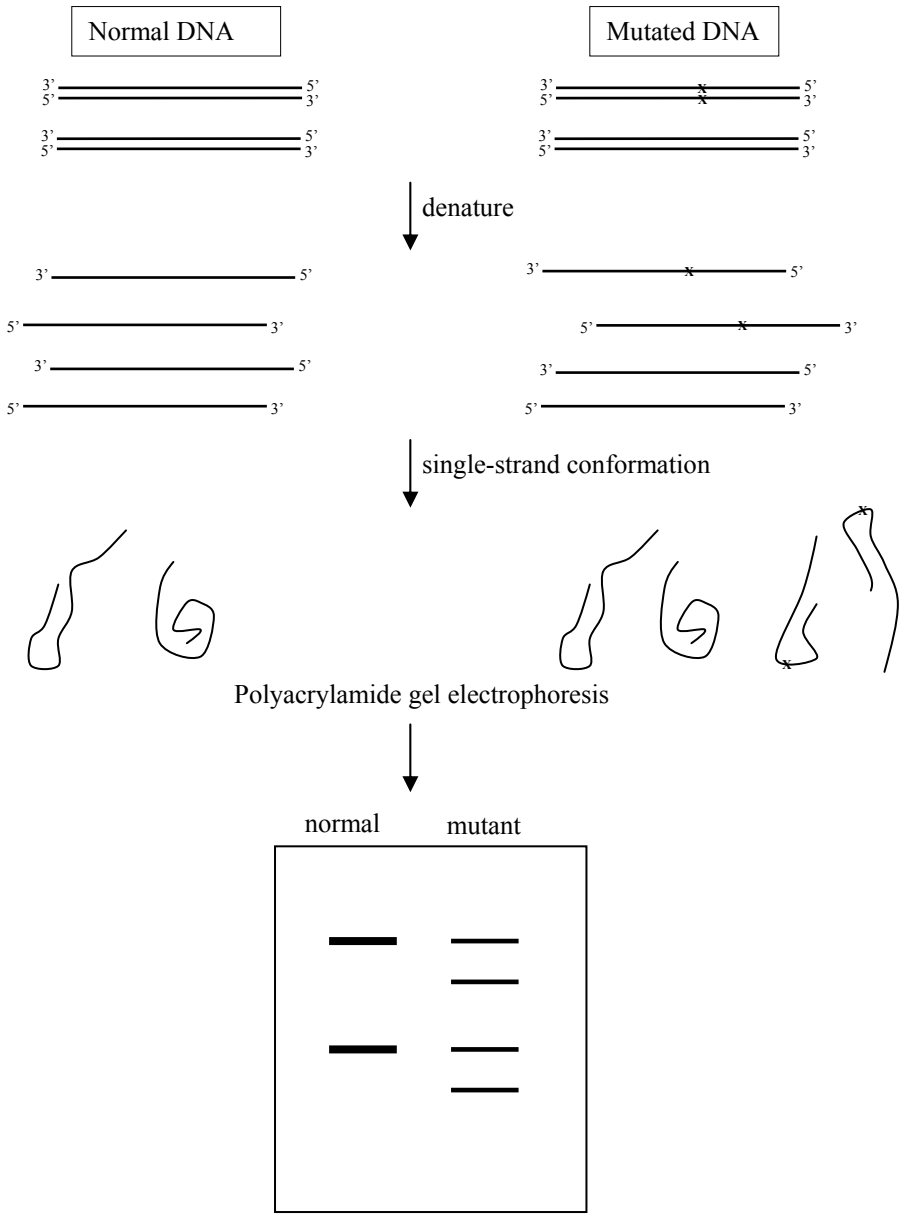

Figure 6. Detection of PCR fragments with DNA sequence variations using single stranded conformation polymorphism (SSCP).

Statistical analyses

In this thesis several different statistical analyses were applied to related molecular findings with clinical parameters. Chi-square analysis was used to compare differences in proportions. Differences in distributions between subgroups were analyzed with the Mann-Whitney U test. Kaplan-Meier survival analysis and log-rank test were performed to study the prognostic significance of the markers used. Overall survival (OS) was calculated from the date of diagnosis until last follow-up or death. Event free survival (EFS) was calculated from date of diagnosis to death, progression or end of follow-up. Five year survival was calculated from the Kaplan-Meier graphs. Probabilities of less than 0.05 were accepted as a significant value. In order to compare the prognostic importance of different variables Cox proportional hazard multivariate analyses were performed. The statistica 6.1 software (Stat Soft Inc, Tulsa, US) was used for all calculations.

