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CHARACTERISATION OF MOLECULAR ALTERATIONS IN DIFFUSE LARGE B-CELL LYMPHOMA WITH RESPECT TO TRANSFORMATION, PROGRESSION AND PROGNOSTIC MARKERS

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Characterisation of Molecular Alterations in Diffuse Large B-cell Lymphoma with respect to Transformation, Progression and Prognostic Markers.

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“The essential is invisible to the eyes”

Antoine de Saint-Exupéry, “The Little Prince”

To all, whom I loved

ABSTRACT

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoma type, and comprises nearly 500 newly diagnosed cases in Sweden every year. DLBCL is a group of aggressive lymphomas that are extremely heterogeneous and differ in morphology, immunophenotype, molecular and clinical features as well as in response to therapy. The transformation of low malignant lymphoma, most commonly follicular lymphoma (FL), to DLBCL is associated with a progressive disease and poor prognosis. The overall aim of this thesis was to identify the genetic events underlying the progression and transformation of indolent FL to aggressive DLBCL and to outline molecular alterations distinguishing DLBCL with germinal center (GC) and non-GC phenotype.

In search for novel markers in lymphoma, we have investigated an anti-apoptotic protein, HS-associated protein X-1 (HAX-1), at the protein and transcript level in malignant lymphomas (paper I). We found high levels of HAX-1 mRNA and protein expression in B-cell lymphomas. We also identified a positive association between proliferation (Ki67) and HAX-1 expression, as well as an inverse correlation between Bcl-2 and HAX-1 demonstrated on a transcript and protein level in FL, which indicates the potential role of HAX-1 as a Bcl-2 homolog. In papers II and III we studied paired tumours of FL and its transformed DLBCL (tDLBCL) counterparts and compared with *de novo* DLBCL (dnDLBCL) using array-comparative genome hybridisation (array-CGH) in order to outline genetic alteration of importance for the transformation process. We identified a gain of 2p15-16.1 as a potential prognostic marker, and that this alteration may appear already in the FL prior to transformation. This chromosomal region encompasses, among others, genes involved in NF-κB pathway, such as *REL*, *USP34*, *COMMD1*, *OTX1* and of known importance for B-cell development, such as *BCL11A*. We have also performed whole exome sequencing (WES) results in a smaller group of patients with consecutive lymphoma samples. A comparison with array-CGH data showed that these two methods complement and strengthen each other in pinpointing crucial genetic events. Furthermore, our studies revealed that clonal evolution of transformed tumours occurs according to the branching model. A number of mutations identified in the peri-transformation phases – defined as FL appearing prior and tDLBCL directly after transformation - affected genes involved in histone modifications, cell cycle, apoptosis, PI-3 kinase pathway, and Ras signalling pathway. In Paper IV we performed proteomic profiling of DLBCL subtypes *i.e.* non-GC and GC, and identified 91 proteins present exclusively in the non-GC type of DLBCL. We focused on two proteins of importance for the NF-κB pathway (BiP and Hsp90) as well as on Cyclin B2. We observed increased expression of these three proteins in DLBCL and also in non-GC *vs* GC type of DLBCL. These findings suggest a potential relevance of these proteins as prognostic markers and may lead to new treatment options.

LIST OF PUBLICATIONS INCLUDED IN THIS THESIS

I. **Anna Kwiecinska**#, Astrid Ottosson-Wadlund#, Rebecka Ceder, Roland C. Grafström, Erik Björck, Magnus Nordenskjöld, Anna Porwit, Bengt Fadeel*
"HAX-1 expression in human B lymphoma"
Leukemia 2011; 25: 868-897

II. **Anna Kwiecinska**, Koichi Ichimura, Mattias Berglund, Andrii Dinets, Luqman Sulaiman, V.Peter Collins, Catharina Larsson and Svetlana Bajalica-Lagercrantz*
"Amplification of 2p as a Genomic Marker for Transformation in Lymphoma"
Genes, Chromosomes and Cancer 2014; 53:750-768

III. Chenglin Wu#, **Anna Kwiecinska**#, Mattias Berglund, Noel FCC de Miranda, Longyun Chen, Gunilla Enblad, Svetlana Bajalica-Lagercrantz*, Qiang Pan-Hammarström*
"Genetic alterations during transformation of follicular lymphoma to diffuse large B-cell lymphoma revealed by array-CGH and whole-exome sequencing"
submitted manuscript

IV. **Anna Kwiecinska***, Anna Porwit, Nazariy Souchelnytskyi, Ann Kaufeldt, Catharina Larsson, Svetlana Bajalica-Lagercrantz, Serhiy Souchelnytskyi*
"Proteomic profiling of Diffuse Large B-cell Lymphoma subtypes (non-GC and GC type) "
submitted manuscript

shared first authorship
* corresponding author

RELATED PUBLICATIONS

1. Harmann S., Gesk S., Scholtysik R., Kreuz M., Bug S., Vater I., Döring C., Cogliatti S., Parrens M., Merlio JP., **Kwiecinska A**., Porwit A., Piccaluga PP., Pileri S., Hoefler G., Kuppers R., Siebert R., Hansmann ML.
   "*High resolution SNP array genomic profiling of peripheral T-cell lymphomas, not otherwise specified, identifies a subgroup with chromosomal aberrations affecting the REL locus"*
   Br J Haematol. 2010 Feb; 148(3): 402-12

2. Högfeldt T., Bahnassy AA., **Kwiecinska A**., Osterborg A., Tamm KP., Porwit A., Zekri AR., Lundahl J., Khaled HM., Mellstedt H., Moshfegh A.
   " Patients with activated B-cell like diffuse large B-cell lymphoma in high and low infectious disease areas have different inflammatory gene signatures"
   Leuk Lymphoma 2013, May; 54(5): 996-1003

CONTENTS

1 Introduction ... 1
  1.1 TUMOUR GENETICS ... 1
    1.1.1 ONCOGENES ... 2
    1.1.2 TUMOUR SUPPRESSOR GENES ... 2
    1.1.3 DNA REPAIR GENES ... 3
  1.2 EPIGENETICS ... 3
  1.3 TELOMERES ... 5
  1.4 CANCER CYTOGENETICS ... 5
  1.5 THE GERMINAL CENTER ... 7
  1.6 HEMATOLOGIC NEOPLASIA ... 8
  1.7 B-CELL DEVELOPMENT AND RECEPTOR REARRANGEMENTS ... 10
  1.8 GERMINAL CENTER DERIVED LYMPHOMA (FL, DLBCL) ... 11
    1.8.1 FOLLICULAR LYMPHOMA ... 11
    1.8.2 DIFFUSE LARGE B CELL LYMPHOMA ... 16
    1.8.3 TREATMENT ... 22
    1.8.4 TRANSFORMATION OF FOLLICULAR LYMPHOMA TO DIFFUSE LARGE B CELL LYMPHOMA. ... 24
2 AIMS OF THE STUDY ... 31
3 MATERIALS AND METHODS ... 33
  3.1 PATIENTS AND TUMOUR SPECIMENS ... 33
  3.2 METHODS ... 34
    3.2.1 DNA preparation ... 34
    3.2.2 Array CGH ... 34
    3.2.3 Taqman DNA copy number analysis ... 36
    3.2.4 DNA sequencing ... 37
    3.2.5 Proteomics ... 37
    3.2.6 Immunohistochemistry ... 39
4 RESULTS AND DISCUSSION ... 41
  4.1 Paper I: HAX-1 expression in B-cell lymphoma ... 41
  4.2 Paper II: Transformed DLBCL from FL ... 41
  4.3 Paper III Genetic alterations during transformation ... 42
  4.4 Paper IV Proteomic profiling of DLBCL ... 44
5 CONCLUSIONS ... 47
6 FUTURE PROSPECTS ... 48
7 ACKNOWLEDGEMENTS ... 49
8 REFERENCES ... 55

LIST OF ABBREVIATIONS

ABC	Activated B-cell
array-CGH	Array comperative genome hybridization
ASTC	Autologous stem cell transplant
BAC	Bacterial artificial chromosome
bp	Base pair
CAR	Chimeric antigen receptor
CGH	Comperative genome hybridization
Cα	C-region gene α
DLBCL	Diffuse large B-cell lymphoma
DNA	Deoxyribonucleic acid
FL	Follicular lymphoma
GC	Germinal center
HDT	High dose therapy
Ig	Immunoglobulin
kb	Kilo basepairs (10^3 bp)
LOI	Loss of imprinting
Mb	Mega basepairs (10^6 bp)
NHL	Non-Hodgkin lymphoma
p	The short arm of chromosome
PAC	Plasmid derived chromosomes
PMBCL	Primary mediastinal B-cell lymphoma
PCR	Polymerase chain reaction
pter	The end of the short arm of a chromosome
q	The long arm of a chromosome
qter	The end of the long arm of a chromosome
RNA	Ribonucleic acid
SHM	Somatic hypermutation
t	Translocation / Transformation
WHO	World Health Organisation

1 INTRODUCTION

1.1 TUMOUR GENETICS

It is generally believed that tumourigenesis in humans is a multistep process and that the genetic alterations stepwise drive the progressive evolution of normal cells into malignant ones (Figure 1). Pathological observations reveal the lesions that represent intermediate steps during progressive development from normal cells to malignant cancer. Genetic analysis also shows that the genome of tumour cells are altered by both subtle mutations and obvious changes in chromosomes (1).

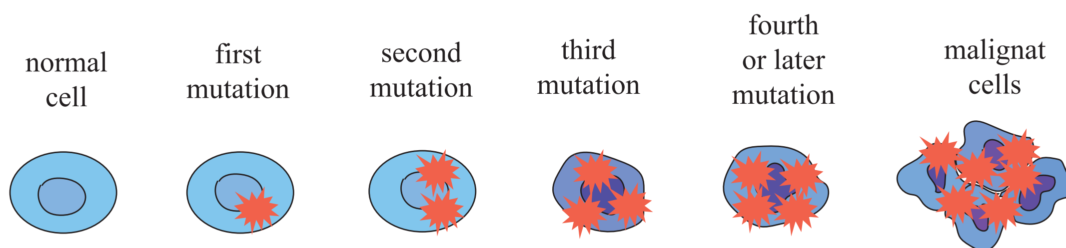

Figure 1. Tumour progression is a stepwise process. Cancer arises through accumulation of genetic alterations, copy number changes and mutations. A combination of selected mutations defines the phenotype of the tumour cells.

Historically, the genetic damage was divided into two kinds: a dominant one, affecting genes named as “proto-oncogenes” and recessive one, referring to tumour suppressor genes. The first ones are usually associated to gain of function while the latter ones are typically affected by loss of function (2).

These terms rather reflect the historical background related to the genes discovery than their normal function (2).

1.1.1 ONCOGENES

What does make a gene an oncogene? And when a good guy turns bad…

Proto-oncogene can be any gene that normally encodes proteins responsible for cell growth, differentiation and proliferation. In this category we can find growth factors with their receptors, signal transducers, transcription factors and regulators of apoptosis. The ”activation” of a protooncogene into an oncogene is a pathological process resulting into increased growth and proliferation leading to tumourigenesis (2, 3).

This ”activation” can occur through different mechanisms, typically involving bp mutations, amplifications or chromosomal rearrangements.

Malignant transformation is related to “dominant” effect of protooncogene activation over normal cells, where ”evil overrides good”. However it may be restrained in two manners. Firstly, limitation is a threshold below which gene expression had very little or no consequence. Secondly, the effect of expression of an oncogene may be modulated by other proteins (2).

1.1.2 TUMOUR SUPPRESSOR GENES

“The guardians of the genome”

Tumour suppressor genes are the guardians of the genome, involved in control of crucial activities in cell life such as DNA repair, cell cycle control and apoptosis. They regulate protein degradation, cell specification, differentiation, migration and angiogenesis (3).

Tumour suppressor genes can be divided in two groups: gatekeepers and caretakers. Caretaker genes act as protectors of the genome against damage, gatekeepers inhibit growth by inducing cell death and cell cycle arrest of already damaged cells (4).

Thus, caretakers are not regulating cell proliferation but rather increase the genome stability. It has been suggested that increased activity of caretaker genes is important for genome stability and reduces cancer risk as well as postpone aging (4).

Inactivation of caretakers often leads to cancer cell development. Our genome is constantly at risk of acquiring mutations, which can be caused by exposure to products of cell metabolism like reactive oxygen species or environmental chemical factors. Despite protecting mechanisms like antioxidant defense system, the cell will be still at risk to accumulate DNA damage. That is where DNA repair systems play crucial roles (4-6).

1.1.3 DNA REPAIR GENES

There are a number of lesions in the DNA acquired by one or both strands that can be repaired by base or nucleotide excision repair. DNA double strand breaks can be repaired by nonhomologous endjoining or homologous recombination. Other lesions like mismatches and crosslinks are repaired by mismatch and crosslink repair genes, respectively (4).

The genes responsible for coordination of these processes with activation of cell cycle checkpoints such as *ATM* and *BRCA1/BRCA2* are also well known caretakers (4).

Inactivation of DNA repair system within tumour cells, results in genetic instabilty, accompanied usually with loss or gains of partial or complete chromosomes. Different types of instability are the result of disorganisation in different repair systems (4).

Within the category of gatekeepers, the *TP53* gene is the most known. *TP53* regulates the expression of genes responsible for apoptosis, cell cycle, DNA repair and angiogenesis (7). *TP53* is also the most frequently mutated gene in human cancer. About half of tumours show alteration in *TP53* alleles (8).

The path to discovery of the *TP53* as tumour supressor gene was filled with a few twists and turns (3) as *TP53* was initially regarded as a dominant oncogene, due to its ability to transform cells in culture (9, 10). We know now, that these first experiments detected mutant p53, called dominant negative effect, creating a situation where a heterozygous mutation in *TP53* causes a deficiency in the function of this gene, mimicking the effect of homozygous mutation in tumour supressor gene. It took a few years until Finlay and coworkers (1988) demonstrated that p53 acts as a suppressor of transformation (11).

Backing up in time, the cardinal features of tumour suppression were first shown in studies on retinoblastoma and Wilms tumour before p53 was identified. Alfred Knudson's legendary ”two hit mutation theory” was a result of his perspective and work as pediatrician (12), (Knudson 1971). He postulated that retinoblastoma might be caused by two mutations; one inherited (germline) and one acquired. Thus children who inherited an affected gene, developed disease earlier in life and it affected both eyes. In non-hereditary cases, requiring two *de novo* mutations, the disease would be rare, develop later and would be unilateral.

Inactivation of a tumour suppressor gene and loss of its normal function can be a result of different changes including large chromosomal alterations, mutations and epigenetic modifications.

1.2 EPIGENETICS

Pathological epigenetic changes are alternatives to mutations and chromosomal aberrations in disrupting gene functions (13).

Historically, the word ”epigenetics” was used to describe events that could not be explained by genetics (14). Conrad Waddington who is believed to coin this term described epigenetics as ”the branch of biology which studies the casual interactions between genes and their products, which bring the phenotype into being” (Waddington, 1942).

In 1957, Conrad Waddington proposed the concept of an epigenetic landscape to represent the process of cellular decision-making during development. At various points in this process (represented in the original picture by a ball) can take specific permitted trajectories leading to different outcomes (15).

Genetic and epigenetic changes occur together in the development of cancer. Feinberg et al. proposed a three step model where the first event is an epigenetic disruption of a progenitor cell which triggers a population of cells with ability to grow. The second step involves an initiating mutation within the population of epigenetically disrupted progenitor cells at the beginning of cell growth. This is a first step in cancer development. The third step is genetic and epigenetic instability, associated with clonal tumour evolution (13, 16).

Epigenetic changes involves changes of global DNA methylation patterns, such as chromatin alterations and loss of imprinting (13). Altered DNA methylation is a striking feature of tumorigenesis. Most CG dinucleotides are methylated on cytosine in human genome. (13).

It has been suggested that hypomethylation is important in the earliest stages of tumorigenesis while hypermethylation is of greater importance for tumour progression. Epigenetic modifications in cancer are typically related to hypermethylation of promotor region of genes especially associated with tumour suppressive function. This leads to silencing of tumour suppressor genes and promotes tumourigenesis (13, 16-18). All the above described mechanisms apply also to lymphoma and other hematological malignancies.

The nature of epigenetic lesions may be very variable. The abnormal cell may switch its epigenotype and silence normally active genes and activate the silent ones by methylation changes, histone modifications and chromatin proteins. Epigenetic lesions may involve alterations in heterochromatin pattern and changes of methylation in gene promoters (16).

