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Regulation of Molecular Processes in Diffuse Large B-cell Lymphoma

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To the patients

Abstract

The molecular understanding of diseases has advanced rapidly due to the use of gene expression profiling. However, these methods have been hampered by the limitation to use frozen tissue specimens. Formalin fixation and paraffin embedding (FFPE) is a standard procedure for long time storage of tissues. FFPE tissues are available in large numbers and are of value for molecular research with the main challenge of low RNA quality compared to fresh frozen (FF) tissues. This thesis showed important aspects on laboratory methods of gene expression using FFPE material analyzing gene regulation and environmental factors in patients with Diffuse Large B-cell Lymphoma (DLBCL). In **Study I**, we evaluated RNA extraction and gene expression of long-term preserved FFPE Non-small Cell Lung Cancer (NSCLC) specimens using quantitative PCR (qPCR) and microarray. High quality gene expression signatures could be recognized in long time stored FFPE tissues. According to the results of Study I, FFPE tissues were further used in Studies II, III and IV.

Different countries of the world have varying prevalence of microbial infections. It should be of interest to study patient populations originating from regions with different infectious and environmental exposures with the same disease. Sweden and Egypt are countries defined as low and high endemic infectious disease areas respectively. DLBCL is the most common type of Non Hodgkin's Lymphoma (NHL) and accounts for approximately 40% of newly diagnosed lymphomas worldwide. The ABC subgroup of DLBCL (ABC DLBCL) has a poor prognosis with short survival. NHL has been associated to viral infections as Epstein Barr virus (EBV) and Hepatitis viruses B and C (HBV, HCV). To understand if differences in environmental exposure are associated to the activated B-cell type (ABC) of DLBCL, we analyzed the expression of genes, regulatory factors and microbial agents of Swedish and Egyptian ABC DLBCL patients using microarrays. In **Study II**, we compared the global gene expression profiles of Swedish and Egyptian patients. Signal transducer and activators of transcription 3 and 5 (STAT3 and STAT5b) were differently expressed. STAT3 was significantly upregulated in Swedish compared to Egyptian patients and controls. The opposite expression patterns was demonstrated for STAT5b. The difference in STAT3 and STAT5b expression was confirmed at the protein level. Based on these results, we investigated microRNA (miRNA) expression profiles in **Study III**. miRNAs are non coding RNAs targeting mRNA modulating their expression at the post-transcriptional level. We found that miRNA-1234 (miR-1234) was significantly upregulated in Egyptian compared to Swedish patients. The expression level of miR-1234 correlated inversely to the expression of STAT3. Furthermore, the Stat3 protein was downregulated in cells transfected with miR-1234, suggesting that STAT3 might be a potential target for miR-1234. In **Study IV**, we analyzed the presence of microbial agents in Swedish and Egyptian ABC DLBCL patients using a microbial detection array (MDA). JC polyoma virus (JCV) was detected in both Swedish and Egyptian patients and the complete HBV genome in Egyptian patients. Study IV supports the notion that viral agents such as JCV and HBV may be involved in the tumorigenesis of DLBCL in high infectious disease regions. ABC DLBCL patients originating from areas with different environmental exposures have altered gene and miRNA expression profiles and a different viral load, which may be of importance for the development of ABC DLBCL. STAT3 may be regulated by miRNA and associated to the presence of viral infections. These results may be of potential importance for the development of STAT targeted therapy.

List of publications

I. **Jacobson TA**, Lundahl J, Mellstedt H, Moshfegh A: Gene expression analysis using long-term preserved formalin-fixed and paraffin-embedded tissue of non-small cell lung cancer. *International Journal of Oncology*, 38(4):1075-81, 2011.

II. **Högfeldt T**, Bahnassy AA, Österborg A, Prokovskaja Tamm K, Porwit A, Zekri A-R. N, 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. *Leukemia & Lymphoma*, Epub ahead of print Nov 8, 2012.