RESULTS AND DISCUSSION

DLBCL (papers I-II)

Diffuse large B-cell lymphoma is a very heterogeneous group of lymphomas and is now considered a composite group of different types of lymphomas [10]. Much work has been done recently in order to subtype and predict outcome for this lymphoma entity using methods such as microarray, cytogenetics and immunohistochemistry [31, 34, 109, 142-144].

Molecular cytogenetic findings

We investigated chromosomal imbalances by CGH in 54 tumors from 40 patients diagnosed with DLBCL. Immunohistochemical expression analyses of bcl-2 and bcl-6 were performed on all cases. Furthermore a material of 37 DLBCL tumors was later investigated for Ku70 expression.

Table 4. The most frequent CGH alterations found by us and/or others and corresponding candidate genes.

Chromosomal region	CGH alteration gain/loss	Candidate gene	Normal function
1p34-pter	lost	*p73*	TSG
6q23-qter	lost	*PRDM1*	B-cell differentiation
		ZAC/PLAGL1	Cell proliferation inhibition
8p22-pter	lost	*FEZ1*	-
12c-q14	gained	*CDK2*	Cell cycle kinase
		CDK4	Cell cycle kinase
		MDM2	P53 inactivation
13q22	gained	*FBXL3A*	F-box protein
		PAM/KIAA0916	-
17p12-pter	lost	*TP53*	TSG
18q21	gained	*BCL2*	Apoptosis
22q	lost	*Ku70*	DSB repair
		NF2	TSG
Xq25-26	gained	*CD40L*	B-cell proliferation

TSG= tumor suppressor gene, DSB= double stranded break.

Copy number changes were detected in 94% of the diagnostic tumor samples and in all the relapses. None of the recurrent alterations were detected as a single abnormality suggesting that other genetic lesions such as balanced translocations, point mutations or small deletions below the detection level of CGH may be the initiating event in the tumorigenesis of DLBCL. The most commonly gained or lost regions are summarized in table 4. In the present study band 18q21 was significantly more often gained in relapses as compared to diagnostic tumors, however, the expression of bcl-2 did not correlate with a gain of band 18q21. This indicates that genes other than *BCL2* are involved in recurrence of these tumors. Here the expression of bcl-2 was interpreted as negative if less than 20% positive cells, and highly expressed in the tumor if more than 80% of the cells showed a positive staining. Nevertheless, tumors with high level amplifications of 18q21 showed high expression of bcl-2, indicating that gene amplification may be the

mechanism for bcl-2 upregulation in these cases. Mechanisms other than translocation for tumors to over express bcl-2 and bcl-6 are gene amplification [28], mutations in regulatory units [30, 107] and methylation [145]. A loss of 22q was significantly more commonly seen in the diagnostic tumor samples with more advanced clinical stage i.e. stage III-IV compared to stage I-II. *Ku70*, a gene involved in repair of double stranded DNA breaks is located at 22q and was expressed in 35 of 37 (95%) cases investigated. This suggests that loss of other genes on 22q than *Ku70* may be involved.

Relapses

The clonal relationships as well as the cytogenetic evolution were investigated in 11 pairs of matched diagnostic tumors and relapses by *IGH* gene rearrangement analysis and/or the CGH profiles. Clonal relatedness was demonstrated in 9 of 11 cases. In one of the two remaining cases different *IGH* gene rearrangements were detected, while the last case could not be analyzed due to technical reasons. Furthermore the time interval between the diagnostic tumor and relapse was in both cases short which makes therapy induced lymphomas less likely. Earlier studies on lymphoma have shown that disease progression could either be clonally related or represent clonally unrelated neoplasms [146, 147]. Clonally unrelated relapses are uncommon, but have been reported [146, 147]. Even if no clonal relation could be proven, it does not exclude a common early ancestral B-cell. From the CGH analysis of one case we could follow the development from the diagnostic tumor to the 3rd relapse and we observed that the tumor progression was not cytogenetically linear (Figure 8). This indicates a clonal heterogeneity where ancestral cells are still present. This presents a problem for the clinical treatment of these patients since the ancestral cells may be less sensitive to chemotherapy. These cells may then gain more mutations which eventually transform them to a more aggressive phenotype.