The chromosomal aberrations such as rearrangements and mutations can cause widespread chromatin disruption. One example is the *MLL1,* which is rearranged and also activated in acute leukemia and also in some B-cell lymphomas. It activates gene expression by methylation of *H3K4* . Another example of epigenetic lesion is the increased expression of the *EZH2* gene, observed in many tumours including lymphomas (19).

Are the epigenetic changes a cause or consequence?

It is widely discussed that epigenetic changes may be a result of gene expression alterations. Good evidence for causal argument would be if the constitutional epigenetic changes were linked with a increased cancer link. We know for example that LOI (lost of imprinting) of the *IGF2* gene is significantly associated with increased risk for cancer in children with

Beckwith–Wiedemann syndrome. Conversely, we know that epigenetic alterations occur in normal tissue like for *e.g.* hypermethylation of *p16* that occurs with aging in normal tissue of women with breast cancer. However, such alterations has been not shown to increase cancer risk (16).

1.3 TELOMERES

Each chromosomal end consists of long stretches of (TTAGGG)n tandem repeats composes the telomeres. Telomeres function is to protect chromosomal ends from erosion, fusions and rearrangements with other chromosomes. Telomeres shorten with every cell division because the DNA polymerase is unable to replicate these ends due to lack of templates. Telomerase are the enzyme complexes that are responsible for maintaining the telomeres in cells undergoing multiple divisions such as B- and T-cell lymphocytes. Also, cancer cells may activate these enzymes in order to take advantage of continuous cell division (20).

1.4 CANCER CYTOGENETICS

When, why and how do chromosomal aberrations arise?

Tumourigenesis is a process associated with acquisition of specific genetic abnormalities, some resulting in activation of oncogenes and inactivation of suppressor genes that are essential for tumour formation. The acquisition of these changes is a multi-step process, where characterisation of primary and secondary aberrations is of a great interest (21).

The primary aberrations are mostly found as the sole karyotypic abnormalities and refer to the first changes occuring in neoplastic cells, and are fundamental for the establishment of a neoplastic clone (21).

The primary aberrations are seen in cells already carrying the primary alterations and thus never appear alone. They may even be so numerous in later disease-stages that they completely dominate the picture (21). Secondary aberrations are considered to be less specific than primary changes and depend on the primary aberration. They may occur during the disturbed mitotic process and sometimes may be facilitated by the primary aberration. This leads to a genetic diversity within a tumour, where different tumour subclones undergo evolutionary selection (21). The subclones with proliferative advantage expand gradually over less fitted subclones. During this dynamic process, the total tumour karyotype may evolve towards a greater or smaller complexity according to a divergent or convergent model. Numerous data suggest that this process may be monoclonal or oligoclonal at this early stage (21).

The concept of the primary and secondary acquired chromosomal aberration refers to functional distinction where primary alterations are considered as more specific (like specific

gene rearrangements) and secondary aberrations are the result of large genomic imbalances (22). According to this theory, the large chromosomal abnormalities would always be secondary to the primary mutations. While primary and secondary alterations are by definition clonal, the terminology "*cytogenetic noise*" is used for extensive but non-clonal abnormalities (23).

The question remains whether the primary and secondary aberrations appear by chance in a random manner or there is a predilection of specific lesions (preferably primary abnormalities) to specific genomic sites. The hypothesis of randomly occurring alterations, that occur along the genome with following selection for the more "evolutionary fit" clones with proliferative advantage, is a logical and possible explanation (21). However, it is also easy to believe that certain genomic rearrangements (especially the primary ones) may tend to occur in a certain genomic localisation and as a result of interaction between a carcinogenic agent and specific genomic site. This theory is supported by the fact that several toxic agents have been found to increase the risk of particular translocations and genetic abnormalities (21).

Moreover, for the external agents, the internal factors play a role in origin of specific chromosomal alterations. The susceptibility and tolerance to DNA brakes are common feature of tumour cells, increasing the possibility of tumourigenic recombination and mutations (24). Common chromosomal fragile sites that are involved in formation of DNA breaks can be considered as predisposing factors for chromosomal rearrangements (21, 25) .

One should not forget the role of the three-dimensional chromosome architecture within a nucleus, where the chromosomal localisation is quite well defined (26-28). That localisation affects the way that various chromosomal aberrations arise and DNA double strand breaks are probably involved in this process. Another logical requirement for chromosomal rearrangements is a physical proximity between breakpoint regions. Several loci recombined in specific translocations, such as *BCR/ABL;* t(9;22) in chronic myeloid leukemia, *IGH/MYC*; t(8;14), *IGH/CCD1* ; t(11;14) and *IGH/BCL2*; t(14;18) in B-cell malignancies, are closely located in the corresponding normal cell types (21, 29) . However, this is not enough to explain the origin of all rearrangements. Another factor that may facilitate the illegitimate recombination is the shared sequence motifs at the chromosome breakpoints (30-33).

Still a lot needs to be explained concerning why and how the chromosomal alterations occur. For the time being we tend to believe that the primary and secondary genetic aberrations arise as stochastic events (21).

1.5 THE GERMINAL CENTER

"Everything comes at a price"

Germinal centers (GC) are the histological structures dedicated to the generation and the selection of B-cells that produce high affinity antibodies (34, 35).

B-cells originate from pluripotent stem cell in bone marrow and continue to differentiate until a functional B-cell receptor is expressed. After starting migration from bone marrow, B-cells encounter the antigen, that initiates further differentiation in a lymph node. In the lymph node B-cells are localised in the follicles, while T-cells are located in the interfollicular areas. Some follicles form germinal centers and these are involved in the selection of B-cells secreting high affinity antibodies (35). Antigen specific memory B-cells and plasma cells appear within 1 week after antigen encounter, which indicates that the GC reaction is remarkably efficient (36). In the GC reaction, a naive B-cell is activated by antigen receptor stimulation from T helper cell and transforms into centroblast that proliferates in the "dark zone" of the GC. The "dark zone" is a place where B-cells undergo somatic hypermutation (SHM), where a single base-pair changes are randomly introduced (1x 10^{-3} bases per generation into the IgV regions) in order to select for immunoglobulin with higher affinity for a specific antigen (37). After proliferative expansion and SHM in the dark zone, B-cells move to the "light zone" and the morphological change from centroblast to centrocyte. Here their fate depends on other GC resident cells like T-cells and follicular dendritic cells (35). In the light zone the B-cells are selected depending on their ability to bind antigen presented to them by follicular dendritic cells and T-cells. This process is a negative selection, where most B-cells acquire deleterious mutations in the IgV regions and then undergo apoptosis. The cells with highest affinity are selected for further differentiation. A fraction of B-cells also switches Ig classes (from IgM and IgD to other classes) by isotype switch recombination. This process enables to produce antibodies with different effector functions due to replacing *IGH* constant C-region by a downstream C-region gene *i.e.* Cγ, Cα, Cε, which allows expression of IgG, IgA, IgE without loosing the specificity (38)

The germinal center is also a place of the origin of most human B-cells lymphomas (35). Moreover, the same genetic mechanisms that are involved in the fundamental germinal center function are involved in lymphomagenesis.

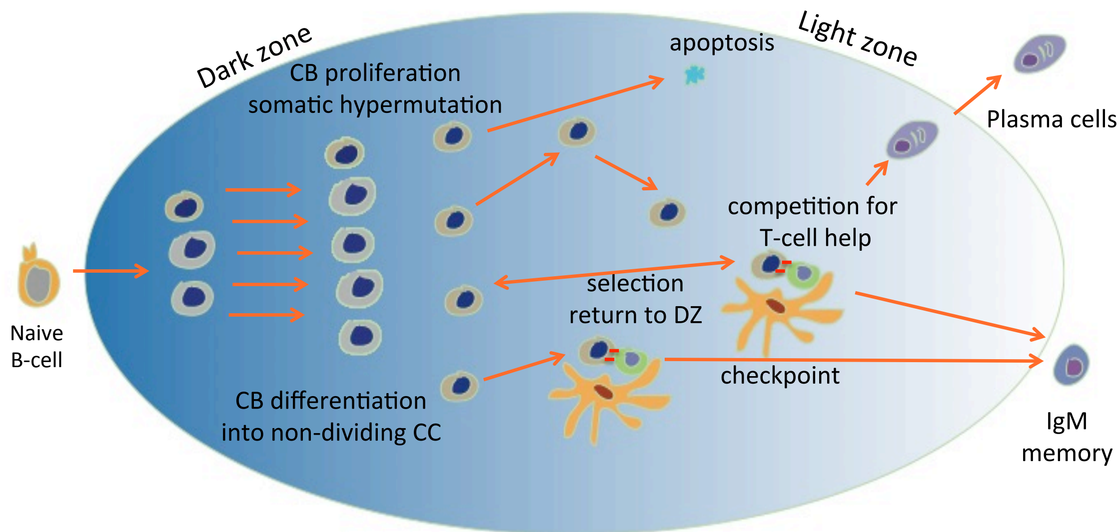

Figure 2. Germinal center – a figure illustrating processes during B-cell differentiation. Abbr.: CC, centrocyte; CB, centroblast; DZ, dark zone. (Modified according to Basso *et al* (35)).

1.6 HEMATOLOGIC NEOPLASIA

The total number of patients who currently suffered from a hematologic neoplasm were approximately 30 000 for the year 2012. Lymphoma is the 7th most common malignant disease diagnosed in Sweden encompassing approximately 2 600 newly diagnosed cases each year. The prognosis and clinical course vary tremendously from indolent forms to highly aggressive tumours with rapid manifestation and progression (Cancerfonden's report 2014).

Lymphomas can be of B- or T-cell origin and the malignant transformation may occur at any stage of differentiation. The vast majority of lymphomas (85%) are of B-cell origin. Here we can distinguish B-cells disorders arising from precursor B-cells to mature B-cells. We further divide mature B-cell lymphomas in two subgroups of Hodgkin and non-Hodgkin lymphomas with a dominance of the latter ones.

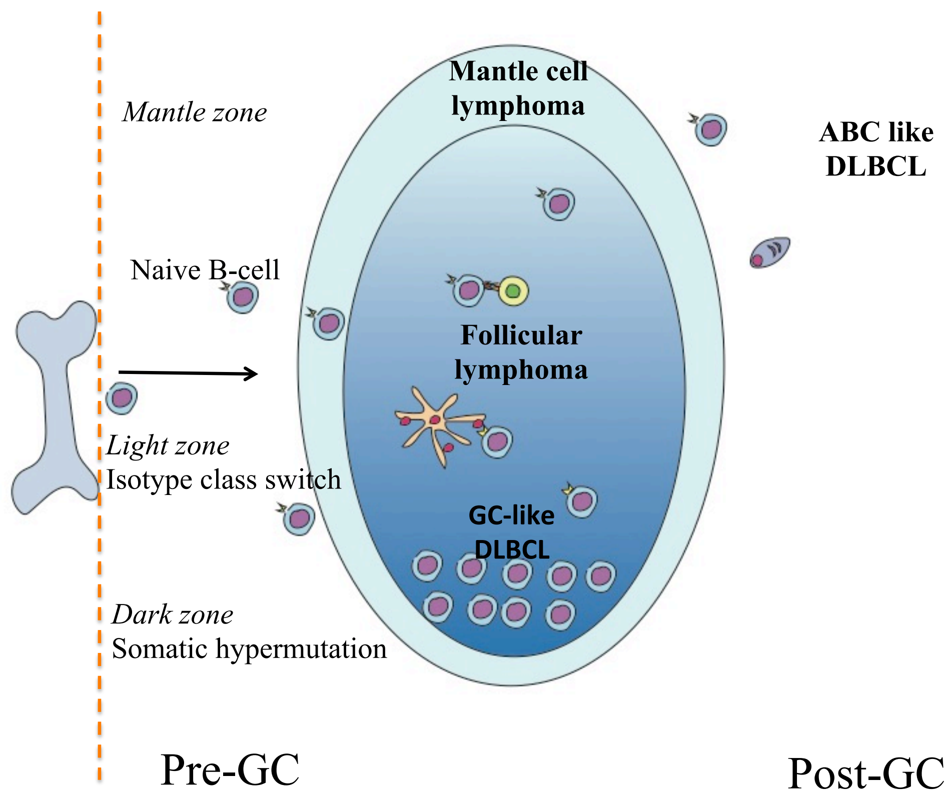

Figure 3. Picture illustrating the origin of mature B-cell lymphomas

Mature B-cell lymphomas are a heterogeneous group of malignancies regarding histological, clinical and morphological features. They may arise in bone marrow but also in other organs, mostly lymph nodes and spleen, but also non-lymphatic organs such as skin or central nervous system may also be their primary site of origin.

Following the first discovery of Manolov and Manolova (1972) of +14q as a marker for Burkitt lymphoma, Zech *et al* (1976) defined the marker as t(8;14)(q24;q23) as pathognomonic for this disease, and the first characteristic translocation was identified in B-cell lymphomas (39, 40). Today, molecular cytogenetic approach using fluorescence *in situ* hybridisation (FISH) and array comparative genome hybridisation (array-CGH) has become a daily diagnostic routine. The identification and characterisation of recurrent chromosomal aberrancies as hallmarks of lymphoid neoplasm has its reflection in the current WHO classification of hematologic malignancies (41).

The WHO classification divides the lymphoid neoplasms based on their morphologic, immunophenotypic, genetic features and clinical presentation. For each neoplasm a cell of origin is postulated, reflecting a differentiation stage rather than a real cell in which neoplastic transformation actually occurs (41).

1.7 B-CELL DEVELOPMENT AND RECEPTOR REARRANGEMENTS.

Immunoglobulin (Ig) molecules, constitute of Ig heavy chains (IgH) and light chains, of either kappa (Igκ) or lambda (Igλ) type. These proteins are encoded by the *IGH* locus at the 14q32, while *IGK* is located at the 2p12 and *IGL* at the 22q11 position (21). The *IGH* gene is consisted of V, D, J and C segments. *IG* light genes lack D segments (21).

B-cell development begins with a rearrangement of *IGH* and *IGK/L* in B-cell progenitors in the bone marrow. V(D)J in *IGH* and VJ in *IGK/L* recombination form a functional Ig (21).

Notably, the *IGH*, *IGK* and *IGH* loci are commonly affected by chromosomal translocations often resulting in activation of oncogenes. This mechanism is observed in many mature B-cell lymphomas. The frequency of translocations varies a lot depending on the lymphoma subtype (21).

For some lymphomas, the specific *IG* translocation is considered as pathognomonic for the disease and is detected in more than 95% of cases *e.g*. Burkitt lymphoma is associated with t(8;14) (q24;q32) involving the *MYC* oncogene and mantle cell lymphoma is associated with t(11;14) (q13,q32) involving the Cyclin D1 gene (CCDN1) (21).

In other lymphomas such as DLBCL, *IG* translocations are detected roughly in half of the cases (42). Translocations in DLBCL may be multiple and involve both *IGH* as well as a light chain locus, the most common is t(14;18)(q32;q21), a hallmark of FL seen in tDLBCL (23, 43)

As *IG* loci are subject to multiple physiological breaks as a part of the recombination during B-cell development, chromosomal translocations involving these regions are believed to be a result of mistakes in the recombination processes. *IG* translocations may lead to constant activation of oncogenes most typically through deregulation mediated by enhancers within *IG* loci. Rarely, the fusion transcripts of the *IG* and oncogenes are observed (21).

Interestingly, enhancers may keep their activity over distances of several hundred kilobases. Such a situation is observed in the case of *MYC* translocations, where breakpoints affecting the *MYC* gene can be located up to 1 Mb centromeric of *MYC* in t(8;14) and telomeric of *MYC* in t(8;22) and t(2;8) (21) .

The most commonly observed rearrangement on non-*IG* locus in mature B-cell lymphoma is rearrangement affecting the *BCL6* locus at 3q27. The translocation results in promotor substitution and causes deregulation of the *BCL6* gene (44-47).

Other oncogenes known to be targets of *IG* translocations are known to be juxtaposed sometimes directly next to each other through a translocation, *e.g. MYC* and *PAX5* through t(8;9)(q24;p13) and *BCL6* and *MYC* through t(3;8)(q27;q24) in t(14;18) positive FL (48, 49).