III. **Högfeldt T**, Johnsson P, Grandér D, Bahnassy AA, Porwit A, Österborg A, Zekri A-R.N, Lundahl J, Khaled HM, Mellstedt H, Moshfegh A: Expression of miRNA-1234 related STAT3 in patients with diffuse large B-cell lymphoma (DLBCL) from high and low infectious disease areas. *Resubmitted following revision 2013*.

IV. **Högfeldt T**, Jaing C, Mc Loughlin K, Thissen J, Gardner S.N, Bahnassy AA, Gharizadeh B, Lundahl J, Österborg A, Porwit A, Zekri AR, Khaled HM, Mellstedt H, Moshfegh A: Differential expression of viral agents in lymphoma tissues of patients with ABC diffuse large B-cell lymphoma from high and low endemic infectious disease regions. *Manuscript.*

Publication performed during the PhD studies not included in the thesis

Olsson J, **Jacobson TA**, Paulsson JM, Dadfar E, Moshfegh A, Jacobson SH, Lundahl J. Expression of neutrophil SOD2 is reduced after lipopolysaccharide stimulation: a potential cause of neutrophil dysfunction in chronic kidney disease. *Nephrology Dialysis Transplantation*, 26(7):2195-201, 2011.

List of abbreviations

ABC	Activated B-cell like
BCR	B-cell receptor
BL	Burkitt lymphoma
cDNA	Complementary DNA
CHOP	Cyclophosphamide, doxorubicin, vincristine, prednisone
CLL	Chronic lymphocytic leukemia
CSR	Class switch recombination
C_T	Cycle threshold
DLBCL	Diffuse large B-cell lymphoma
dsRNA	Double stranded RNA
EBV	Epstein Barr virus
FF	Fresh frozen
FFPE	Formalin fixed and paraffin embedded
FL	Follicular lymphoma
GCB	Germinal center B-cell
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBsAg	Hepatitis B surface Antigen
HBV	Hepatitis B virus
HBx	Hepatitis B x protein
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
Hek	Human embryonic kidney
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HPyV	Human polyoma virus
HTLV	T-cell lymphotropic virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPA	Ingenuity pathway analysis
IPI	International prognostic index
Jak	Janus kinase
LLMDA	Lawrence Livermore MDA
MCL	Mantle cell lymphoma
MDA	Microbial detection array
miRISC	miRNA-induced silencing complex
miRNA	microRNA
ncRNA	non coding RNA
Nf-κB	Nuclear factor kappa-light chain enhancer of activated B-cells
NHL	Non Hodgkin's lymphoma
NSCLC	Non small lung cell cancer
OS	Overall survival
PCR	Polymerase chain reaction

PFS	Progression free survival
PIAS	Protein inhibitors of activated STATs
POL	Polymerase
Pre-mRNA	Precursor mRNA
PyV	Polyoma virus
qPCR	Quantitative polymerase reaction
R-CHOP	Rituximab-cyclophosphamide, doxorubicin, vincristine, prednisone
RAG	Recombination activation gene
REAL	Revised European American classification of lymphoid neoplasms
ROR	Receptor tyrosine kinase –like orphan receptor 1
S1PR1	Sphingosine-1-phosphate receptor
SH2	Src-homology 2
SHM	Somatic hypermutation
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SOCS	Suppressor of cytokine signaling
STAT	Signal transducers and activators of transcription
stRNA	Short temporal RNAs
TAD	Transcriptional activation domain
TF	Transcription factors
U-STAT	Unphosphorylated STAT
UTR	Untranslated region
WHO	World health organization

Contents

1 INTRODUCTION TO CANCER

1.1 The hallmarks of cancer

There are more than 200 types of cancer diseases, which show the complexity of the disease. Although all types are different, they share several common features. These features have been stated as the six hallmarks of cancer: self sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis [1]. The authors of the six hallmarks also published an updated version and added features as: avoiding immune destruction, enabling replicative immortality, tumor promoting inflammation, genome instability and mutation [2].