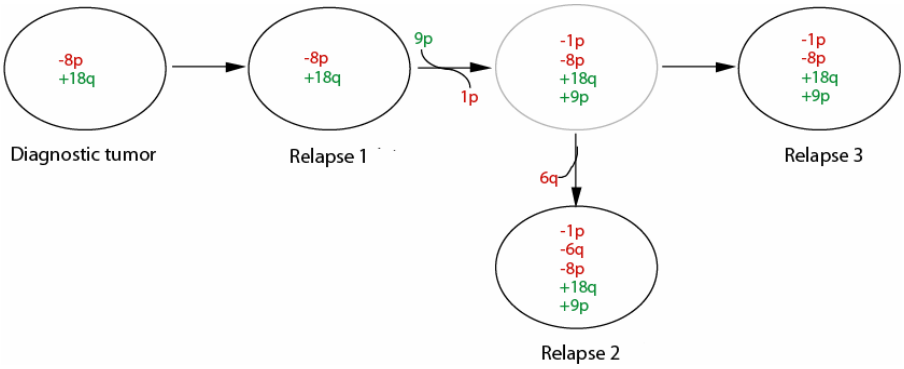

Figure 8. CGH alterations associated with tumor progression in a patient with *de novo* DLBCL. The scheme includes the diagnostic tumor and three subsequent relapses as well as a hypothetical intermediate cell (dotted). A plus (+) indicates chromosomal gain, and a minus (-) denotes losses.

Protein expression

Using gene expression profiles, a great amount of molecular data have been made available for further studies. Since gene expression profiles are difficult to incorporate in routine diagnosis, the ideal way to go would be to use the information from gene expression and then identify a few genes that could be used to subgroup patients based on immunohistochemistry, as was shown possible in a recent study [34]. The aim of the present study was to confirm the use of CD10, bcl-6 and IRF-4 in order to subgroup DLBCL into a GC and a non-GC subgroup as well as to investigate the impact of bcl-2 as a prognostic marker.

The most common definition in the literature of the GC-group is concomitant positive staining for bcl-6 and CD10 [71, 74, 148], whereas a more intricate model described by Hans *et al* (2003) also includes IRF-4. In this three marker model, DLBCL tumors are subclassified based on the expression of two GC markers (CD10 and bcl-6) and a post-GC marker (IRF-4). The classification procedure is outlined in figure 9. If the tumor is positive for CD10 a GC phenotype is assessed independent of the other markers.

However, if CD10 is negative, cases with bcl-6 positive and IRF-4 negative are considered as GC and all others as non-GC (Figure 9).

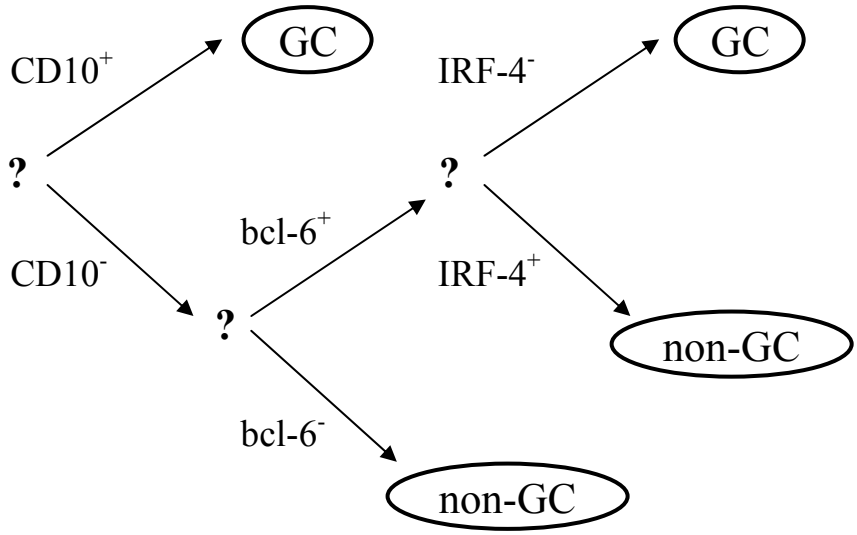

Figure 9. A schematic illustration of the subclassification of DLBCL suggested Hans et al (2003). A (+) indicates positive IHC staining while (-) denotes negative staining.