Moreover, one of the most common alterations detected in all types of mature B-cells lymphoma, is a deletion of the long arm of chromosome 6. The incidence varies from 5% in

B-CLL up to 30% in DLBCL (21). The deletions are mostly terminal and the band 6q21 is one of the most commonly affected. The second most frequently affected is region 6q25-27 and the third most common 6q23. Previous studies have shown inactivation of the *PRDM1/BLIMP1* gene located in 6q21 in ABC-DLBCL (50, 51). Then, a commonly deleted region 6q23.3 encompasses *TNFAIP3* (*A20*) and *PERP* genes, has been detected in FL and marginal zone lymphoma (MZL). Moreover, mutations in *A20* have been described in classical Hodgkin lymphoma as well as primary mediastinal B-cell lymphoma (52).

The general rule is that the prognosis is inversely related to the karyotypic complexity (21). The most common cytogenetic aberrations in FL and aggressive B-cells lymphomas are listed in Table 1.

Neoplasm	Cytogenetic aberration	Frequency (%)
FL	t(14;18)(q32;q21) and variants involving *BCL2*	80-90%
	translocations involving 3q27/*BCL6*	<10% FL g1-2,55% FL g3b
	+X, +7, +12/12q, +18/18q, +der (18)t(14,18), del6q, del10q, del17p, dup1q, der(1p)	>10%
DLBCL	translocations involving 3q27/*BCL6*	20-40%
	t(14;18)(q32;q21)	20-30% (GC)
	t(8;14) (q24;32)/t(8q24)	5-15% (GC>ABC)
	+3/3q, +18/18q, +19q, del6q, del9p	10-40% (ABC)
	+1q, +2p13-16, +7,+11q,+12/12q	10-40% (GC)
	+9/9p23-24	50-90% (PMBCL)
	+2p13-16	50-90% (PMBCL, GC)
B-cell lymphoma UNS with features intermediate between DLBCL and B; Burkitt lymphoma	translocations involving 8q24/*MYC*	40-60%
	t(14;18)/*IGH-BCL2*	35-50%
	t translocations involving 3q27/*BCL6*	
	t(8,14)(q24,q32) and variants	100% (Burkitt lymphoma)
	dup 1q	30-50%
	+7, +12	10-30%

Table 1. Overview of frequent and clinically relevant chromosomal aberrations in the most common mature B-cells lymphomas (21). (Modified from Mitelman *et al* 2015 (21)).

1.8 GERMINAL CENTER DERIVED LYMPHOMA (FL, DLBCL)

1.8.1 FOLLICULAR LYMPHOMA

Follicular lymphoma (FL) is the second most frequent subtype of nodal lymphoid malignancies in Western Europe. FL is the prototype of indolent lymphoma, characterised by typically slow progression. Nevertheless a number of patients develop increasingly resistant disease over time with 3% per year risk of transformation to an aggressive lymphoma, associated with poor outcome (53).

1.8.1.1 Pathology of FL

FL is a neoplasm composed of germinal center B-cells, typically both centrocytes and centroblast/large transformed cells, which usually has at least partially follicular growth pattern (41).

The proportion of centroblasts and centrocytes underlies the grading system: *grade 1* comprising low number of centroblasts (<5 per high power field (HPF)), *grade 2* 5-15 centroblasts/HPF, *grade 3a* >15 centroblasts/HPF , whereas in *grade 3b* FL is composed of solid sheets of centroblasts. Grade 3b FL resemble *de novo* DLBCL and harbor a different molecular profile compared to grade 1-3a FL (41).

Diagnosis of FL should be based on a surgical excision or lymph node biopsy. Considering the heterogeneity of FL, core biopses should be only applied to patients without accessible lymph nodes (*e.g.* retroperitoneal tumours) (Figure 6). FL grading is difficult to assess on core biopsies and may not be representative for the whole tumour (54).

1.8.1.2 Cell of origin

In follicular lymphoma, tumour cells are organized in follicles and express germinal center markers such as Bcl6 and CD10. Lymphoma cells show also gene expression profiles typical for centrocytes and centroblasts, which suggests that FL cells undergo differentiation characteristic for GC cells (53, 55). The first hit in a cascade leading to FL is believed to be t(14;18) based on the frequency of this alteration and its function in pathogenesis (53).

It has been suggested that since VDJ recombination occurs in early B-cell development in bone marrow, the 14;18- translocation as the first genetic hit may therefore occur in the bone marrow (53). Naive B-cells carrying the t(14;18) exit the bone marrow and colonise a secondary follicle where they undergo the germinal center reaction (53). Constitutive expression of the anti-apoptotic protein Bcl2, which is normally absent in normal GC, gives lymphoma cells a survival advantage. (56). Moreover, Bcl2 helps the neoplastic B-cells to escape apoptosis, which would normally occur due to weak B-cell receptor affinity (53, 56).

The origin of FL is a matter of debate. The hypothesis which is recognising naive t(14;18) positive B-cells as potential FL progenitor cells is complicated by the fact that very rare t(14;18) positive B-cells (at frequency $10^{-5} – 10^{-7}$) are detected in about 50-70% of healthy population without any risk of developing lymphoma (53, 57-59). However, we have to consider that most of these t(14;18) positive circulating B-cells in healthy individuals are not truly ”naive” as studies show. Instead they are germinal center educated IgD+ CD27+ (or IgM+CD27+) memory B-cells (56, 60, 61). It has also been shown that FL can arise in both the donor and recipient after allogenic stem cell transplantation showing matching complex genetic profile (62). This report also indicated that progenitor lymphoma cell may have far more complex genetic alterations than previously suspected (53).

FL may be proceeded by an *in situ* phase - FLIS, which may progress to a clinical disease in a subset of patients (63). However, it may be difficult to distinguish this entity from very early stage of incidentally detected FL and a lymph node with partial FL involvement (53).

1.8.1.3 Genetic landscape of FL

Around 80-90% of FL show t(14;18)(q32;q21) or very rare variants t(2;18)(p12;q21) and t(18;22)(q21;q11) (21).

In molecular terms t(14;18)(q32;q21) in FL juxtaposes the *BCL2* oncogene next to *IGH* locus (64). The breakpoints in the *IGH* locus affect he region containing the J-segments. This translocation originating from failed VDJ rearrangement, is an argument for the theory saying that a lymphoma progenitor cell originate in bone marrow rather than germinal center

The t(2,18)(p12;21) and t(18;22)(q21,11) that juxtapose the *BCL2* gene next to *IGK* and *IGL* loci are rare variants (21).

Only in about 10% of t(14;18) positive cases, the translocation is the single cytogenetic change (65). Otherwise, the recurrent secondary alterations comprise +X, +1q21-44, +7, +12q, +18q, del(1)(p36), del(6q), del(10)(q22-24) and development of ploidy (21, 65). The most frequent secondary event arising after t(14;18) is a duplication of the der(18)t(14;18).

In t(14;18)-negative FL, the most common changes concern chromosome 3 (66). Trisomy 3 seems to be more frequent among t(14;18) negative than positive FL and the occurance of t(3q27) has been reported significantly higher (up to 55%) in FL3b (21, 66). The target gene in this region (3q27) is *BCL6* (known even as *BCL5* or *LAZ3*), which encodes a transcriptional factor important for germinal center formation (29, 45).

Another common alteration in FL is non-random loss of 1p36. This region encompasses the *TNFRSF14* gene, which has been shown to be recurrently mutated resulting in a truncated protein and decreased surface expression of the receptor (67, 68). Inhibitory or stimulatory signals to T-cells can be transmitted to the receptor depending on ligand interactions. Mutations in this receptor are recurrent event in FL and act as a link between tumour cells and microenvironment (53).

A variety of cytogenetic and molecular studies have linked loss of 6q as being with unfavorable outcome (67). These studies revealed that *TNFAIP/A20* (6q23.3), a negative regulator of NF-κB signaling and receptor tyrosine kinase *EPHA7* (6q16.1), have a tumour suppressing role in FL (69).

Recent studies have shown that germinal center derived lymphomas show a high frequency of mutations in histone modifying genes (53). FL in particular, is characterised by recurrent mutations in the histone methyltransferases *MLL* (89%), previously mentioned *EPHA7* (deletion + methylation 70%), histone acetylases *CREBBP* (33%), *EP300* (8,7%) and *MEF2B* (15,3%), suggesting that it is a disease of the epigenome as well as the genome (19, 53, 70-74).

One of the best studied histone modyfing genes in lymphoma is *EZH2* gene coding enzyme that is a catalytic unit of polycomb repressive complex 2 and acts as H3K27 methyltransferase that catalyses histone H3 Lys 27 methylation (H3K27me3). It has a function as transcriptional repressor and also contributes to embryonic stem cell properties. *EZH2* is essential for normal germinal center formation. Knocking down *EZH2* gene leads to a total loss of germinal centers (75). Conversely, upregulation of *EZH2* induces extent follicular hyperplasia. With help of immunohistochemistry, one may appreciate EZH2 nuclear stain in centroblasts (75). Also, key *EZH2* targets (*CDKN1A/p21*) were shown to be differentially repressed and *H3K27* trimethylated in centroblasts vs naive B-cells (76). *EZH2* controls proliferation through *CDKN1A* and blocks GC exit and differentiation program (76).

EZH2 gene is a common subject of mutations in lymphoma. Mutations in Tyr 641 in *EZH2* have been identified in 7% of FLs and 22% of GC-like DLBCLs leading to increase histone H3 Lys27 methylation (H3K27me3) (19). It is a gain of function mutation and one of the most common mutations seen in DLBCLs.

While in the normal germinal center the expression of wild type *EZH2* is similar to *BCL6* showing a decreasing gradient associated with B-cell differentiation towards plasmacells, the *EZH2* mutant is upregulated in B-cells during germinal center reaction suggesting that activating *EZH2* mutations lock the B-cells in GC program favoring their proliferation (77) Somatic mutation of *EZH2* cooperates with co-occurring genetic lesions to accelerate lymphomagenesis and disease progression (19, 70, 75).

EZH2 inhibition as a therapeutic strategy has been shown successful in killing lymphoma cells carrying *EZH2* activating mutations. Interestingly, it has been shown that *EZH2* inhibitors kill DLBCLs of GC type regardless of mutation whereas non-GC DLBCLs are resistant to this treatment (78).

1.8.1.4 Clinical correlations and disease progression in FL.

It is possible to distinguish at least two very distinct cytogenetical groups of FL.

The first group encompassing FL grade 1 and 2 with t(14;18) can progress to FL grade 3a which is often associated with acquisition of tertiary cytogenetic aberrations of *e.g*. 17p (*TP53*), 9p21(*CDKN2A*) or 8q24 (*MYC*). This process is associated with unfavorable prognosis (21).

The second group involves mostly FL grade 3b and lack the t(14;18). It often shows a translocation involving 3q27 (*BCL6*) and can progress to DLBCL (21).

Gene expression profiling studies show distinct differences between FL grade 1,2 and 3a as a group compared to grade 3b, indicating a different biological background. Grade 3b FL show larger similarities to DLBCL and is in a clinical aspect regarded as such.

1.8.1.5 Genetic landscapes and implications for therapy.

High-throughput sequencing has revealed the genetic landscapes of many malignancies, leading to growing interest in ”personalised medicine” (63).

Mutations that arise during early ”genetic evolution” are common in cancer cells and are attractive targets for therapy (63). However, mutations arising during disease evolution are rather restricted to a subclone and thus less suited to targeted therapy. Thus the interest in contribution of ”common progenitor cells” (CPCs) in disease relapse and development of therapy resistance increases. Understanding the clonal evolution and ability to trace back the initiating events of tumourigenesis had become an important issue in cancer biology (63).

1.8.1.6 Tumour microenvironment.

The role of the microenvironment for tumour development and its evolution cannot be underestimated, however this is when the question like ”which came first, the chicken or the egg” appears. It sill remains unclear if tumour cells drive the changes in the microenvironment, allowing the lymphoma cells to establish the clone, or if the changes in the microenvironment proceed the ones in tumour cells? The current opinions are suggesting that genetic alterations in tumour cells are driving the genetic changes in the tumour microenvironment. Another type of correlation seen in the case of lymphoma is that a more aggressive genetic/epigenetic pattern is associated with more aggressive changes in the microenvironment.

Microenvironment in germinal center derived lymphomas, and FL in particular, involves interactions of lymphoma B-cells with T-cells and macrophages as well as follicular dendritic cells.

The microenvironment can support tumour growth and survival, and suppress the anti-tumoural immune response. FL cells express high levels of cytokines (CXCR4 and CXCR5) and are attracted to follicles by cytokines such as CXCL13 released by follicular T-helper (FTh) and follicular dendritic cells (FDC) (53, 79). As antigen presenting cells the FDCs contribute to BCR signaling. FTh cells secrete cytokines like IL-4, which binds to its receptor on lymphoma cells triggering the ERK and STAT6 signaling. It has been demonstrated that increased number of tumour associated macrophages is associated with poor prognosis (53, 79). Increased microvessel density and angiogenic activity have also been associated with poor outcome in FL. Another important mechanism concerns the adaptive immune response. Malignant B-cells induce T-cell exhaustion through constant IL-12 secretion and programmed cell death-1 receptor (PD-1 receptor) stimulation, which results in impaired T-cells function and decreased T-cell motility (80). B-cells affect the function of many T-cells subsets through inducing the conversion of regulatory T-cells (T-regs) that in result suppress the activity of other effector T-cells (of both CD4+ and CD8+ subsets) (80).

Tumour microenvironment is an important and promising target for therapies. Drugs like Lenalidomide targeting angiogenic activity and largely understood microenvironment have

been shown to present good response to a treatment of lymphomas in combination with standard chemotherapy. Promising results are also seen in anti-PD1L drugs that are able to reduce PD1L expression on tumour cells and reverse the exhaustion of T-cells regaining their normal function and motility (81).

1.8.2 DIFFUSE LARGE B CELL LYMPHOMA

Diffuse large B-cell lymphoma (DLBCL) is the most common B-cells lymphoma that comprises around 500 newly diagnosed cases in Sweden every year. DLBCL is a diagnosis that encompasses a group of lymphomas that fulfill a WHO definition but are extremely heterogeneous and differ in morphology, immunophenotype and molecular as well as clinical characteristic and behaviour. Patients who suffer from DLBCL respond differently to treatment, some are cured and achieve complete remission (CR) but some succumb to the disease.

DLBCL is according to the WHO definition 2008 : " a neoplasm of large lymphoid cells with nuclear size equal to or exceeding normal macrophage nuclei or more than twice a size of a normal lymphocyte and that has a diffuse growth pattern" (41). Biological and clinical studies have subdivided DLBCL into morphological variants with immunohistochemical subgroups representing distinct disease entities. However, the large number of cases remains biologically heterogeneous, unable to be classified according to clear and accepted criteria of division. These tumours are classified as DLBCL not otherwise specified (NOS) and this group comprises the vast majority (90%) of all diagnosed DLBCLs (including the tumours studied in this Thesis). The remaining 10% of the DLBCLs are associated with specific characteristics as listed in the Table 2.

In spite of its heterogeneity, most of the patients continue to be treated in a uniform fashion, where R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone), is the most commonly used regimen.

Why to perform molecular diagnosis of DLBCLs?

Molecular studies are done in order to delineate "grey zone" lymphomas, identify prognostic subgroups, but most important of all, to identify molecular subtypes and pathways that could be relevant for targeted therapy.

Gene expression profiling (GEP) studies have identified in DLBCL NOS, at least 2 distinct molecular subtypes, referred to as germinal center B cell (GCB or GC) and activated B cell (ABC). However, even with this approach, about 15% of patients remain unclassifiable. The GC and ABC groups represent lymphomas arising from different stages of B-cell differentiation. This molecular approach has shown to have prognostic implications where the

ABC subtype is characterised by poor prognosis and inferior outcome following R-CHOP therapy (3 year progression free survival of 40% as compared to 75%, $p<0,001$) (55) (82).

What is also important from a clinical point of view, is the fact that the molecular subtyping shows that the lymphomas are driven by very different oncogenic signalling pathways, which could be differentially exploited for therapeutic benefit.

WHO classification of DLBCL
I. ***Diffuse large B-cell lymphoma not otherwise specified (DLBCL NOS)***
II. ***Diffuse large B-cell lymphoma subtypes:*** • Primary DLBCL of the CNS, • Primary cutaneous DLBCL, leg type • EBV positive DLBCL, of the elderly • DLBCL associated with chronic inflammation
III: ***Other B-cell lymphomas of large cells:*** • Mediastinal large B-cell lymphoma • Intravascular large cell lymphoma • Lymphomatoid granulomatosis • ALK positive LBCL • Plasmablastic lymphoma • LCBL arising in HHV8-positive multicentric Castleman disease • Primary effusion lymphoma • T-cell/histiocyte rich large B-cell lymphoma
IV: ***"Grey zone" lymphomas:*** • B-cell lymphoma unclassifiable with features intermediate between DLBCL and Burkitt • B-cell lymphoma unclassifiable with features between DLBCL and classical Hodgkin

Table 2. The short summary of classification of DLBCL NOS, DLBCL subtypes, and other B-cell lymphomas according to WHO 2008 (41). (Modified according to Klapper 2015).