1.2 Causes of cancer

Inherited genes may cause chronic illness but, genes are far from the most important factors. Instead, our lifestyle and environment accounts for 90-95% of all chronic illnesses. Environmental factors that play an important role in the development of cancer are tobacco use, diet, radiation and infectious organisms [3]. Only 5-10% of all cancers are caused by an inherited gene defect, which makes lifestyle factors of great importance for cancer development [3, 4]. Figure 1 shows the distribution of environmental and genetic factors causing cancer.

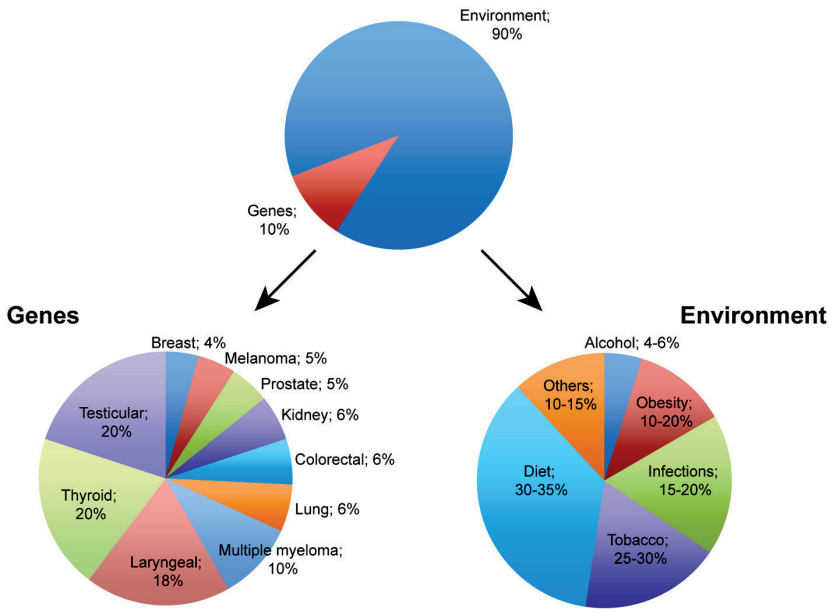

Figure 1. Schematic picture showing how genetic and environmental factors contribute to cancer. Adapted and modified from [3].

The use of tobacco increases the risk of many types of cancer due to at least 50 carcinogenes found in tobacco. A decrease in the prevalence of smoking has been demonstrated in developed countries while developing countries of the world show an increase in smoking [3]. Smoking and cancer development are associated with dysregulated signaling pathways and chronic inflammation [5]. Inflammation has also been associated with other factors causing cancer, such as use of alcohol [6], diet and obesity [3]. Obesity is a growing problem with an increased prevalence of overweight linking abnormal signaling pathways and inflammation to cancer [7, 8].

Infectious agents have during the past decades been found to be associated to cancer [9]. Viruses account for most infection-caused cancers while bacteria account for a smaller part. The most prevalent agents are Human papillomavirus (HPV), EBV, Kaposi´s sarcoma-associated herpes virus, Human T-lymphotropic virus 1 (HTLV-1), Human immunodeficiency virus (HIV), HBV and HCV and the bacterium Helicobacter pylori. These agents are associated to cervical cancer, anogenital cancer, skin cancer, nasopharyngeal cancer, Burkitt lymphoma (BL), Hodgkin´s lymphoma, Kaposi´s sarcoma, adult T-cell leukemia, B-cell lymphoma and liver cancer (Table 1). In 2002, it was estimated that 18% of all neoplasms are associated with infections, ranging from less that 10% in western countries to 25% in African countries [3, 9]. One possible mechanism in the development of infection related cancer is that oncogenic viruses target the Nf-κB signaling pathway that plays a crucial role in the regulation of inflammation, cell growth and apoptosis [10, 11]. Other environmental factors also play an important role in the development of cancer. Populations of the world have different exposure of environmental factors depending on where they live. Even though this is generally known, few studies compare patient populations with different environmental exposures.