In this study, we found that both models identified a subgroup of patients with a favorable prognosis, i.e. the GC group. The model by Hans et al is preferable since it defines a larger group of GC-patients (Figure 10). Furthermore, although no difference in clinical presentation was found between patients in the GC vs. non-GC group, the GC group demonstrated a significantly better survival (Figure 10).

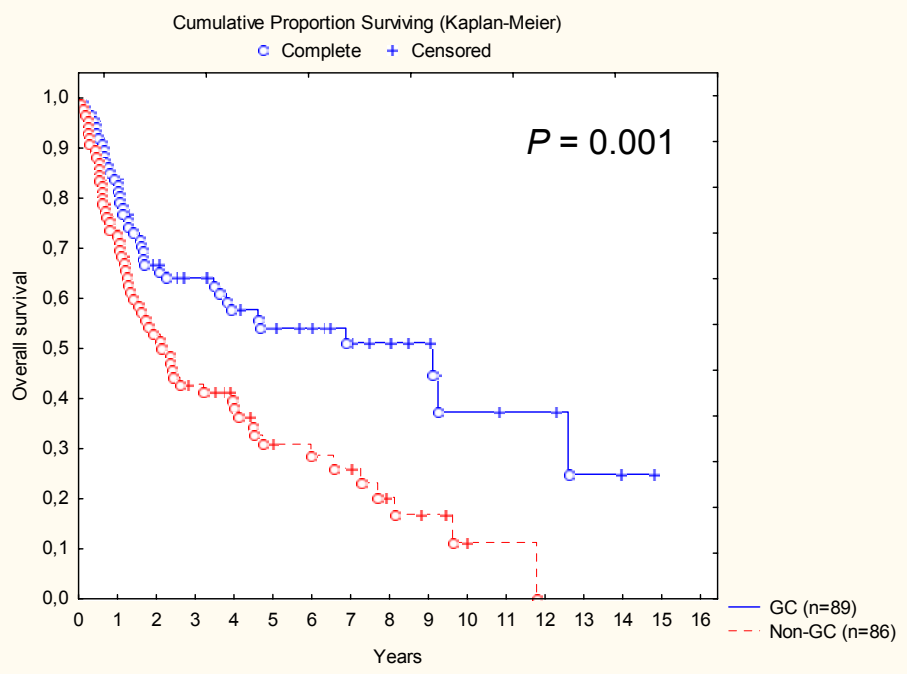

Figure 10. A Kaplan-Meier curve showing significantly longer overall survival for DLBCL patients with GC compared to non-GC tumors.

In the present study bcl-6 expression was found to be an important prognostic factor, associated with better EFS and OS (P=0.000001 and P=0.0002 respectively). CD10 predicted prognosis especially well in the group with low IPI, which was also found in another study [35]. We could not confirm any prognostic value for IRF-4 alone. However, a few other studies show contradictive results [31, 34], and also shows that IRF-4 is more commonly expressed in the ABC and non-GC group that are associated to a worse outcome. In another study IRF-4 expression was not associated with clinical outcome, but with immunoblastic morphology and primary nodal presentation [55]. Very little is known about the relationship between bcl-6 and IRF-4 but it has been suggested that bcl-6 could abrogate the effect of IRF-4.

Here the poor prognostic impact of bcl-2 expression was confirmed and the predictive capacity was maintained for both the GC and non-GC group. Further, bcl-2 expression was found to be more important for EFS than OS (P=0.001 and 0.06 respectively), Expression of bcl-2 was significant for patients with IPI ≤ 2 but no difference was found for patients with IPI 3 or higher. Patients with high IPI have been shown to have lower rate of complete responses and a higher rate of relapse from complete response [22], indicating that other factors than bcl-2 expression are responsible for the worse EFS in patients with IPI > 2. When the following factors were included in a multivariate analysis, GC vs. non-GC, IPI (0-5), bcl-2 positivity, all were demonstrated as independent prognostic factors for EFS and OS.