1.8.2.1 GC DLBCL

GC DLBCLs are considered to originate from lymphoid cells residing in the germinal center and expressing *CD10*, *BCL6* and *LMO2* (LIM domain only-2) (82).

About 30-40% of GC DLBCLs possess a t(14;18), 30% have *C-REL* amplifications, 20% carry mutations of *EZH2* and 10% have a deletion of *PTEN*. All of the above mentioned alterations are characteristic for DLBCLs of GC type and are practically never seen in ABC DLBCL (Figure 4) (82). EZH2 is known to be "a master regulator" of germinal center differentiation. However, it is also involved with BCL6 in B-cell lymphoma development. (77). As described before, somatic point mutations within exon 15 of the *EZH2* gene result in a replacement of a single tyrosine (Tyr 461) within the EZH2 protein. This change leads to a

gain of function and an increased methylation of histone 3, which promotes lymphomagenesis by transcriptionally silencing key regulator genes (82). Therefore, both BCL6 and EZH2 have the potential to become important targets for therapeutic agents.

The other main pathway activated in GC DLBCL and other types of lymphoma is phosphatidylinositol 3 kinase (PI3K)/AKT/MTOR signaling pathway which is crucial for cell growth and metabolism (82). A deletion of the *PTEN* tumour suppressor gene has been detected in 10% of these lymphomas, and the loss of PTEN protein expression detected by IHC has been seen in 50% of GC DLBCLs compared to 14% among non-GC cases, indicating other mechanisms involved in loss of PTEN function. The constitutive activation of PI3K/AKT/MTOR pathway in GC DLBCL may be a possible target for inhibitory therapy (82).

The anti-apoptotic protein Bcl2, is commonly overexpressed in both GC and non-GC DLBCL, although the mechanisms are different. In GC type of DLBCL, overexpression is mainly due to presence of t(14;18). However, in non-GC DLBCLs it is due to transcriptional upregulation or gene amplification (82, 83). The use of Bcl2 inhibitors is a promising option for treatment of DLBCLs, especially of GC type (53).

Just as a clarification, the term ABC subtype refers to GEP profiling, while the term non-GC is used in the aspect of immunohistochemistry (IHC). Both terms are therefore differentially used in the text depending on context. Even though these groups overlap to an extent a direct translation is not complete. This aspect is further discussed below.

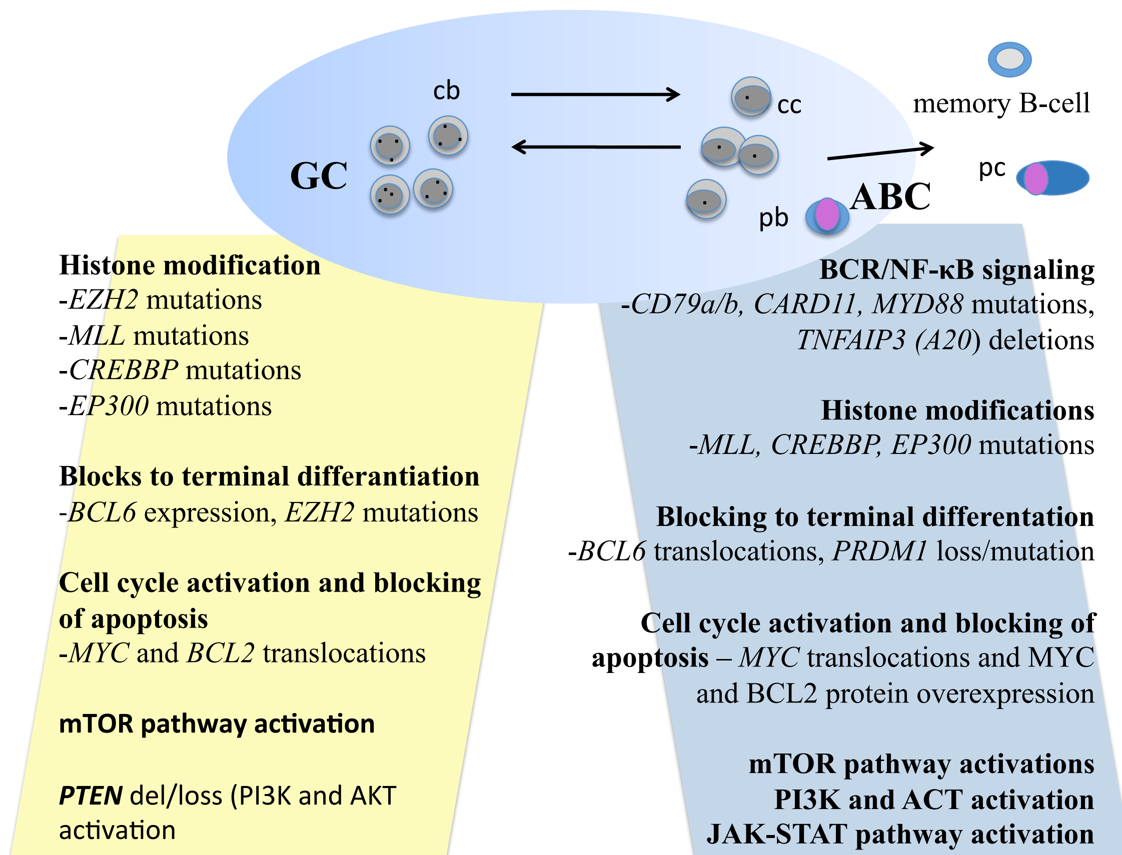

Figure 4. Key oncogenic pathways in DLBCL (GC and ABC type), Abbr: cb, centroblast; cc, centrocyte; pb, plasmablast; pc, plasma cell. (Modified from Sehn and Gascoyne, Blood 2015 (82)).

1.8.2.2 *ABC DLBCL* and *non-GC DLBCL*

ABC DLBCLs originate from B cells at the plasmablastic differentiation stage, prior to germinal center exit. The ABC type of DLBCL relies on NF-κB pathway constitutive activation, which is a pathogenetic hallmark of this ABC subtype and promotes cell survival, proliferation and inhibition of apoptosis (82). It occurs to a large extent due to constitutive activation of the CBM signaling complex (composed of CARD11, Bcl10 and MALT1), which is normally active in response to antigen stimulation (Figure 5). In ABC DLBCL the genetic aberrations may activate CBM complex with 10% of cases show activating mutations of *CARD11* or chronic activation of B cell receptor (BCR) signaling. B cell receptor harbors mutations in CD79a or CD79b in about 20% of cases (38, 82). Mutations in *MYD88* are detected in >30% of ABC DLBCL, resulting in NF-κB upregulation. Also loss of function of *TNFAIP3* (*A20*) occurs often via mutation and deletion, resulting in activation of NF-κB signaling. The drugs targeting NF-κB pathway and its oncogenic drivers are promising therapeutic agents (82).

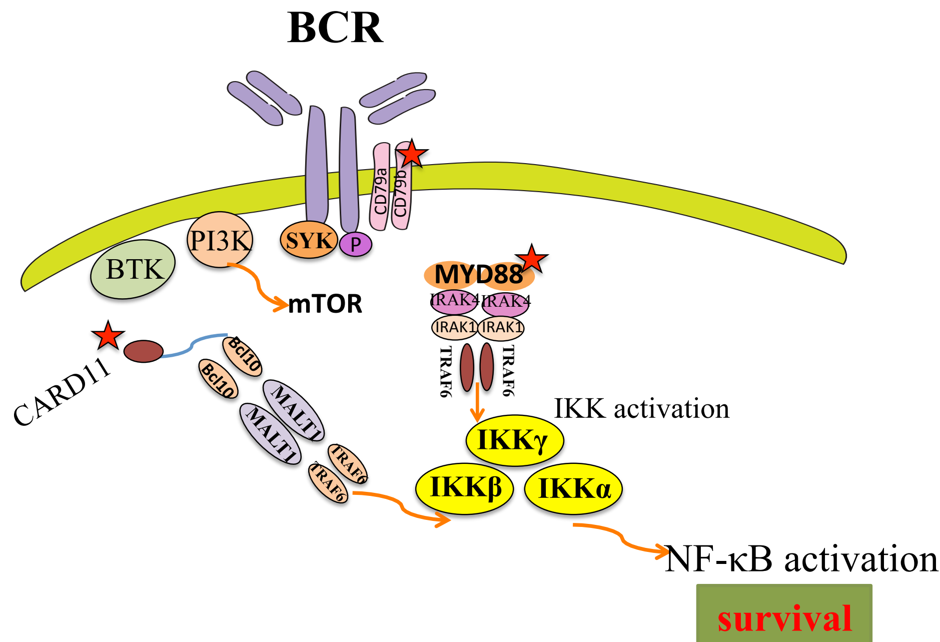

Figure 5. Chronic B-cell receptor activation and MYD88 mutations in ABC DLBCL. Multiple mutated BCR signalling pathway agents have been identified in BCR (CD79b) or downstream the pathway CARD11 leading to chronic NF-κB activation. A star marks mutations occuring in various adaptors in the BCR signaling pathway. (Modified from Bachy, Seminars in Hematology 2015 (38)).

1.8.2.3 Determining the cell of origin

The cell of origin has a prognostic and predictive meaning, which makes it a potential therapeutic targets, there is a great need for reliable method in assessing cell of origin and subtype of DLBCL (82). Gene expression profiling (GEP) is not routinely available and hence there is a need for a more practical well-functioning IHC based algorithm. The Hans *et al* algorithm is commonly used for distinguishing GC and non-GC patients based on the presence of 3 IHC markers (CD10, Bcl6 and MUM-1) (84). Different algorithms have been proposed since then (Choi, Muris, Nyman, Natkunam, Tally and Visco-Young) (85). All of them were very promising at the beginning but turned out having various levels of concordance with GEP analysis. However, the Hans algorithm remained the most commonly established in the clinical routine for a decade. Having a practical approach to algorithms used in clinical diagnostics, one may make an observation that CD10 is a reliable marker of germinal differentiation, distinguishing GC subtype with >95% concordance with GEP analysis. A problem appears as far as other surrogate biomarkers are concerned, giving overall agreement rating from 80-93% depending on a study. In conclusion – we are able to

diagnose a GC DLBCL with very good confidence (>95%), if it shows expression of CD10 but otherwise IHC based subclassification still is a challenge, where we have even about 20% misclassification (Klapper W. at EHA meeting 2015). In search for practical application of GEP studies in diagnostic routine, the 20 gene assay using NanoString technology in paraffin embedded tissue (Lymph2Cx) is a method currently undergoing validation (82).

1.8.2.4 Role of MYC and BCL2 expression in DLBCL, "double hit lymphomas"

The *MYC* oncogene rearrangment is as hallmark of Burkitt lymphoma, but can also be identified in about 10% of DLBCL patients (82). MYC overexpression promotes cell growth and proliferation, and many studies have shown poorer outcome in patients with MYC expression. A number of studies have also revealed the impact of MYC under influence of BCL2. The aggressive character of these tumours stresses the role of early diagnosis of "double hit" lymphomas in diagnostic and clinical practice. Concurrent *MYC* and *BCL2* translocations are identified in 5% of DLBCLs and represent a treatment resistant subgroup (82). The median survival in this group is about 8 months (82, 86, 87). However, more studies are needed to identify the impact of the corresponding part involved in *MYC* rearrangement, as not all of the patients with "double hit" lymphomas may have such a poor outcome (82). Importantly, the overexpression of the MYC protein is more commonly due to an upregulation by additional mechanisms such as amplification and can be demonstrated by IHC in 25-30% of DLBCLs (82). Negative prognostic impact of high MYC protein expression is observed first of all in patients with coexpression of the Bcl2 protein. Patients with coexpression of MYC and Bcl2 proteins are commonly referred as "dual expressers". This group comprises about 25% of DLBCL patients (82). Double expression of MYC and Bcl2 is associated with significantly poorer outcome of these patients as compared to those with expression of one or neither protein (5 year progression free survival (PFS) is about 25% following R-CHOP) (82, 87, 88). Notably, the "dual expressers" belong more often to the non-GC type of DLBCLs, which may also be related to an inferior survival of these patients (82). According to these findings, MYC and Bcl2 expression should be routinely assessed at diagnosis (both the presence of translocations and protein overexpression) to identify a poor risk subgroup and to choose between various treatment options. The overview of studies showing the assessment of prognostic effect of MYC and Bcl2 is illustrated in Table 3.

Study	**Therapy**	**Result**
Johnson *et al.* (JCO 2012)	R-CHOP	Negative prognostic effect if Bcl2 is coexpressed
Green *et al.* (JCO 2012)	R-CHOP	Only analysed in combination with Bcl2
Hu *et al.* (Blood 2013)	R-CHOP	Negative prognostic effect if Bcl2 is coexpressed
Valera *et al.* (Hematologica 2013)	R-CHOP and similar	Poorest if Bcl2 is co-expressed
Horn *et al.* (Blood 2013)	CHOP and R-CHOP	Poorest if Bcl2 is co-expressed

Table 3. Clinical relevance of MYC and Bcl2-expression in DLBCL (according to Klapper EHA 2015).

Although the MYC and Bcl2 “dual expressers” are well studied, MYC and Bcl6 “dual expressers” are less well characterized and more studies concerning the impact of the coexisting effect of these proteins are needed. Moreover, “triple expressers” involving MYC, Bcl2 and Bcl6, although rare, do occur.

1.8.3 TREATMENT

1.8.3.1 Treatment of FL

First line treatment for the patients with limited disease stage I-II, radiotherapy, has a curative potential (54). “Watch and wait” approach can also be considered in asymptomatic patients. Another option for these patients is the monoclonal antibody, rituximab (anti-CD20 monoclonal antibody) in monotherapy if "watch and wait" is not acceptable. (54). When patients become symptomatic the choice of treatment varies from single to combination therapy. The addition of rituximab to several chemotherapy regimens was shown to improve progression free survival (PFS) and overall survival (OS). The CVP regime (cyclophosphamide, vincristine and prednisone) was for years one of the standard treatments followed by versions including rituximab (R-CVP) that is now used in many countries. R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) is a very active therapy, but some studies shown that R-bendamustine is as effective in patients with FL grade 1-2, although for patients with FL grade 3, R-CHOP is preferable (54).

1.8.3.2 Standard therapies in newly diagnosed DLBCL

DLBCL is usually treated with rituximab in combination with the chemotherapy regime R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone) and about 60-70% of patients are cured. Those, that relapse and are refractory to treatment receive salvage therapy and high dose therapy followed by autologous stem cell transplantation.

Other regimens used in treatment of newly diagnosed DLBCL include R-ACVBP consisting of rituximab, doxorubicin, cyclophosphamide, vindesine, bleomycin, and prednisone with subsequent consolidation with high dose methotrexate and holoxan plus ifosfamide and cytarabine (38, 89).

Another dose dense regimen showing efficacy in DLBCL treatment is DA-EPOCH based on continuous infusion of dose adjusted etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicine with rituximab (38, 90, 91).

Although the R-CHOP remains the main core for the therapeutic approaches with the benefit of most of the DLBCL patients, there is still a group of patients that relapse underlining the need for optimization of front line therapy and development of new more effective salvage strategies (82).