Table 1. Major human infection associated malignancies

Malignancy	**Agent**
Carcinomas:	
Bladder	Schistosoma haematobium
Cervical	HPV
Hepatocellular	HBV
	HCV
Bile Duct	Opisthorchis viverrini
Nasopharynx	EBV
Stomach	Helicobacter Pylori
Lymphomas:	
Adult T-cell	HTLV-I
Burkitt	EBV
Hodgkin	EBV
Sarcoma:	
Kaposi	HHV8

HPV: human papilloma virus, HBV: Hepatitis B virus, HCV: Hepatitis C virus, EBV: Epstein-Barr virus, HTLV1: Human T-cell lymphotropic virus type 1, HHV8: Human herpes virus 8

Adapted and modified from [9].

1.3 Tumor suppressor genes and oncogenes

Two categories of genes play a central role in cancer development: tumor suppressor genes and oncogenes. These genes are important during cancer development with regulating capabilities acquired through mutational events during tumor initiation. Tumor suppressor genes functions as negative regulators of cellular proliferation, but their inactivation by mutation may result in tumor growth. An activated oncogene may lead to increased cell growth and is capable of promoting cancer [12].

1.4 Viruses causing cancer

Viruses have been classified as small infectious agents that pass through fine-pore filters (unlike bacteria and cells). Peyton Rous suggested an association between cancer and viruses in 1911 showing that filtered cell free tumor extracts could be used to spread tumors in chickens. Rous was awarded the Nobel Prize in 1966 for his discovery of tumor-inducing viruses [13]. The current human viruses classified as carcinogenic for humans are listed in Table 1. HIV-1 may also be listed as cancer-causing agent, but is primarily known to cause immune suppression.

In 1965, EBV was the first human virus to be classified as carcinogenic and was discovered by electron microscopy of BL cell lines, showing herpes-like particles different from other known viruses. The fact that BL patients had elevated levels of antibodies to EBV antigens indicated a causal link between EBV and BL. More recent studies have shown that EBV provides a key survival signal in the early stages of tumor development [13].

The outbreaks of hepatitis in 1930-1940's among individuals that had received vaccines containing human sera or plasma were the first evidence of a transmissible agent causing hepatitis. Baruch Blumberg was awarded the Nobel Prize for his discovery of the HBV surface protein and the association with hepatitis [13]. HBV infection is often chronic and persistent and combined with chronic inflammation it may cause liver diseases and hepatocellular carcinoma (HCC). Patients with HBV infection have a higher risk of developing HCC compared to uninfected individuals [14]. The decline of HCC incidence by the introduction of anti-HBV vaccine strongly indicate that HBV is involved in HCC [15].

Human warts have been discussed to be associated with cancer since 1907 when an Italian physician claiming that cell-free filtrate derived from common warts could transfer disease. It was later discovered that papilloma virus caused skin carcinoma in rabbits in 1935 [13, 16]. Since then, HPV has been demonstrated to be present in cervical cancer biopsies as well as in cervical cancer derived cell lines. HPV is now recognized as a human cancer virus causing cervical cancer [17, 18]. Vaccines targeting high-risk HPV strains are now available limiting the spread of HPV as well as the incidence of HPV-related cancers. Harald zur Hausen was awarded The Nobel Prize in 2008 for his discovery of HPV causing cervical cancer [13].

The first human polyoma viruses (HPyV), BK (BKV) and JC (JCV) were isolated in 1971 [19, 20]. BK and JCV were recently classified as "possibly carcinogenic to humans" by a WHO international cancer research working committee [21] but the relation of JCV to

human malignancies is controversial. Studies have proposed JCV to be related to human cancers as colorectal-, gastric-, lung- and brain cancers [22-26].

Even though viruses may cause cancer, not all infections by an oncogenic virus may lead to cancer. A viral infection might be acting as an initiating or promoting factor but may not be sufficient to cause cancer. Involvement of other factors in the process of transformation is important as accumulated genetic and epigenetic changes [13, 27]. The majority of individuals infected with a tumorigenic virus as EBV or HPV do not develop cancer unless the patient is immunocompromised. However, in some geographical areas, the risk of cancer development in viral infected individuals is higher. This has been demonstrated in Chinese and Japanese populations with chronic HBV infection who have a higher risk of HCC development compared to other geographical areas [14]. Equatorial African population with EBV infection in combination with malaria has a higher risk of developing endemic BL compared to other geographical areas [27].