It is well known that the stage of differentiation of the originating B-cell will affect the phenotype of the resulting lymphoma [10, 11]. During the GC reaction B-cells are rapidly differentiated and cells in different stages coexist during a short interval. The knowledge of the different subpopulations in the GC is still scarce but heterogeneity within populations of GC cells has been detected [149]. DLBCL tumor cells are thought to derive from GC or post-GC cells, and in general DLBCL is a very heterogeneous group of lymphomas both clinically and molecularly [13]. Therefore it is reasonable to suggest that these differences are the results of the subtle changes occurring during the GC reaction, meaning that a GC or a non-GC phenotype is just a start to subclassify this group of lymphomas. Interestingly, in this material a group of 12 patients with a GC phenotype also showed expression of IRF-4, a post-GC marker, and this group also showed better event free survival (EFS). The same was also found for 15 patients in the non-GC group that were positive for bcl-6, which is in fact a GC marker. This suggests that further subclassification of DLBCL is possible.

Varying cut-off levels for determining positive stainings for bcl-6, CD10 and bcl-2 have been applied in different studies to study prognostic impact, which could be one explanation for the divergent results in earlier studies. The most common levels are 10-30% for bcl-6 and CD10, and 20-50% for bcl-2 [29, 34, 36, 53, 71, 143, 150]. We applied cut-off levels according to Hans et al [34], i.e. 30% positive cells for CD10, bcl-6 and IRF-4 and 50% for bcl-2, in order to confirm their results but other cut-off levels were also evaluated. For our series of tumors the cut-off values used by Hans et al gave the best results.

Cytogenetic characterization of HL (paper III)

Cytogenetic studies of HL are impaired by the fact that only 1-3% of the cells in the tumor are neoplastic cells, and the majority constitutes reactive inflammatory cells. Analysis of established cell lines is one way to overcome this problem, but it may then be questioned whether these cell lines are representative for *in vivo* HL tumors. Establishment of such cell lines have been proven to be difficult, and only about 10 cell lines have been described. The results of the present study indicate that many of the numerous abnormalities are also seen in tumor material from HL patients. In our study of HL derived cell lines chromosomal imbalances were detected by CGH and SKY. The CO cell line was found to be overgrown by a T-ALL cell line CCRF-CEM. By CGH a loss of chromosomal region 9p as well as chromosome 20 and gain of chromosome X was found in two of the cell lines (Table 5). Based on the SKY analysis loss of chromosome 20 was found in all cell lines, furthermore recurrent breakpoints at 8q22, 14q10, 14q24 and 18p11.2 were found in the two HL cell lines of NS subtype (HDLM-2 and L-540; Figure 11). All cell lines showed numerous imbalances indicating chromosomal instability.

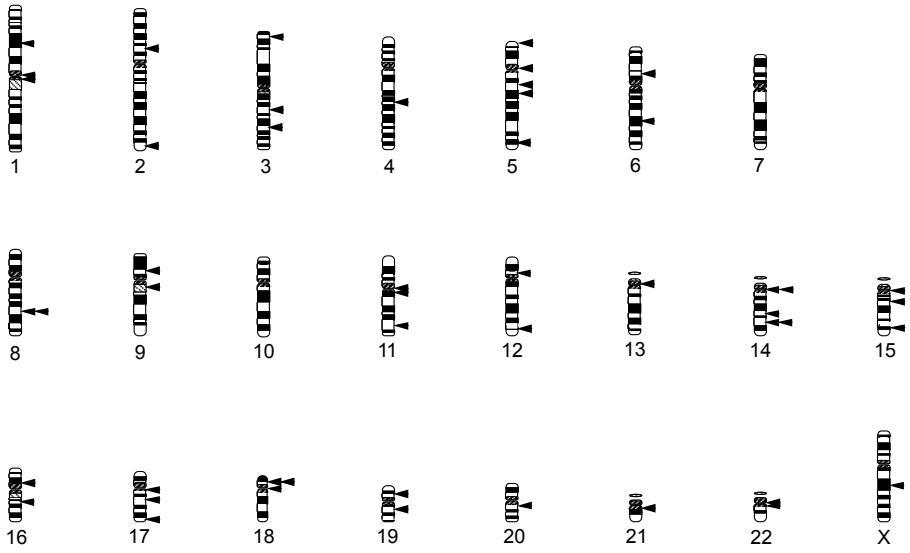

Figure 11. Breakpoints found in two cell lines derived from patients with HL of NS subtype. Each breakpoint is indicated by an arrow and was only counted once per cell line.

Table 5. Chromosomal aberrations in the three HL derived cell lines.