1.8.3.3 Novel therapies in DLBCL

Novel therapies have been introduced during the past few years taking into consideration molecular subtypes of DLBCL, and thus targeting the specific pathways involved in the pathogenesis of this heterogeneous group of diseases. Examples of some prototypic drugs associated with a specific molecular pathway are listed in table 4. Multiple therapies targeting NF-κB pathway or the B-cell receptor signaling are under the evaluation process. These drugs would be most suitable in ABC DLBCL as these pathways are constantly active in this subtype (82). Bortezomib, a proteasome inhibitor blocking degradation of IκB (inactivating protein for NF-κB) has been shown to bring benefit in treatment of ABC DLBCL in combination with DA-EPOCH in patients with relapsed DLBCL (82, 92). Current trials are investigating the combination of bortezomib with R-CHOP in untreated patients (82). Therapies targeting B-cell receptor pathway (BTK, SYK, PI3K) are under investigation and bring a promise of targeting multiple components at once (82). Ibrutinib (Bruton's tyrosine kinase inhibitor) has been shown to induce response in 41% of ABC DLBCL patients as compared to 5% GC DLBCL patients (82, 93). The SYK inhibitor fostamatinib disodium has demonstrated the response in more than 20% of patients with refractory DLBCL (94, 95). Notably, Idelalisib (PI3K-δ) has shown promise in indolent NHL but has not been fully investigated in DLBCL (96, 97). Based on the frequent activation of PI3K/ACT/MTOR pathway in GC DLBCL, this drug could be used in the treatment of this subgroup of lymphomas. (82). Another type of drugs showing promising results in combination with R-CHOP therapy is lenalidomide, which is an immunomodulatory drug with antiangiogenic activity and inhibitory effect on NF-κB pathway (98). In a retrospective study of patients with relapsed DLBCL lenalidomide appeared to have better effect on ABC-DLBCL as compared to GC type with 52% *vs* 9% response rate (99). Lenalidomide has been successfully combined with R-CHOP giving promising results in terms of overcoming the negative prognostic impact of ABC-DLBCL phenotype (100, 101). The combination of rituximab with CHOP has improved the outcome in DLBCL dramatically and therefore further attempts of improving the targeting of CD20 are investigated. Obinutuzumab and ofatumobab as compared to rituximab are under evaluation. Additional monoclonal antibodies targeting various B-cells surface markers (for ex. CD79b) are currently investigated (102). Taking into consideration the favorable outcome with R-CHOP treatment, novel therapies are likely to represent the additives to this backbone (82). The identification of prognostic markers and development of molecular assays providing the information on the most suitable treatment for individual patient would be a suitable attempt for future routine clinical use.

Agent	Target	Molecular subgroup
Bortezomib	NF-κB	ABC
Fostamatinib	SYK	ABC
Ibrutinib	BTK	ABC
Enzastaurin	PKCβ	ABC
Idelalisib	PI3K	GC? ABC?
ABT-199	BCL2	GC, “dual expressers”
EZH2 inhibitors	EZH2	GC
BCL6 inhibitors	BCL6	GC
Lenalidomide	NF-κB, microenvironment	ABC
Obinutuzumab	CD20	all
Ofatumobab	CD20	all
Polatuzumab vedotin	CD79b	all

Table 4. Selected novel targeted agents under evaluation in DLBCL with target and patient subgroup that may more likely benefit from this therapy (adapted from Sehn *et al*. Blood 2015 (82)).

1.8.4 TRANSFORMATION OF FOLLICULAR LYMPHOMA TO DIFFUSE LARGE B CELL LYMPHOMA.

1.8.4.1 Clinical aspects

Histologic transformation (HT) defines the transformation from indolent lymphoma to more aggressive form. (103). Transformation is a well known aspect of natural history of the indolent lymphomas. It is most commonly occuring in FL with subsequent transformation to DLBCL but has also been described for other indolent lymphomas like marginal zone lymphoma, lymphoplasmocytic lymphoma, lymphocyte predominant Hodgkin lymphoma and in small lymphocytic lymphoma/chronic lymphocytic leukemia (Richter syndrome) (103).

The risk of transformation for FL varies in different studies from 24% to 70% and about 30% in 10 years (104). These differences may depend on the patient cohorts, follow-up time and diagnostics. An risk for transformation is estimated to occur 2% to 3% per year, as observed in several studies (104). This incidence remained the same even in the era of rituximab, which is suggests that rituximab itself may not diminish or influence the risk of transformation (103).

The risk of transformation is higher for the patients with high Follicular Lymphoma International Prognostic Index (FLIPI). Other adverse clinical factors may be advanced age or high lactate dehydrogenase (LDH) level (103).

The impact of treatment on transformation is a subject of debate (105). It has been suggested that a more aggressive treatment of the low malignant lymphoma is associated with lower risk of transformation but it was not confirmed by other studies (106). Also, a controversial

aspect of introducing early treatment was shown to be associated with lower transformation risk in some studies but has not been confirmed by others (103, 107).

Clinical features of transformation include increased LDH, presence of B-symptoms, hypercalcemia, and a localised growth or presence of new extranodal sites of disease. A number of studies have shown clinical utility of positron emission tomography (PET) scan to detect transformation, High standardised uptake value (SUV) on a FDG-PET correlates with more aggressive histology (103).

The overall survival (OS) of patients with transformation was about 1-2 years. However, several studies suggest the survival may be more favourable during the rituximab era. Link *et al*. shown in 2013 that outcome of the patients who underwent transformation from FL is even similar to those with primary DLBCL when treated with R-CHOP (103, 108) .

The treatment approach for a patient is usually individualised. There is no standard therapy for patients with tDLBCL. Therefore there is a need for randomised studies that can be used to guide this practice (103).

There is also a need to determine biomarkers that can be useful for predicting transformation as there are very few useful markers that can be reliably used in clinical practice. In the era of targeted therapies where the treatment is based on molecular profile of the disease, identification of the subclones potentially responsible for predicted transformation already at the diagnosis can guide treatment and have direct clinical implications.

1.8.4.2 Histopathological and molecular aspects of transformation

The definition of histological transformation may seem clear but it is complicated by the fact that diagnosis is often based on histological analysis made on core biopsy, fine needle aspiration or on clinical ground only (103). The diagnostic gold standard says that the suspicion of histological transformation should be confirmed, if possible, on excisional biopsy or representative core biopsy followd by repeated biopsy at progression is recommended (103). Another problem is the complex clonal architecture of FL which is composed of different subclones either mixed or restricted to specific sites, and undergoing constant evolutionary changes during progression and transformation or even “multiple transformations”, that can be limited to specific sites (Figure 6) (103).

Heterogenous distribution of subclones at progression and transformation requires that selection of the biopsy site is based on clinical features and PET scan, so that site of the aggressive subclone is sampled rather than a low-grade subclone (109).

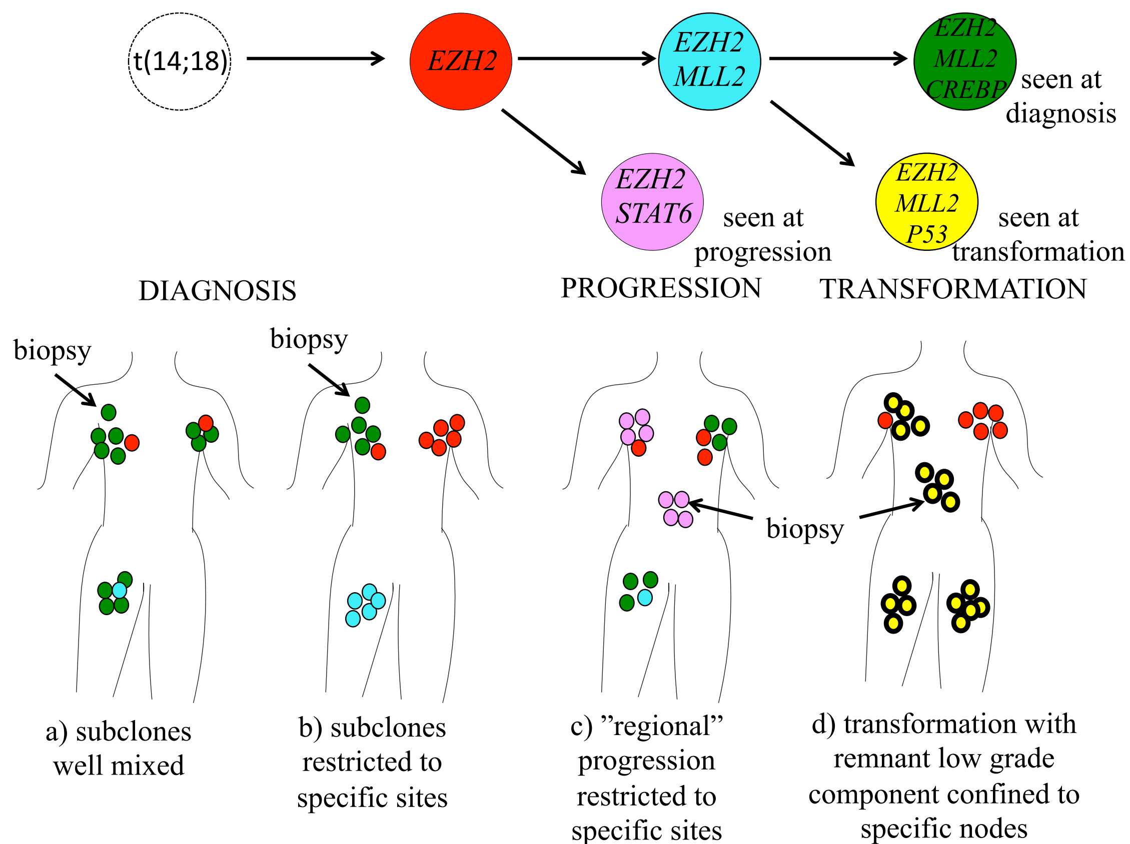

Figure 6. Different scenarios of distribution of subclones in FL to tDLBCL a) subclones well mixed, b) subclones restricted to specific sites, c) regional progression limited to specific sites d) transformation with remnant low grade component confined to specific nodes. Proposed clonal evolution above. (Modified and adapted according to Casulo *et al* 2015 (109)).

Genetic analyses indicate that there is no single mechanism driving transformation from FL to DLBCL, and that several mechanisms are affected during this process (109, 110).

Changes in chromosomal copy number *i.e* "chromosomal number variation" (CNV) have been suggested to be the key mechanisms driving transformation and permitting mutations to take place. Pasqualucci *et al* found that an increase in genomic instability accompanied transformation based on CNV analysis (110).

Recent efforts to identify genetic alteration associated with transformation have identified events occurring early in life history of FL. The most common genetic alterations associated with FL, the t(14;18)(q32;q21) translocation, results in overexpression of the antiapoptotic protein Bcl2 . Moreover, mutations in the histone modyfing gene *MLL2* (as well as other histone modifiers such as *CREBBP* and *EZH2*) occur in > 80% of the early indolent FL cases and are present in nearly all the tumour cells, suggesting that they are early driver events. During transformation they remain stable and clonally dominant which suggests that they may represent key events funding the common progenitor cell of FL (111). Moreover, Pasqualucci *et al* suggested that these mutations may encourage the "mutator" phenotype of FL, allowing AID (activation induced cytidine deaminase) to access inappropriate regions of

the genome leading to aSHM (aberrant somatic hypermutation) and genetic instability driving transformation, suggesting that intraclonal variation due to a SHM may be a predictor of transformation (109, 110).

Known molecular markers with a role as predictors for transformation are few and their clinical application is limited. Several recurring DNA copy number alterations in FL have been associated with transformation (112). Among them gains of 2p, 12q13-14, 18q21 and Xq as well as loss of 1p36, 6q and 17p have been suggested to be associated with FL transformation.

Transformation into DLBCL involves regulators of tumour suppression, cell cycle control, cell proliferation and show changes in *TP53*, *MYC* and *CDKN2A* which is associated to more aggressive phenotype (111).

The most frequent genetic aberration seen in transformation is the loss of *CDKN2A/B* in about 45% of DLBCL transformed from FL. *CDKN2A/B* encodes two tumour suppressor genes whose protein products (p14-ARF, p16-INK4A and p15-INK4B) have important function in cell cycle regulation and the stabilising of *TP53* and they are mutually exclusive (111). *MYC* oncogenic effect is a result of chromosomal translocations, gains, amplifications and mutations, encompassing about 40% of cases. Deregulated MYC oncogenic activity may give advantages to the transformed clone concerning its proliferative ability and metabolism.

A number of gene expression profiling studies using paired tumours of FL and subsequent DLBCL have been published. However, these studies are based on small numbers of paired samples (113-115). In 2002, Lossos and colleagues, have studied 12 pairs of matched FL and DLBCL tumours (113, 116) and they have described two subgroups; one with increased *MYC* and its targets, that appeared to lose proliferation control; and the other with decrease in *MYC* and downstream targets associated with loss of apoptotic signalling (113, 116). One year later, Elenitoba-Johnson and colleagues suggested the p38 MAP kinase as important pathway during transformation (114). Davies *et al*. in 2007 studied 20 paired samples of FL with corresponding DLBCL and could show that *TP53* mutations, *TP53* loss, *CDKN2A* loss and *c-REL* amplification were associated with increased proliferation and transformation (113, 115). In 2009, Gentles and colleagues have described a "pluripotency signature" related to specific embryonic stem cell, in transformed DLBCL but not in FL- counterparts (113, 117). Last year, Brodtkorb and colleagues presented an integrated analysis of gene expression and copy number changes, which revealed that genes involved in the NF-κB pathway (*BTK*, *IGBP1*, *IRAK1*, *ROCK1*, and *TMED7-TICAM2*) are significantly associated with FL transformation (118). The most common genetic alterations associted with transformation are illustrated in Figure 7.

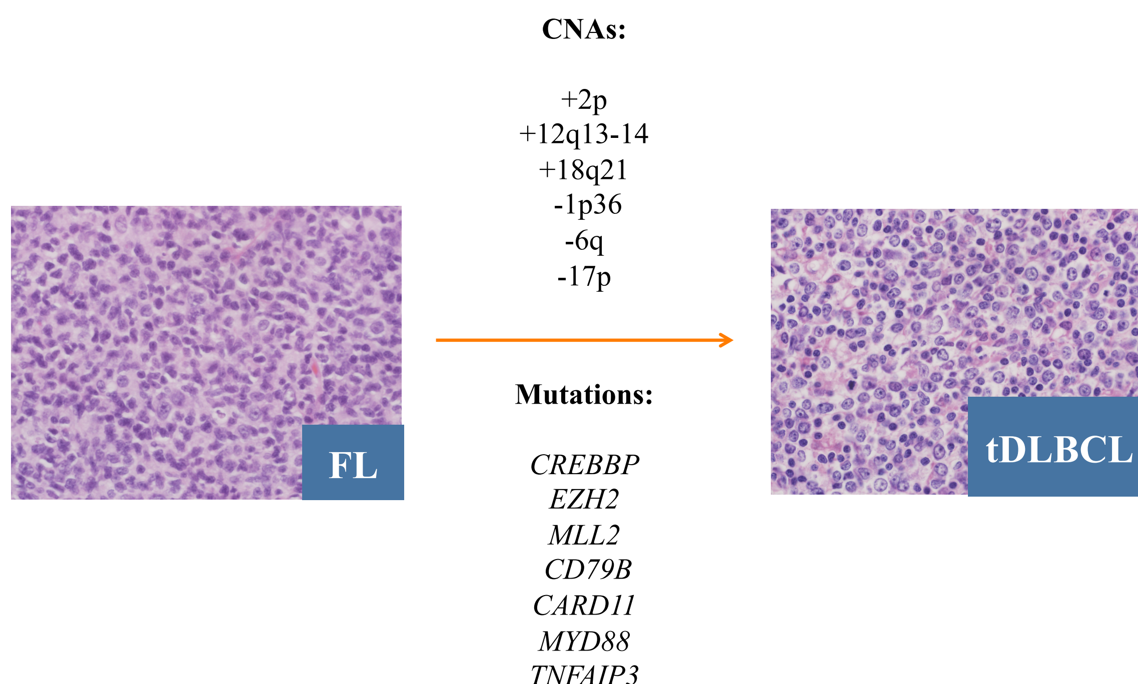

Figure 7. Common genetic alterations underlying histologic transformation of FL to DLBCL. (According to Bouska *et al* 2014 (112)).

The nomenclature of tumours transformed from FL to DLBCL has also been a subject of debate. In latest publications of Pasqualucci and colleagues and Okosun *et al*, the authors refer to transformed lymphomas as "transformed follicular lymphoma" (tFL) pointing out their origin (110, 119). As a pathologist, however, I share the opinion that these tumours are already classified as DLBCL and behave as such, so the term "transformed DLBCL" or tDLBCL is more correct and such the term is used in the Thesis.