The human tumor viruses display different mechanisms of cell transformation and there does not seem to be a general mechanism. Still, viruses may be divided into direct and indirect acting viruses. Direct acting viruses carry one or more viral oncogenes, which is not the case for indirect acting viruses. Examples of direct tumor viruses are HPV, HPyV and EBV infecting cells into a supportive state of viral gene expression and replication. Their oncoproteins target cellular tumor suppressor genes [27]. An example of an indirect acting tumor virus is HBV using both indirect and direct mechanisms. Persistent HBV infection may lead to chronic liver injury, necrosis, inflammation and liver regeneration and HCC. HBV carries a potential oncoprotein, the X protein, which is not analogous to the oncoproteins of HPV, EBV and HPyV. The X protein indirectly contributes to the process of carcinogenesis by activating cell signaling cascades [27-29].

1.5 Inflammation associated to cancer development

Cancer has since long been linked to inflammation. This is based upon observations that tumors often arise at sites of chronic inflammation and that inflammatory cells are present in tumor samples [30]. Activation of triggers of chronic inflammation as microbial infections, autoimmune diseases and other inflammatory conditions increases the risk of developing cancer. Anti-inflammatory treatment has been shown to decrease the incidence and mortality of several tumor types [31, 32]. Inflammatory cells, chemokines and cytokines in the tumor microenvironment are other proof of that cancer may be linked to inflammation. The targeting of such inflammatory mediators and key transcription factors (STAT3 and NF-κB) may decrease the spread and incidence of cancer. Signal transducers and activators of transcription (STAT3) and Nuclear Factor κ-light chain enhancer of activated B-cells (NF-κB) will be discussed in more depth in this thesis. Drugs targeting cancer-related inflammation has evolved recently [31].

The link between cancer and inflammation may be viewed as two pathways, one intrinsic pathway and one extrinsic. The intrinsic pathway is driven by genetic alterations such as activation of oncogenes and the extrinsic pathway is driven by inflammation or infection, that increases the risk of cancer development at certain sites (e.g. colon, prostate, pancreas) [31]. The intrinsic pathway involves one of the most frequently mutated oncogenes in human cancers: RAS [33]. The activation of signaling pathways downstream of RAS

induces production of tumor-promoting inflammatory chemokines and cytokines that support the development of a tumor tissue microenvironment [33]. Key factors of both the intrinsic and extrinsic pathways are STAT3 and NF-κB [34, 35]. NF-κB and STAT3 are both involved in the inhibition of apoptosis. More specifically, NF-κB can promote cell survival by inducing gene expression of BCL2 (an anti-apoptotic gene) and STAT3 may also inhibit maturation of dendritic cells, resulting in tumors evading the immune system [31, 36].

2 THE LYMPHATIC SYSTEM

2.1 Introduction

A network of vessels (lymphatics), lymph nodes and lymphoid organs are together known as the lymphatic system. The primary functions of the lymphatic system are to transport interstitial fluid and to facilitate the immune response. The lymph from the tissues is filtrated and returned to the blood. There are both primary and secondary lymphoid organs. The primary organs are the thymus and the bone marrow where the immune cells, lymphocytes, are produced. The secondary organs include the spleen, Peyer's patches, appendix, the tonsils and the lymph nodes. They are responsible for the maturation of lymphocytes and the initiation of an immune response [37, 38]. Figure 2 shows the overall lymphatic system and the immune system of the human body.