Cell line	Subtype	Stage	Ploidy	CGH
HDLM-2	NS	IV	Hypodiploid	+X,+9p23-24,-18,-20
L-540	NS	IVB	Hyperdiploid	-X,-1p,-2q33-37,+2q24-32,+6p,-9, -10,+12p,+14q21-32,-20
DEV	NLPHL	II	Hypotriploid	+X,-3p21-q29,+6p21,-9pter-q22, -11p12-15,-12p,-15p11-q15,+19p13

NS= Nodular sclerosis HL, NLPHL= Nodular lymphocyte predominant HL, B= B-symtoms (fever, weight loss and night sweats), (+) indicates a gain and (–) a loss

Transformed DLBCL from HL (papers III-V)

Histological transformation from HL to DLBCL do occur but is considered a very rare event [101, 151]. A common B-cell precursor with possible GC origin has been proven [101], but still information about this type of transformation is scarce. Two cell lines were established from patients with a cHL who later developed a DLBCL. A transformation is hypothesized but could not be proven due to lack of material. However, by studying cell lines, chromosomal events involved in the transformation from HL to DLBCL could be identified. Four cell lines established from patients with HL (DEV, HDLM-2, L-540 and CO) were compared with the transformed cell lines.

Of the HL derived cell lines studied, two were of NS subtype (HDLM-2 and L-540) and one was of NLPHL type (DEV). Since both U-2932 and U-2940 were of NS subtype the focus will be on comparing HDLM-2 and L-540 with the two transformed cell lines.

U-2932 was established from DLBCL tumor cells obtained from ascites of the patient. The time interval from the first presentation of HL to the first confirmed DLBCL was 16 years, and in between the patient relapsed four times with HL. U-2932 was shown to be of B-cell phenotype with a strong expression of CD10 and bcl-6 and thus of GC-phenotype. Cytogenetically U-2932 was found to be very complex, showing numerous breaks and balanced as well as unbalanced translocations as outlined by the SKY analysis (Table 6). The 18q21 region was highly amplified and involved in multiple translocation events as shown by FISH analysis. Also chromosomal region 3q27 was highly involved and CGH also showed this region to be highly amplified. By immunohistochemistry bcl-2, bcl-6 and p53 was shown to be over-expressed, while CD30 was found to be negative. A point mutation in

TP53 exchanging cysteine (aa 176) to tyrosine was found by sequence analysis. The *IGH* gene rearrangement analysis revealed a V_H4-39 gene with 92% homology to the germline sequence, thus displaying 8% of SHM. Both the ascites and U-2932 had identical V_H4 rearrangement patterns on the SSCP analysis. No evidence for ongoing SHM was found, and due to lack of material, the clonal relationship with the HL tumors could not be determined.

The U-2940 cell line was established from a pleural effusion of a DLBCL in a patient previously diagnosed with cHL. The time interval between the initial HL diagnosis to the diagnosis of DLBCL was only eight months. U-2940 was shown to be of B-cell phenotype but negative for CD10 in contrast to U-2932. A GC origin could not be determined since bcl-6 and IRF-4 were not investigated. Cytogenetically U-2940 was shown to be less complex than U-2932 (Table 6). Chromosomal aberrations concerning 7q, 16p, and Xq as well as a strong CD19 expression may suggest deregulation of the CD19-PI3K-BTK pathway. No mutation was found in the *TP53* gene. The *IGH* rearrangements from both the pleural effusion and the established cell line showed an identical hypermutated V_H3-23 rearrangement proving the origin of U-2940 cells from the neoplastic B-cells from the pleural effusion, no evidence of ongoing SHM was found. No evidence of *IGH/BCL2* rearrangements was found but about 20% of the cells showed expression of the bcl-2.

Although the two cell lines show differences concerning mutated *TP53*, expression of CD10 and bcl-2, similarities that may be responsible for the transformation is detected. Especially a translocation with materials from 2q and 7q and similar breakpoints as well as loss of material on 6q was found in both cell lines.

Table 6. Composed karyotypes of the two DLBCL cell lines from patients with transformed HL.