The vast majority of DLBCL cases transformed from FL present as GC subtype. However, there are subsets of tDLBCL that present as ABC-tDLBCL or unclassifiable, according to some studies encompassing about 30% of tDLBCL cases (112). The frequencies of underlying copy number alterations and specific mutations differ significantly between these two groups (GC and ABC DLBCL) as illustrated in Figure 8. The ABC-tDLBCL type show more frequently gains of chromosome 3, 3p and 3q and 1q44, and losses of 1p13.1 and 1p36.11-p35.3 encompassing *CD58* and *ARID1A*, and loss or double loss of 6q including the negative regulator of NF-κB pathway *TNFAIP3*. However, the non-GC type of tDLBCL shows more commonly loss of 19p13.3-p13.2 including *CD70*. On the other hand gains of chromosomes or arms of 7 and 12 were more frequent in GC type of tDLBCL (112). Also the mutational pattern differs between the non-GC and GC types of tDLBCL. Mutations more

typical for non-GC encompasses genes *CD79B*, *CARD11*, *MYD88*, *TNFAIP3*, while the GC type more often is associated with mutations in genes *SOCS1*, *BCL2*, *MLL2* (Figure 8).

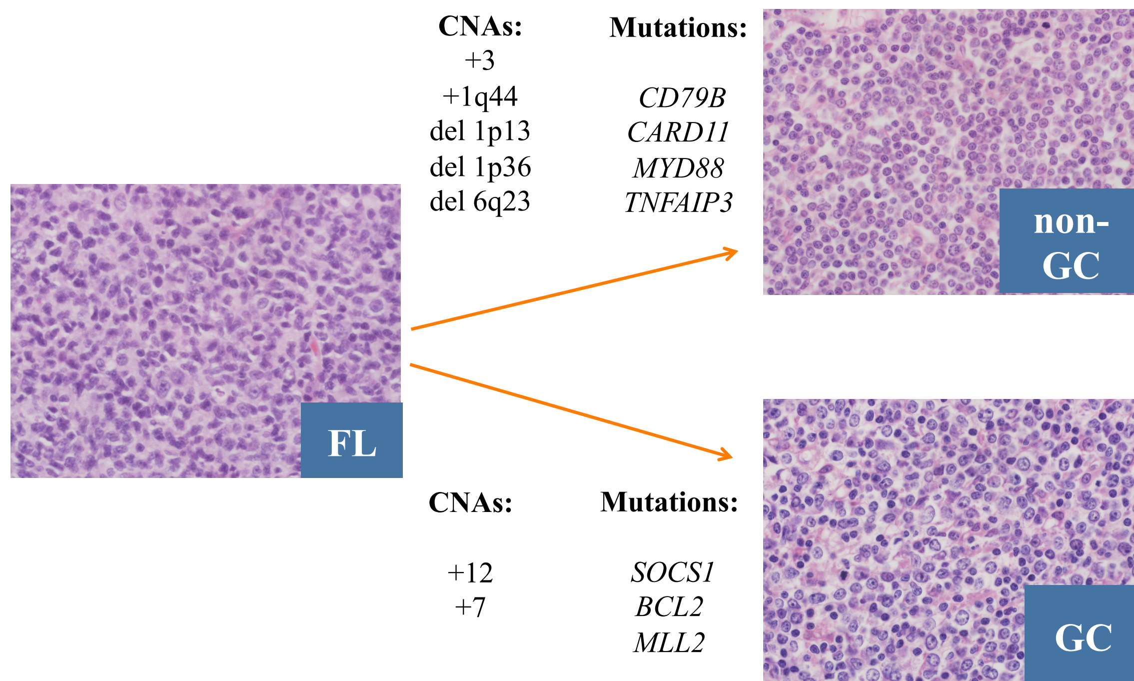

Figure 8. Molecular subtypes associated with transformation from FL to DLBCL. (According to Bouska *et al* 2014 (112)).

1.8.4.3 The microenvironment in FL transformation

Gene expression profiling (GEP) have identified pattern corresponding to the role of microenvironment in FL progression and transformation. Two general aspects are widely discussed: the microenvironment's role in supporting proliferation and blocking apoptosis and its antitumoral immunologic effect (109).

Also the microenvironment of FL may have impact on predicting transformation risk and survival. The loss of B2M expression, also known to be associated with transformation, could be associated with immunomodulatory changes of the microenvironment proceeding transformation. It has also been suggested that immune changes in microenvironment may possibly induce "mutator phenotype" by for example affecting the expression of AID (Activation Induced (Cytidine) Deaminase) in FL cells that may result in aberrant somatic hypermutations (aSHM) in FL progenitor cells (109).

Several changes in microenvironment have been described to accompany the transformation. The typical changes described in non-neoplastic cells include loss of follicular dendritic cells,

increased interfollicular CD4+ T-cells, decreased number of regulatory T-cells and PD1+ T-cells, increased microvessel density, all of which have been linked to an increased risk of transformation (120). However, many of these studies refer to patient cohorts not uniformly treated and treated without routine use of rituximab. These observations must be re-evaluated in the current era of rituximab treatment, possibly in the context of uniform therapy and randomized controlled clinical trials (113).

1.8.4.4 Clonal evolution

The clonal evolution of a tumour may occur through either a linear model in which the predominant clone expand acquiring novel genetics changes leading to progression of through branching model in which the common progenitor cell evolves to the initial tumour and then progresses stage by stage through different clone evolution and through distinct genetic pathways (111).

Transformation of follicular lymphoma occurs almost exclusively through branching rather than linear model. The dominant clone arises in most patients from an ancestral mutated common progenitor cell evolving through acquisition of genetic events shared partially between the indolent and transformed forms (111). This is different from CLL to DLBCL transformation occurs mostly through a linear model of evolution where the DLBCL is a final stage of progressive expansion of the original founder CLL clone, accumulating genetic lesions in an additive way (111).

1.8.4.5 Treatment challenges and future directions

The treatment approach for a patient with transformation is very often individualised and there have been no randomized studies in a modern time that can be used as guidelines (103). There is therefore no standard therapy for patients with transformation and treatment options are based on previously received regimens and response to them. The historically poor prognosis for these patients resulted in the treatment approach based on high dose therapy (HDT) and autologous stem cell transplant (ASCT), the treatment approach shared by many centers (103).

The promising results are seen in treatment with autologous T-cells expressing and anti-CD19 chimeric antigen receptor (CAR), also in case of patients with DLBCL who has progressed after autologous stem cells transplantation (121). Chimeric antigen receptors (CARs) are fusion proteins with antigen-recognition domains and T-cell activation domains. T-cells expressing anti-CD19 CARs recognize and kill CD19+ target cells. The infusions of CAR T-cells can be administrated together with various chemotherapeutic regiments in treatment of DLBCL, as showed by Kochenderfer and colleagues (121). A prerequisite for effective treatment of lymphoma with T-cells is infiltration of malignant tumour mass by T-cells. The toxicity is the most troublesome side effect experienced by patients including neurologic toxicity and hypotension but it has been shown released completely after a short time period (121). Infusion of anti- CD19 CAR T cells remains the potentially powerful new treatment for chemotherapy refractory B-cell malignancies.

2 AIMS OF THE STUDY

The overall aim of this thesis was to identify the genetic events underlying the progression and transformation of indolent follicular lymphoma (FL) to the more aggressive diffuse large B-cell lymphoma (DLBCL) and to outline molecular alterations distinguishing DLBCL with GC and non-GC phenotype.

More specifically, the aims were to identify:

- distinct molecular events marking progression from FL to DLBCL that could be developed as prognostic markers for application in clinical practice.
- proteome changes characteristic for GC and non-GC DLBCL lymphomas in order to identify new diagnostic and prognostic markers as well as potential therapeutic targets.

3 MATERIALS AND METHODS

3.1 PATIENTS AND TUMOUR SPECIMENS

3.1.1.1 Paper I

The tumour samples were obtained from 50 patients diagnosed with various lymphoma subtypes between 1996 and 2008. The samples were retrieved from the archives of the Department of Pathology-Cytology at Karolinska University Hospital, Solna and the diagnostic material was reviewed according to the WHO 2008 classification (41). The tumour material encompassed a spectrum of indolent and aggressive lymphomas including B-cell chronic lymphatic leukemia (B-CLL) (no=8); immunocytoma (no=2); mucosa associated lymphoid tissue lymphoma (MALT lymphoma) (no=6); mantle cell lymphoma (no=6); follicular lymphoma (FL) grade 1-3a (no=6); diffuse large B-cell lymphoma (no=9); Burkitt lymphoma (no=5);and classical Hodgkin lymphoma (no=8). Anonymised biopsies from reactive tonsills and lymph nodes were used as controls. The FL cases were included in a study performed previously by one of the co-authors (122).

3.1.1.2 Papers II, III and IV

In Paper II, we studied 81 tumours comprised of 21 FL, 31 transformed DLBCL (tDLBCL) - all of them showing germinal center (GC) related immunophenotype - and 29 *de novo* DLBCL (dnDLBCL: 10 showing GC and 19 of non-GC phenotype), collected from a total 60 patients diagnosed between 1985 and 2007. Paired tumour samples were available from 15 patients, of which 3 patients contributed with more than 2 consecutive samples. Fresh frozen tumour tissues were identified from medical files of the Departments of Pathology-Cytology at Karolinska University Hospital, Solna and Uppsala Academic Hospital, Sweden. The diagnostic material was reevaluated according to the WHO 2008 classification (41). All the tumours from Karolinska University Hospital (1-47) were presented for the first time, whereas the tumours from Uppsala Academic Hospital (48-81) were previously reported in a series of cases investigated by conventional CGH (123).

In Paper III, 12 samples were obtained from 4 patients with an indolent FL and a tDLBCL counterpart, first diagnosed with FL between 1982 and 1997. Three patients (P1, P2 and P3) contributed with paired FL-tDLBCL tumours, whereas in case of one patient (P4) – a total of 6 consecutive tumours could be evaluated (4 FL and 2 tDLBCL). The detailed clinical charateristics of the patients is described in supplementary data of the Paper III. The material from all the patients (P1-P4) was obtained from Department of Pathology at Uppsala Academic Hospital and previously studied by conventional CGH (123) and array CGH (124).

In Paper IV, a cohort of 31 patients diagnosed as DLBCL (15 GC and 16 non-GC; 11 tDLBCL and 20 dnDLBCL) at the Department of Pathology, Karolinska University Hospital, Solna between 1999 – 2007 was studied. For two of the patients with tDLBLC, the FL

component was also available. TMA was constructed from 30 DLBCL, 2FL and 2 normal lymph node controls. IHC on one of the dnDLBCL was performed separately, since this tumour was not available for TMA. The patient characteristics are available in Table 1 in Paper IV. The patient cohort consisted of 19 men and 12 women with a median age about 60. The vast majority of the patients received R-CHOP therapy and 16 of 31 patients were in complete remission (CR) at the latest follow-up in 2014.

The patients' material studied in Paper IV is also a part of the tumour material investigated and reported in Paper II .

3.2 METHODS

3.2.1 DNA preparation

In this thesis DNA was prepared from fresh frozen tissue and used for array-CGH and WES studies. The same quality DNA was also used for copy number PCR analysis with the exception of one case where the fresh frozen material was not available and instead DNA material from formalin fixed paraffin embedded tumour tissue was obtained. A standard protocol was applied based on proteinase K digestion, phenol-chloroform separation and ethanol precipitation. Good quality DNA was obtained and the quality and concentration of the DNA was measured by spectrophotometry.

3.2.2 Array CGH

The comparative genome hybridisation (CGH) was introduced by Kallioniemi *et al* in 1992 and is a molecular cytogenetic technique allowing screening of whole genomes and revealing chromosomal regions of gains and losses (125). In **Paper II** of this thesis, we used the array-CGH technique, which has developed from the classical metaphase CGH (126, 127). The array-CGH was performed at Prof. V. Peter Collins and dr Koichi Ichimura lab at Addenbrooke's Hospital, Cambridge, UK. DNA extracted from tumour samples were labelled with red fluorochrome and each tumour sample was co-hybridised wih normal reference DNA (labelled green) to the arrays. Prior to hybridisation, the array slides were competitively hybridised with Cot-1-DNA to allow blocking of repetitive sequences. The arrays consisted in our case of totally 3038 clones: 2555 BAC clones, 477 PAC clones and 6 cosmids distributed over the entire genome with a mean average distance of 0,97 Mb, printed on a glass slide. The ratio of fluorescence intensity of the tumour to the reference DNA was then calculated to measure the copy number changes for a particular location in the genome. The ratio in favor of green indicated loss and in favor of red meant excess of tumour DNA, hence gain (Figure 9).

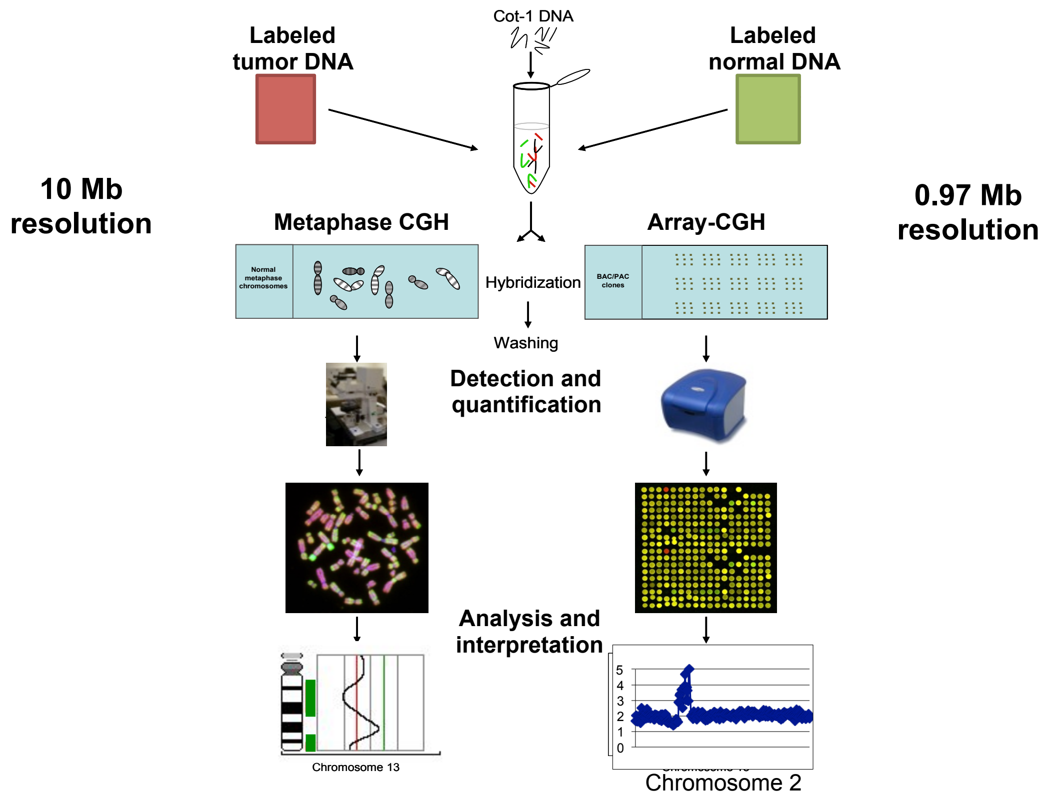

Figure 9. Schematic illustration of metaphase CGH and array-CGH. The main difference concerns the hybridisation target. In metaphase CGH – the chromosomes are spread on slides while in array-CGH they are replaced by BAC/PAC clones corresponding to genomic regions (adapted by permission of dr Emma Flordal Thelander from her doctoral thesis “Genetic Characterisation of Hematological Malignancies with Focus on Mantle Cell Lymphoma” 2007 (128)) .

The resolution of arrays depends generally on their design, the number of DNA target sequences, their individual lengths and the distance between them (129). The resolution can be for example increased by using tile path arrays, that are design to leave no gap between the clones and the clones are spotted on the arrays in order to overlap each other (129) (Figure 10).

The remaining disadvantage of genomic array techniques is their inability to detect completely balanced aberrations like inversions, insertions and translocations. However, the typically high resolution of genomic arrays, the possibility of fast detection of genomic imbalances in archival material, quickly made this method important tool in molecular cytogenetics (21).

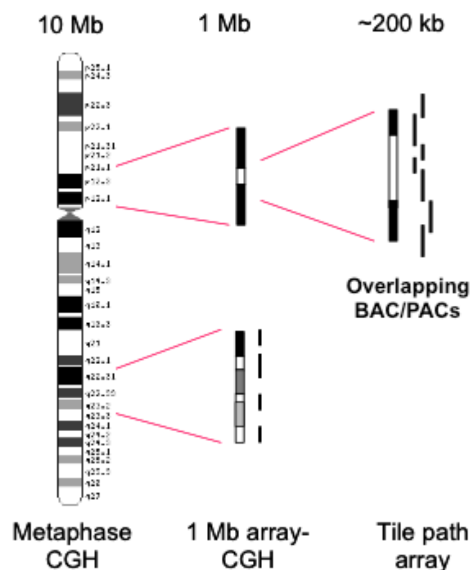

Figure 10. A schematic illustration of different array-CGH approaches and resolutions. (adapted by permission of dr Emma Flordal Thelander from her doctoral thesis "Genetic Characterisation of Hematological Malignancies with Focus on Mantle Cell Lymphoma" 2007 (128)).