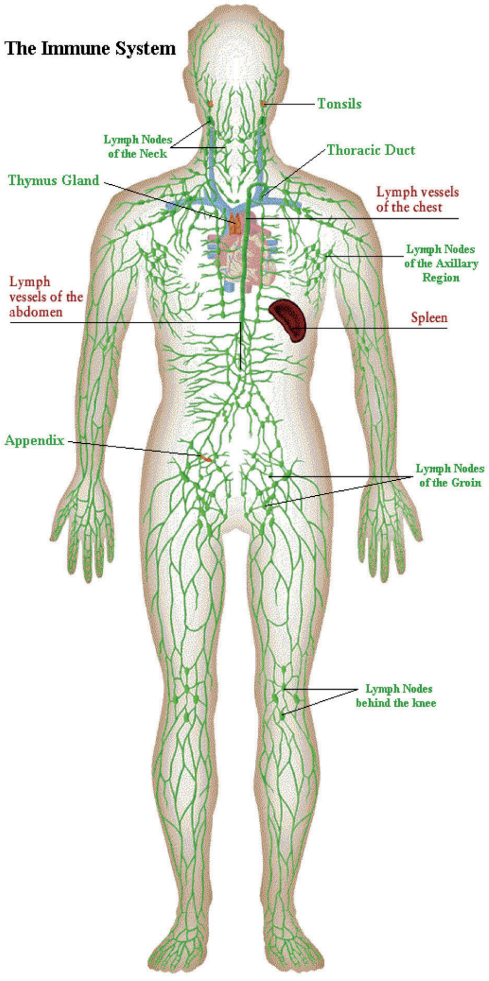

Figure 2. Picture showing the parts of the immune and the lymphatic system of the human body. Source: http://www.naturalhealthschool.com

2.2 Lymphocytes

Lymphocytes are important cells of the immune system. They consist of Natural killer cells (NK-cells), B-lymphocytes (B-cells) and T-lymphocytes (T-cells). All lymphocytes arise from the bone marrow. B-cells mature in the bone marrow and T cells mature in the thymus. NK-cells have the ability to kill infected host cells and are a part of the innate immune response (first line of defense). B-cells and T cells are the major cells of the adaptive immune response (second line of defense) [39].

2.3 B-CELLs

The B-cell carries a receptor molecule called B-cell receptor (BCR) or immunoglobulin (Ig) when secreted. The BCR detects external stimuli (antigens) and trigger a response of the adaptive immune system. To be able to detect a large variety of antigens, the receptors must be able to bind and distinguish between many different antigens. Each clone of a B-cell has a specificity with a unique receptor. After the B-cell has been activated by recognition of an antigen, it differentiates to either an effector B-cell or a memory B-cell. The effector B-cell (plasma cell) has the ability to secrete the differentiated Ig molecules, which in secreted form also may be called antibodies. Memory B-cells are long-lived B-cells that upon stimulation of the same antigen, can activate a secondary immune response. There are different subsets of B-cells depending on where they reside. Follicular B-cells reside in the follicles of lymphoid organs. Marginal zone B-cells are located in the marginal zones of the splenic white pulp and B-1 B-cells reside in the mucosal tissues and the peritoneum [39]. The different stages of B-cell development are: lymphoid progenitor cell, pre B-cell, resting naïve B-cell, activated or memory B-cell, mature memory B-cell, germinal center B-cell, IgM secreting B-cell and plasma cell [38].

2.3.1 B-cell Receptors and antibodies

Each B-cell has a BCR with a single specificity. This means that the human total B-cell repertoire have millions of antigen receptor specificities. The specificity arises through genetic mechanisms during lymphocyte development in the bone marrow [38]. The gene segments that encode the Ig molecule undergo recombination to generate the receptor specificity. The gene segments are Variable gene segments (V), Diversity gene segments (D) and Joining gene segments (J). The diversity of the Ig molecules is generated through combinations of these segments. The result is variability in the nucleotide sequences giving each Ig molecule its own specificity. The developed Ig molecule consists of four polypeptide chains, two heavy- and two light chains. Each chain has a constant region and a variable region. The variable region is the recognition site for antigens [39] (Figure 3). In total, there are five classes of antibodies: IgM, IgD, IgG, IgA and IgE. Immature B-cells that have not yet met an antigen express IgM as monomers on the surface and IgD is expressed on the mature B-cell. The B-cells are then activated by antigens leading to clonal expansion, the process of differentiation of antigen specific cells and plasma cells that secrete antibodies.