Cell line	Composed karyotype
U-2932	45,X,-X,der(1)del(1)(q23)dup(q12q23),del(2)(q11), der(3)ins(3;18)(q2?7;q21)?hsr(18)(q21),der(6)t(6;18)(q16;q?), der(7)t(7;2;15)(q22;q24q34;q22),t(9;19)(q34;q13.1),der(10) t(7;10)(q22;q21),der(11)t(1;11)(q12q23;q23)?hsr(11)(q21q23), del(13)(q12),der(14)(14;3;18;3)(q32;q13.2q2?7;q21;q2?7)?hsr (18)(q21),der(18)t(1;18)(q23;q12),der(18)t(3;18)(q2?7;q23)?hsr (18)(q21),der(21)t(15;21)(q22;p11) [cp12]
U-2940	45-46,X,-X,del(3)(p13-p21),del(6)(q14-q16),del(7)(p11), +i(7)t(2;7)(q23;q31),dup(12)(q12-q21),der(14)t(X;14)(q21;p11), t(16;16)(p12;p13.3) [cp11]

DLBCL and HL show similarities such as a GC origin, highly mutated immunoglobulin genes [103, 107] and frequent chromosomal aberrations [109, 142]. However, these similarities do not explain differences in morphology and clinical behavior. Chromosomal aberrations studied by CGH and SKY may indicate candidate regions for genes involved in the transformation (Table 7). However, the genomic instability of DLBCL and HL will certainly complicate the identification of key genes involved in the transformation. Furthermore the important genetic events could both involve structural rearrangements as well as net alterations resulting from several different abnormalities.

Alterations of chromosomal regions 6q14-16 and 7q11-31 were found in both cell lines. Recently a B-cell specific transcription factor BACH2 located in 6q15 was found to be lost in about 20% of DLBCL [152]. Interestingly BACH2 has been shown to be of importance for the effect of some anticancer drugs such as doxorubicin, etoposide and cytarabine [153] from which the two first are frequently used for treatment of HL. Loss of BACH2 expression may lead to less sensitive cells and a higher risk for relapse and transformation.

Table 7. A summary of chromosomal imbalances found in *de novo* DLBCL, cHL and transformed HL*.

Chromosomal region	CGH alteration gain/loss	*de novo* DLBCL	cHL	Transformed HL (U-2932, U-2940)
1p34-pter	loss	+	-	-
6q14-16	loss	+	-/+	+
8p22-pter	loss	+	-	-
17p12-pter	loss	+	-	-
22q	loss	+	-	-
Xpter-q21	loss	-	-	+
2p13-16	loss	+	+	-
7q11-31	gain	+	-	+
12c-q14	gain	+	+/-	+
13q22	gain	+	-	-
18q21	gain	+	-	+
Xq25-26	gain	+	+	-

*Data from the database at http://www.progenetix.de/Aboutprogenetix.html

Gain of chromosomal region 7q11-31 in HL is not a frequent event but was found both in the two transformed cell lines (U-2932 and U-2940) and in *de novo* DLBCL. Therefore, genes at 7q11-31 may be involved in the HL to DLBCL transformation. A possible candidate gene *CUTL1* regulates normal hematopoesis, in part by modulating the levels of survival and/or apoptosis factors expressed by the microenvironment [154].

Transformed DLBCL from FL (paper VI)

Transformation of FL to DLBCL is a very common event in FL. Recurrent chromosomal gains have been detected at 1q, 2p, 3q, 7, 8q, 12, 17q, 18q and X while losses have preferentially been seen at 4q, 6q, 8p, and 13q (Table 8) [155-159]. Here 28 patients, 11 men and 17 women, suffering from DLBCL, all with a previous history of FL, were studied regarding chromosomal copy number alterations using CGH. The DLBCL tumors showed more chromosomal imbalances compared to FL tumors (P=0.014). This is in agreement

with previous reports [159], and indicates that the DLBCL tumors are more cytogenetically unstable resulting in a scattered picture of chromosomal alterations [160]. Some regions were found to be gained upon transformation such as 7pter-q22, while other regions were specifically lost in the DLBCL tumor such as 4q13-21, 6q16-21 and 8p22-pter, suggesting the location of putative genes that may be involved in the transformation.

Table 8. Comparison between studies concerning chromosomal aberrations identified in transformed DLBCL tumors and absent in the FL counterpart detected by CGH.