3.2.3 Taqman DNA copy number analysis

Taqman DNA copy number analysis is used for a copy number prediction at a specific locus. It is a quantitative PCR based Taqman method, that was applied in **Paper II** in order to validate the results of array-CGH. The Taqman DNA copy number analysis present a similar approach as real time qPCR. The target and reference primers are labeled with FAM and VIC dyes respectively and both are run in a duplex-real time PCR. The reference primer is designed to detect genomic sequence with known diploid copy number, which acts as internal control. Each reaction is run in triplicates using a qRT-PCR machine. The number of copies of the target gene in each test sample is determined by relative quantification using the comparative Ct (ΔΔ Ct) method (130). This method measures the Ct difference (Δ Ct) between target and reference and compares the Δ Ct of the test probe to a calibrator sample, that has two copies of the targeted gene. The copy number of the target is then calculated. **In Paper II**, we have extracted raw data from RT–PCR by means of Sequence Detection Software SDS (Applied Biosystems) and DNA copy number prediction was calculated by CopyCaller software (Applied Biosystems).

3.2.4 DNA sequencing

The DNA sequencing technology was introduced in 1977 by Fred Sanger, who in 1980 was awarded the Noble Prize for his invention. Originally, the method used modified ddNTPs (dideoxonucleotides) lacking 3'-OH group and terminating the DNA strand elongation in PCR. That required four reactions run per sample, as one of four ddNTPs (ddATP, ddGTP, ddCTP, ddTTP) was used in each PCR experiment. The classical reaction required a single stranded DNA template, a DNA primer, a DNA polymerase, normal dNTPs and one of four modified ddNTPs. As the ddNTP would terminate the DNA strand elongation, a PCR product consisting of a mixture of DNA fragments with different length was obtained. The DNA fragments were then separated by gel electrophoresis and DNA sequence was decoded (131). The technology then developed by using various fluorescent dyes in order to separate the signals from different bases (dye terminator sequencing), which enabled to run one reaction per sample (132, 133). Later the method was improved through utilisation of capillary electrophoresis for separation and detection of results in chromatograms as individual peaks for each base in the sequence (134). The automated Sanger method is known as "first generation", while the newer methods are called "next generation sequencing" (NGS) (135). Sequencing technologies offer a broad variety of methods grouped according to the template preparation, sequencing method, imaging and data analysis. The unique and specific protocols determine the differences in data output from various platforms.

In our **Paper III**, we have used the Illumina platform HiSeq 2000 (San Diago, CA, USA). In this method, the procedure starts with the preparation of libraries from DNA or RNA. Then the single stranded molecules in the library are hybridised to oligonucleotides attached to a glass surface of a flow cell. The complementary strand is synthesised by a polymerase. There are now two types of olignoucleotides attached to the surface, one of them available as a free end. During the next step called "bridge amplification", the free end of the newly synthesised strand falls over, and attaches to another oligonucleotide forming a single stranded (ss) bridge (135). Then, the other complementary strand is synthesised forming a double stranded (ds) bridge. The two strands are then denaturated, and the amplification is repeated. This process is called "clustering". A cluster is a clonally amplified fragment of target DNA, bound to the Illumina flow cell. In the end, each cluster of ds DNA is denaturated, with removal of the reverse strand, so that only the forward strand is left. In total, about 100-200 million molecular clusters are formed, where each cluster corresponds to a single molecule from the library (135). During sequencing a primer is hybridised to the forward strand. A reversible terminator, that is fluorescently labelled, is bound to each dNTP, and then cleaved to enable incorporation to the next base (136).

3.2.5 Proteomics

The successful expansion of genetic methods has raised the level of opportunities for studies on proteins and this area of interest with the all–embracing name "proteomics" (137). Proteins are the crucial elements of cellular machinery, performing tasks in all the biological processes. In short, one may say, that life depends on proteins. (138). The crucial role of

proteins was recognized early in life sciences. The name “protein” comes from a Greek word “*proteios*”, which means “the first rank” , and was introduced by Berzelius in 1838 pointing out the meaning of these molecules (137). It is estimated that more than (numbers vary from 10,000 to 500,000, with up to 50,000 polypeptide chains) 9000 proteins are involved in a various biological functions per nucleated cell (137).

Moreover, posttranslational modifications, such as phosphorylation, hydroxylation, methylation, glycosylation *etc*) create a large diversity within the proteins. Studying of proteins is of great interest and importance, and the popularity of protein analyses including mass spectrometry is also increasing.

There are two main approaches for mass-spectrometry based proteomics, that can be described as “top-down” and “bottom-up” analysis (138). In the “top-down” approach, the intact proteins are separated by various techniques, *e.g*. by 2DE, and identified by mass spectrometer. The most common example of “top-down” mass spectrometry method is matrix-assisted laser desorption/ionization (MALDI). In the “bottom-up” methods proteins are enzymatically digested into peptides using enzymes like trypsin or pepsin, and are in solution after chromatography or electrophoretic separation (138). This peptide mixture is then introduced to a mass-spectrometer. An example of “bottom up” method is liquid chromatography tandem mass-spectrometry (LS-MS/MS). Both these unique methods have a shared mode of analysis, which is mass-spectrometric analysis. In mass-spectrometric analysis, the mass-to-charge ratios (m/z) of analysed molecules are obtained. By collecting this data, the analysed compounds can be identified by comparison against the standard database of molecules with known masses (138). In the whole protein analysis in “top-down” proteomics, and in the peptide analysis in “bottom-up” proteomics, each compound is given m/z signature detectable by the mass spectrometer. A combination of the different techniques creates additional possibilities for proteomic analysis.

3.2.5.1 MALDI-TOF MS

Matrix associated laser desorption/ionization (MALDI) is an ionization mass-spectrometry based method, in which the studied peptides or proteins are ionized by a laser impulse, and then generated ions are measured. MALDI-TOF-MS (Matrix associated laser desorption/ionization time of flight mass spectrometry) was applied for protein identification in **Paper IV**.

First the proteins are separated through a two dimensional electrophoresis (2-DE) in polyacrylamide gel. The proteins are digested and separated by their isoelectric point (first dimension) followed by separation according to molecular mass (second dimension). The gels with protein spots are visualized by silver stain of fluorescent dyes. Stained 2-DE gels are scanned and the staining intensities of all protein spots are analysed by an image analysis software. By applying multivariate statistics analysis, the protein spots for identification by MS are selected. The selected proteins spots are then excised, destained, and undergo trypsin digestion. The generated mixture of peptides is then embedded in a matrix compound, that

had the capability to absorb the laser energy and then ionise the molecules. The samples are loaded to a mass spectrometer, where the peptides are ionised under the effect of laser beam. The ions are separated according to their mass-to-charge ration, typically by accelerating them in an electric or magnetic field, and are measured by a detector capable of detecting charged particles, such as an electron multiplier. The results are in form of spectra of detected ions. The obtained data are then matched against established databases in order to identify corresponding proteins matching up the theoretical peptide masses with experimentally observed.

3.2.5.2 SELDI-TOF-MS and LC MS/MS

Surface enhanced laser desorption/ionisation time of flight MS (SELDI-TOF-MS) differs from the MALDI-TOF-MS by a main distinction, that in the SELDI-TOF the tested proteins are applied on a chip array, which contains different chemicals associated with various properties (hydrophobic, hydrophilic etc). Based on these distinctive properties, the analytes are selected through bounding to a chip surface. All other undesired components are then washed away, which increases the volume of low-abundant molecules in the analysed sample, but on the other hand some information may be missed due to decreased variety of the compounds. The further procedures, including matrix application, ionization, detection of ions, and m/z analysis is similar to MALDI (139).

Liquid chromatography tandem mass spectrometry (LS-MS/MS) is used to identify proteins by physical separation of peptides using isoelectrophocusing, followed by analysis by mass spectrometry (139). Firstly, all the proteins are digested using trypsin, and then labeled with isotops in order to enable quantification. The labelled molecules are then pooled in a tube, undergo isoelectric focusing in an immobilized pH gradient gel, and are analysed by LC-MS/MS, where they undergo the electrospray ionization and peptide fragmentation. The analysis leads to information on peptide's amino acid sequence, and various software tools are available for protein identification and peptide quantification.

Facing the rapid development of MS techniques, some may suggest, that 2DE-method is no longer the method of choice for proteomic analysis. However, at the same time, as recognition of issues related to newer shotgun-MS methods grow, there is the fresh look at maturity and strengths of 2DE-MS as genuine analytical method (140). 2DE-MS strength depends on the fact, that it allows the analysis of intact proteins in a single run, enabling the simultaneous characterization of total protein compartment consisted of thousands of proteins, including isoforms and post-translational modifications (140).

3.2.6 Immunohistochemistry

Immunohistochemistry (IHC) is an analytical method used to detect the presence or absence as well as localisation of proteins. IHC is based on the binding of antibody to a specific antigen in cells or tissues (141). IHC applies commonly to FFPE sections, but can also be

used on fresh frozen tissue and cytological material. According to classical IHC procedure used on FFPE sections, tissue is deparaffinised in 100% xylene, followed by rehydration in graded series of ethanol and water solution. The antigen retrieval is performed by incubation in citrate buffer in order to bind calcium ions, that could interfere during binding of primary antibody to the antigen. Antigen retrieval is performed at high temperature (95-99°C) for the restoration (retrieval) of the "formalin modified" antigen molecular structure. After this step, the sections are pre-treated with hydrogen peroxide, that blocks an endogenous peroxidase, that otherwise may cause a non-specific background staining. Non-specific binding of primary and secondary antibodies is blocked by bovine serum albumin. The primary antibody is then applied on a tissue, where it binds to a target epitope. This step is followed by application of secondary antibody, which is biotinylated and has affinity to avidin . The ABC (avidin/biotin complex) solution is then added and binds to the secondary antibody through reaction between biotin (on the secondary antibody) and avidin. Avidin components interact with biotinylated horseradish peroxidase, that increases the intensity of the signal after addition of a chromogen substrate. The most commonly used chromogen substrate is diaminobenzidine (DAB), which gives finally brown staining as a final result of the above described procedures. The finishing steps include dehydration of tissue sections in increasing grade of ethanol concentrations and 100% xylene followed by counterstain with hematoxylin. In our **Papers I, II and IV** we performed immunohistochemistry according to the above described principles, using method developed, automated and optimised by Bond Max System (Leica, Newcastle Upon Tyne, UK).

4 RESULTS AND DISCUSSION

4.1 PAPER I: HAX-1 EXPRESSION IN B-CELL LYMPHOMA

In our first work, performed in collaboration with Prof. Bengt Fadeel group (Institute of Environmental Medicine, Molecular Toxicology, Karolinska Institutet), we have investigated the expression of the *HAX-1* gene in human B-cells lymphoma on mRNA and protein level. HAX-1, which is a multifunctional protein involved in apoptosis and cell migration, has previously been shown to be involved in different cancer type, but has never been studied in hematological malignancies (142). In paper I, the *HAX1* expression was first evaluated on mRNA level, through search of in two public databases: the *In Silico transcriptomics (IST)* database containing data from 9783 Affymetrix gene expression analysis from 43 normal tissues, 68 cancer types and 64 other entities and the *Human gene expression map* composed of data extracted from 5372 samples and 369 cell types. The results of the IST search showed a high level of *HAX-1* expression in hematological malignancies, especially plasma cells neoplasm (plasma cell leukemia and myeloma) as well as B-cell lymphomas. The *Human gene expression map* search showed *HAX-1* overexpression in anaplastic large T-cell lymphoma. Next, we investigated the HAX-1 protein expression detected by IHC in a panel of B-cell derived neoplasms, that consisted of 50 B-cell lymphomas (including B-CLL, immunocytoma, MALT lymphoma, MCL, FL, DLBCL of GC and non GC type, Burkitt lymphoma and Hodgkin lymphoma). The FL samples were selected based on the previous publications from members of the same group investigating *HAX1* mRNA expression on number of FL cases by microarray analysis (122). The results of IHC evaluation of HAX-1 protein expression were compared with expression of anti-apoptotic protein Bcl-2 and proliferation measured in Ki67 staining. Our IHC results showed a negative correlation between the HAX-1 expression and Bcl-2 expression in various types of B-cells lymphoma. That may suggest the role of HAX-1 as a Bcl-2 homolog, compensating for low Bcl-2 expression in these tumours. Moreover, we could also observe a positive association between HAX1 expression and Ki67 suggesting that there may be other functions involving HAX-1 beyond the anti-apoptotic effect. Interestingly, the gain and amplification of 1q21.3 region being locus of *HAX-1* gene has been reported in aggressive tumours including transformed DLBCL described in our **Paper II (**143) **.**

4.2 PAPER II: TRANSFORMED DLBCL FROM FL

Transformation of FL to DLBCL is a common event associated with poor prognosis and response to treatment. Hence, there is a need for molecular markers of transformation that would enable prediction of this event and adjustment of treatment. In studies on genomic copy number alterations, gains involving +1q, +2p, +3q, +7, +8q, +12, +17q, +18q, +X and lost of -1p36, -4q, -6q, -8p, -13q and -17p were reported as associated with transformation (120, 123, 144-146). Several genes have been postulated to be involved in the transformation FL to DLBCL, including *TP53*, *MYC*, *REL*, *BCL-2* and *MYD88* (147-151). The previous

reports have also described gains of the 2p15-16 region detected by array-CGH. The *REL* gene located in this region has been discussed as a potential driver for the transformation process (144).

In our Paper II project, performed in collaboration with Prof V. Peter Collins and dr Koichi Ichimura (Department of Pathology, Addenbrooke's Hospital, Cambridge, UK), we have studied 81 tumours from 60 patients (29 dnDLBCL, 31 tDLBCL, and 21 antecedent FL) regarding chromosomal copy number alterations using array-CGH. Paired tumour samples were obtained from 15 patients and within this group 3 tumour sets consisted of more than two consecutive samples, allowing us to follow specific genetic changes during the progression, and before and after transformation. Half of the tumours were previously outlined by metaphase CGH by the members of our group (123). In our study, we have observed, that the DLBCLs showed a higher number of imbalances as compared to FL, and tDBLCL as compared to dnDLBCL, indicating that in particular the tDLBCL tumours are more instable cytogenetically. This observation has also been reported in previous studies (144, 152).

In our attempt to outline the chromosomal alterations of importance for the transformation, we have found, that the 2p15-16 region encompassing, among others, the *REL, BCL11A, PEX13, USP34, XPO1, COMMD1* and *OTX1* genes, was the only alteration that showed a high level amplification detected. We have observed, that a gain/amplification of this region was more common in tDLBCL than dnDLBCL ($p<0.001$). Moreover, the amplification of 2p15-16 region could also be seen in FL prior to transformation, and in first tDLBCL directly after transformation, pointing out the possible role of this alteration in the transformation process. We have also performed quantitative real time PCR on the selected, previously mentioned genes within 2p15-16 region, and could observe a higher level of amplification of *REL*, *USP34*, *COMMD1* genes (all of them involved in NF-κB pathway) indicating their potential role as drivers for transformation.

Moreover, we could demonstrate that 17q21.33 amplification was exclusively found in tDLBCL as compared to FL and dnDLBCL ($p<0.04$), and could be associated with late phase of transformation. Likewise, a higher number of losses at 6q16.3, 9p and 14q as well as gains of 6p were found as late events in FL prior to transformation suggesting the potential role of the genes located in these regions in the peri-transformation phase of transformation.