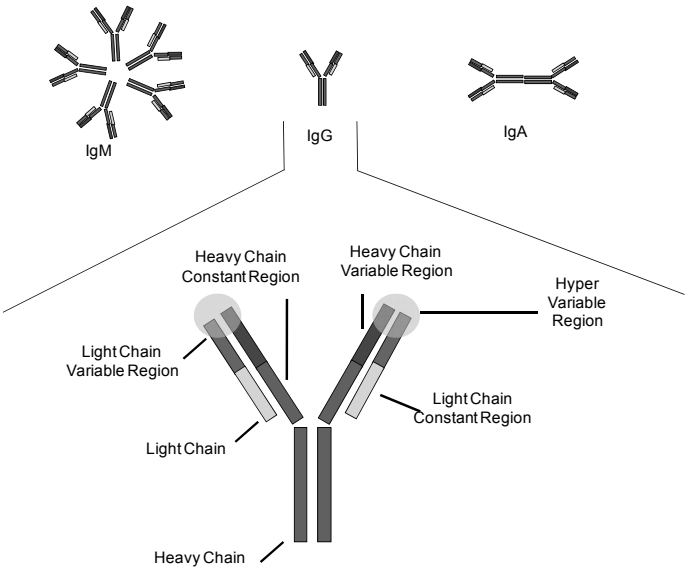

Figure 3. Structure of a secreted antibody showing heavy and light chains as well as constant and variable regions.

2.3.2 Lymph nodes

A pathogen that enters the body may generate an infection. The antigens specific for the pathogen will eventually meet a lymphocyte in a secondary lymphoid organ such as lymph nodes. Antigens are carried to the lymph nodes by macrophages or dendritic cells. The lymph node is a structured compartment collecting extracellular fluid from the tissues and returns it to the blood. There are afferent lymphatic vessels and efferent lymphatic vessels. The fluid from the tissues and antigen-bearing cells from infected tissues are drained to the lymph nodes by afferent vessels. Proliferated and differentiated antigen specific lymphocytes leave the lymph node through the efferent lymphatic vessels. B-cells are localized in the follicles and some of the follicles include a germinal center. T cells are distributed in the paracortical areas or T cell zones [38].

2.3.3 Germinal centers

The germinal centers are part of the follicles in the lymph nodes and the site where B-cells undergo proliferation after meeting an antigen and their corresponding activating T cell. Proliferating B-cells are the majority of cells in the germinal centers but there are also antigen specific T-cells present (10%). Resting B-cells are pushed toward the periphery of the follicle forming a mantle zone of resting cells. At the germinal center, there is intense cell proliferation with rapidly dividing B-cells. Centroblasts are B-cells that have reduced their expression of Ig receptors, especially IgD. Centrocytes are B-cells that have reduced their rate of proliferation and start to express higher levels of Ig receptors. The centroblasts are situated in the dark zone of the center and with further development, the B-cells enter the light zone. The light zone is also filled with follicular dendritic cells [38].

2.3.4 B-cell tumors

Tumors have maintained many characteristics of the cell type they developed from. This is especially illustrated for B-cell tumors that arise from all stages of B-cell development. All B-cells in a B-cell tumor have identical Ig gene rearrangements revealing their origin from one cell. The normal control of B-cell homeostasis is disrupted in B-cell tumors. This is often associated with abnormal Ig rearrangements. One of the Ig loci may be joined to another gene situated on another chromosome. This is known as translocation, which might alter gene expression of genes controlling cell growth [38].

3 LYMPHOMA

3.1 Introduction

Lymphomas is a common name of neoplasms of lymphoid precursor cells [40]. Thomas Hodgkin was the first to report lymphomas in 1832 and named the disease to Hodgkin´s disease [41]. Later, the disease was divided into Hodgkin´s lymphoma and non Hodgkin's lymphoma (NHL). B-cell lymphoma represents the majority of NHL, but there are also T-cell- and NK cell lymphomas. Lymphoid neoplasms are a group of highly diverse diseases and reflect the diversity of the immune system.