CGH	Hough *et al.*	Goff *et al.*	Nagy *et al.*	Boonstra *et al.*	Martinez-Climent *et al.**	Present study
+X	18	0	0	3	2	2
+2p16	4	2	1	0	2	1
+3q	2	0	0	0	1	1
-4q	4	1	0	1	2	2
-6q	0	1	2	2	1	3
+7	7	1	0	5	1	3
-8p	2-3	1	0	0	1	1
+8q	0	1	1	1	0	1
+12q	12	1	0	3	2	1
-13q	2	0	2	1	3	1
+17q	2	0	0	0	2	1
+18q21	1	2	2	1	2	0
N	23	6	5	6	10	11

N= number of patients studied, * Study based on array-CGH.

An effort was made to determine if the chromosomal changes appeared early or late in the tumor progression. The tumors were ordered with increasing number of CGH alterations where early events were considered when occurring in tumors with few CGH alterations. Loss of 4q13-21 and a gain of Xq21-26 appeared as early events in the transformed DLBCL tumors indicating candidate regions for genes involved in the initiation of the transformation to the more aggressive DLBCL. Gain of 18q21 appeared early in the

FL tumors. Deregulation of the bcl-2 gene at 18q21.3 through the t(14;18)(q32;q21.3) translocation is the most common cytogenetic event in FL and an upregulation of the bcl-2 gene is considered to be one of the first events in the genesis of FL [117]. In contrast, a gain of 18q21 seemed to appear later in the DLBCL tumors and may thus not be important for the transformation from FL to DLBCL. However, other studies have shown partly divergent results (Table 8).

The present study was compared with 5 others concerning chromosomal changes detected by CGH in serial samples only (Table 8). Taken together the most common changes include gain of 2p16, 7, 12q and 18q21 and losses in 4q, 6q and 13q.

In one case six consecutive tumors were available. This series of tumors may serve as a model of linear tumor progression (Figure 12). Interestingly 3 previously reported regions, i.e. 6q, 12q and 17q (Table 7), involved in the transformation event is detected only in the DLBCL tumors suggesting that genes in these regions are indeed involved in FL to DLBCL transformation. Furthermore we noted that very little differed between CGH profiles from the two DLBCL tumors, as well as between FL tumors, and that most imbalances occurred during the FL-DLBCL transition.

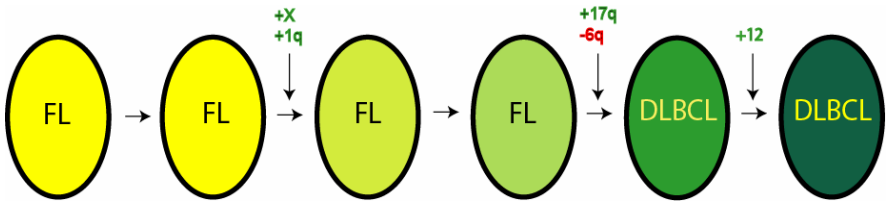

Figure 12. Cytogenetic evolution in a series of 6 tumors from the same patient. Chromosomal regions gained or lost between tumors are indicated. Gains are indicated as (+) and a (-) denotes a loss.

In the present study indications of non-linear tumor progression / transformation was found, similar to that for DLBCL tumor progression (Figure 8). Taken together it can be suggested that transformation from FL to DLBCL may be the sum of changes in different genes giving the same results.

Interestingly, imbalances in regions 6q and 7q were also found in the DLBCL cell lines derived from patients with a previous HL as well as in *de novo* DLBCL. This suggests that transformation from FL and HL to DLBCL may occur in a similar way.

CONCLUSIONS

Loss of genes at 22q may be correlated to advanced clinical stage in diagnostic tumors in DLBCL.

Gain of chromosomal band 18q21 may be more common in relapses.

In DLBCL, a GC phenotype as well as the bcl-6, CD10 and bcl-2 expression are important prognostic factors and may be valuable for the clinical management of the patients.

Loss of chromosomal region 6q14-16 and genes at 2q and/or 7q may be of importance in transformation HL to DLBCL.

Loss of 6q16-21 and gain of 7pter-q22 may be involved in the transformation process from FL to DLBCL.

Amplification of chromosomal band 18q21 is an important mechanism for upregulation of bcl-2 in DLBCL but probably not involved in histological transformation from FL to DLBCL.

Tumor progression detected by CGH in *de novo* as well as transformed DLBCL is not necessarily linear. Non-linear cases occur probably with a higher incidence than previously believed.

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