4.3 PAPER III GENETIC ALTERATIONS DURING TRANSFORMATION

Our third project, related to **Paper II**, was performed in collaboration with Prof. Qiang Pan-Hammarström's group (Division of Clinical Immunology, Karolinska Institutet). In this study, the whole exome sequencing (WES) was performed on paired tumours (FL-tDLBCL) obtained from four patients. One of the patients (patient 4, P4) was presented with 4 consecutive FL and 2 sequential DLBCL tumours. Studies of these samples allowed us to follow the changes acquired during progression and transformation of the disease. Three of

the patients (including P4) have been previously characterised by array-CGH in our second project. We could thus compare results of the WES experiment with the array-CGH data. As a result, we found an overlap between the CNV pattern and the mutation landscape. Moreover, the pathway analysis has shown that pathways (and genes) altered in FL included apoptosis (*IRAK2*, *CSF2RB*), and Wnt signaling (*LPR5*, *PLCB4*), while pathways affected in DLBCL comprised B-cell receptor pathway (*VAV3*, *CARD11*, *PLCG2*). Interestingly, the multi-sample case has shown that *MLL2* and *MEFB2* were the only recurrently mutated genes, and notably, different mutations were detected in these genes in the two early FLs as compared to the six later tumours (FLs and tDLBCLs), corresponding to progressed and transformed disease. We have also investigated the aspect of transformation. For this purpose, we have looked at mutations overlapping the CNV, and present in FL prior and DL right after transformation. As a result, we could suggest, that genes *CHD4*, *NPY5R*, *PPP1R15B* as well as *DOCK4* and *MROH5* may be of interest in the context of transformation process.

To conclude, in **Paper III**, we have shown the strength of integrated analysis combining the mutation pattern identified by WES with the CNV –profile detected by array-CGH. This integrated approach allows outlining of genetic alterations, that may be of importance for the progression and transformation. Also, it strengthens the likelihood, that these alterations are of the somatic origin of these alterations, even in the situations where no normal tissue control is available for comparison. Moreover, we could demonstrate, that the clonal evolution of FL, leading to progression and transformation to DLBCL, occurs according to the branching rather than the linear model (Figure 11).

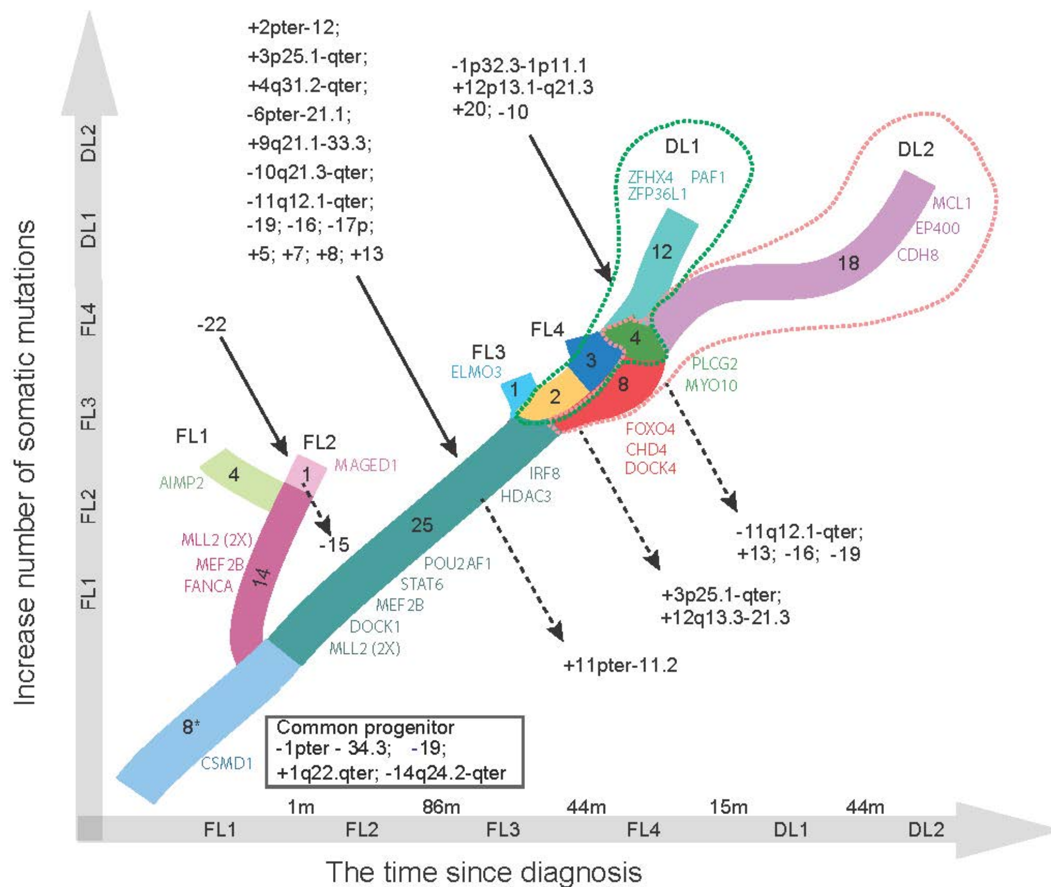

Figure 11. "Evolutionary tree" illustrating clonal evolution of the lymphoma in patient 4. The arrows indicate copy number changes: acquired alterations are listed above the branch and lost below the branch. The number of shared and acquired mutations are shown within the branches (adapted from Paper III, Figure 2B).

4.4 PAPER IV PROTEOMIC PROFILING OF DLBCL

Since the major breakthrough of GEP studies published by Alizadeh *et al* in 2000, that identified molecular subtypes of DLBCL (GC, ABC and unclassified) associated with different genetic, biological and clinical features, there has been a need to translate these results into diagnostic routine and clinical practice (55). The OMICs technologies offer a vast variety of methods, which can be used in search for biomarkers, that could be applied as diagnostic and prognostic tools as well as therapeutic targets. Despite the fact, that proteomic profiling is a well-established method, which allows studying a whole proteome of intact proteins in cells and tissues, little has been reported about protein expression profiles in various DLBCL subtypes. The groups of Deeb and Ruetschi have independently, recently, presented a super-SILAC based approaches leading to a 55-protein and 87-proteins signature respectively, which may allow to discriminate different DLBCL subtypes (153-155). We have performed this study in order to focus on the proteome profiling of DLBCL subgroups

(non-GC and GC) on the level of intact proteins. 31 DLBCL tumours were studied (16 non-GC and 15 GC type), of which 6 samples (3 non-GC and 3 GC) were used for proteomic based analysis. As the result of proteome profiling we found 300 protein spots differentially expressed between the non-GC and GC type of DLBCL. During mass-spectrometry analysis we identified 91 proteins, that were present exclusively in non-GC but not in the GC type of DLBCL. We then performed systemic analysis of identified proteins that led to creation of 19 subnetworks. The subnetworks represented proteins and genes involved in cell proliferation, cell death, cell cycle regulation, NF-κB pathway, MAPK signaling, PI3 kinase pathway, Wnt signaling, B-cell receptor signaling, proteasomal activity, p53 pathway and many others. At the end, we selected three proteins: BiP (immunoglobulin heavy chain binding protein), Hsp90 (heat shock protein 90) and cyclin B2 for validation by IHC. BiP and Hsp90 are proteins involved in the NF-κB pathway, while cyclin B2 is involved in the cell cycle regulation (Figure 12 and 13). We showed that BiP and Hsp90 tend to be over-expressed in the non-GC type of DLBCL. Although positive expression of BiP was observed in less than the half of the DLBCL cases in our study, a positive Hsp90 expression was seen in almost all the cases. Likewise, the overexpression of cyclinB2 was seen in the majority of the cases with more than the half showing moderate staining. Although cyclin B2 was shown to be an adverse prognostic markers in various cancer types, it has not been previously studied in a context of lymphoma. Our findings suggest the potential clinical relevance of these proteins and also implicate the need for further proteomic studies in this field.

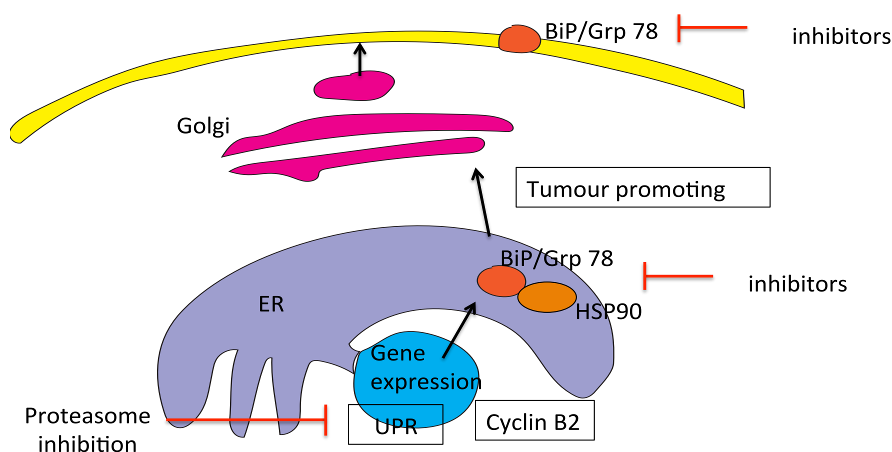

Figure 12. Schematic illustration of functions and relationship of the studied proteins. BiP (GRP78) is a ER chaperon. BiP is stabilised by Hsp90. Normally, BiP protects cells from accumulated unfolded proteins. In tumours, BiP act as a inhibitor of apoptosis. Cyclin B2 is a cell cycle protein, that has been shown to be overexpressed in various tumours. These proteins can potentially be affected by inhibitory treatment. (Modified after Gutierrez *et al* 2014 (156)).

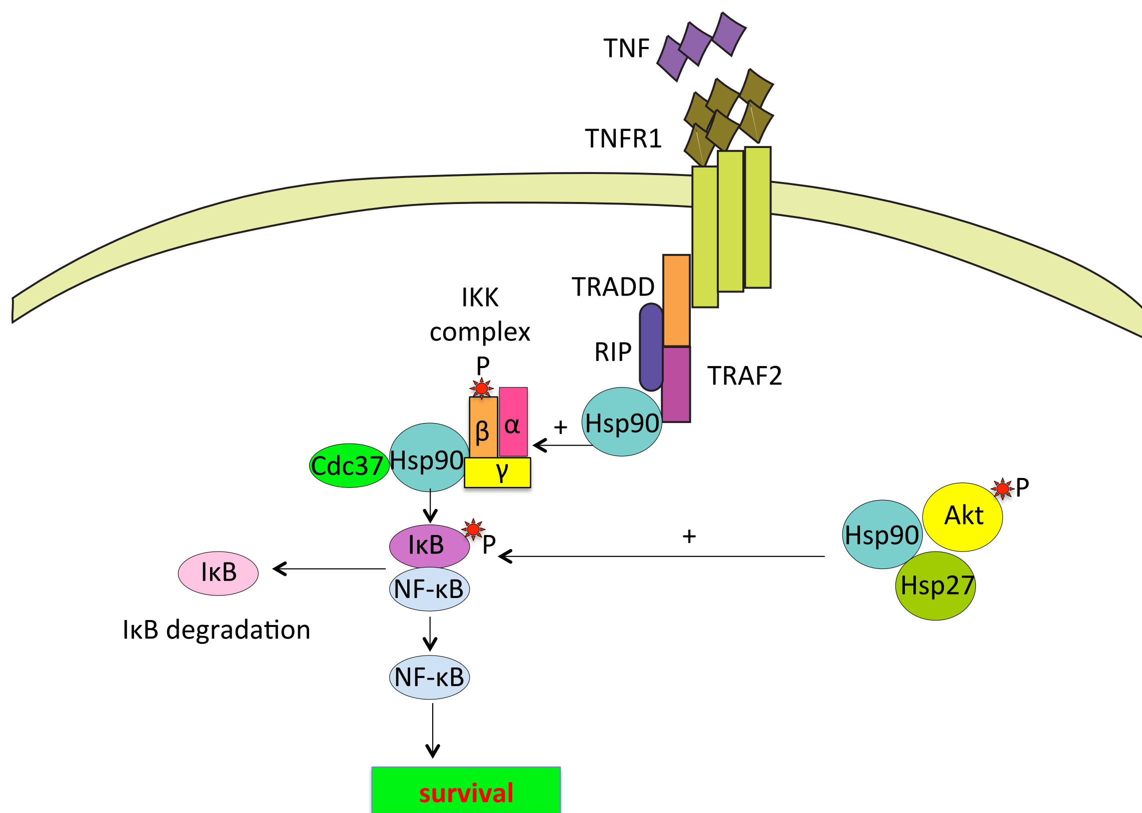

Figure 13. Schematic illustration of regulation of NF-κB pathway by Hsp90. The ability of Hsp90 to regulate cell survival depend most of all of the level of IKK complex. The Hsp90 is forming a complex with Cdc37, which is a co-chaperone and then bind to IKK complex via interaction with regulatory subunit IKKγ (NEMO) and IKKβ. This Hsp90 mediated IKK – dependent mechanism seems to be crucial for NF-κB activation leading to cell survival. (Modified by Beere 2000 (157)).

5 CONCLUSIONS

- Several genomic events are involved in the transformation of FL to tDLBCL, and array-CGH (detecting CNVs) and WES (identyfying mutations) give complemetary information on the pathobiology of this process. Mutations detected within CNVs are more likely to be of somatic origin and could be regarded as of tumourigenetic importance, even without the presence of a normal tissue for comparison.

- The 2p15-16.1 gain/amplification is involved in the transformation from FL to tDLBCL, and is associated with worse prognosis. If noted in FL, this aberration has a potential to serve as molecular marker associated with risk for transformation.

- The 2p15-16.1 region encompasses several genes, such as *REL*, *BCL11A*, *PEX13*, *USP34, COMMD-1*, *XPO-1* and *OTX-1*, of which some are involved in NF-κB pathway, underlying its importance as a target for personalised therapies.

- 17q21-23 was gained only in tDLBCL, and was not found in FL or dnDLBCL, and could therefore be considered a marker for tDLBCL.

- Tumour progression of FL and its transformation to tDLBCL occurs rather according to the branching model than the linear one.

- The protein profiles of non-GC and GC types of DLBCL identified at least 91 proteins differentially expressed between these two groups. These proteins were involved in functional networks representing, among others: the cell proliferation, cell death, cell cycle regulation, NF-κB pathway, MAPK signalling, PI3 kinase pathway, Wnt signalling, B-cell receptor signalling, proteasomal activity, and p53 pathway.

- BiP and Hsp90, involved in NF-κB pathway, as well as Cyclin B2 showed an increased expression in DLBCL, and tended to be related to the non-GC type of DLBCL. These proteins could therefore be a potential clinical markers.

- HAX-1 shows a positive correlation with Ki67 and an inverse correlation with Bcl2, which suggests its role as Bcl-2 homolog. Furhermore, HAX-1 shows a variable expression on protein and transcript level among different subtypes of B-cell lymphomas.

6 FUTURE PROSPECTS

The challenge of the next decade will be to bring research discoveries to a clinical practice. It must be done in an efficient and practical way, and will depend on collaborations among institutions and professionals of various disciplines (158).

First, there is a need of an integrated approach to diagnostics that will combine the data obtained from various molecular methods and allow the selection of the most promising biomarkers for a specific clinical application and individual patients. Second, these biomarkers will need to be validated on *e.g.* drug test assays for a routine clinical approach. The third step will be combining drugs for the best effect, optimal dosing, and least toxicities for the particular individual patient.

Cancer research is a very dynamic field but its success will be measured by the ability to combine the scientific data into practical approach that may be used in the every day clinical practice. They will need a cooperation between research laboratories, clinical diagnostic laboratories and new generation clinical trials, that would allow combination of different drugs with various therapeutic modalities. That will be only possible with open collaborations and a pragmatic approach involving scientists, clinical laboratory medicine specialists and clinicians.

The pathologist in a multidisciplinary team has a critical and vital role for the choice of treatment. Based on the deep understanding of disease and professional expertise, the pathologists provides the clinician not only with the diagnosis, but also with information about individual characteristics of the disease, its histopathological and molecular profile, giving an important perspective in a team considering a patients's case.

Personally, I believe in a close collaboration between Clinical Departments of Hematology and Oncology, Clinical Laboratories and large Research Centers, integrating molecular medicine, diagnostics, research technology and biobanking infrastructure with clinics, enabling the adaptation of personalised medicine in health care. The examples of centers successfully creating such platforms are inspiring and give a promise for future of Karolinska University Hospital, Karolinska Institute and Sci-Life Laboratory. The optimal goal would be a collaboration with other centers on the national and international level.

Looking ahead, we have also started a collaboration with colleagues from University of Oslo, Oslo University Hospital as well as University of Toronto, planning further investigation of potential clinical implications of proteomic markers described in this thesis in a larger cohort of patients. We have performed further experiments and are going to correlate the results with clinical data together with our colleagues in Norway and Canada.

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