Approximately 2 200 patients are diagnosed with lymphoma in Sweden per year and the disease is more common among men than women. Lymphoma corresponds to approximately 3-4% of all cancer cases in Sweden per year [42] (Figure 4).

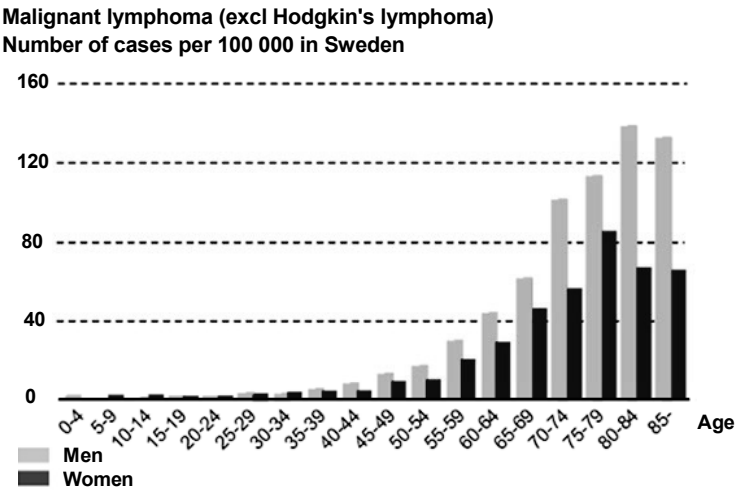

Figure 4. Incidence of malignant lymphoma per year in Sweden defined by age.
Source: Socialstyrelsens statistikdatabas; Cancer i siffror, 2009

3.2 Types of B-cell non Hodgkin´s lymphoma

NHL is clinically divided into high- and low-grade malignant lymphoma. High-grade malignant lymphoma is characterized by fast proliferating cells, rapid growth of tumors and is, in many cases a curable disease with treatment. However, the survival is short without treatment. Examples of high-grade lymphomas are DLBCL, Peripheral T cell lymphoma and BL. Low-grade malignant lymphomas are characterized by slow proliferation of cells and usually slow disease progression, but this disease cannot be cured with the treatment options available today. Patients with low-grade lymphomas with treatment may live long but may never be cured. Examples of low-grade malignant lymphomas are follicular lymphoma (FL) and chronic lymphocytic leukemia (CLL). Mantle cell lymphoma (MCL) has properties similar to both high- and low-grade malignant lymphomas. Some stages of FL are also classified as high-grade malignant lymphoma. Table 2 shows the different types

of NHL and their case distribution. NHL arise by accumulation of genetic aberrations that results in a growth advantage of a malignant clone. B-cell lymphomas develop during B lymphocyte development and represent their malignant counterpart. Figure 5 shows how different stages of B-cell development represent each malignant lymphoma subtype.

DNA modification during B-cell development is essential for the normal immune response but the modifications may also give rise to genetic abnormalities leading to lymphoma. The B-cell development starts in the bone marrow with the V (D) J recombination process recombining genes encoding the variable regions of heavy and light chains that form the BCR. The process gives rise to double strand DNA breaks by recombination activation gene 1 (RAG1) and 2 (RAG2) controlled by end-joining repair processes [43]. The heavy chain genes are brought together from the V, D and J elements and the light chain genes are brought together by the V and J elements. Only cells with both heavy and light chain regions that can be translated into protein will survive [44]. Once a BCR is expressed, the lymphocyte leaves the bone marrow to become mature naïve B-cells. The germinal center reaction in a secondary lymphoid tissue is initiated once an antigen is encountered. Further DNA modifications take place during the germinal center reaction, both somatic hyper mutation (SHM) and class switch recombination (CSR). SHM introduces mutations, small deletions and insertions to the variable Ig region to increase the antibody antigen specificity. CSR is the process of class switching by DNA recombination to switch from IgG to IgM, IgE, IgA or IgD [45, 46]. B-cells develop into memory B-cells or plasma cells following the germinal center reaction. All these processes are heavily controlled but may also go wrong. V (D) J recombination, SHM and CSR are especially critical processes that may give rise to lymphoma [47].

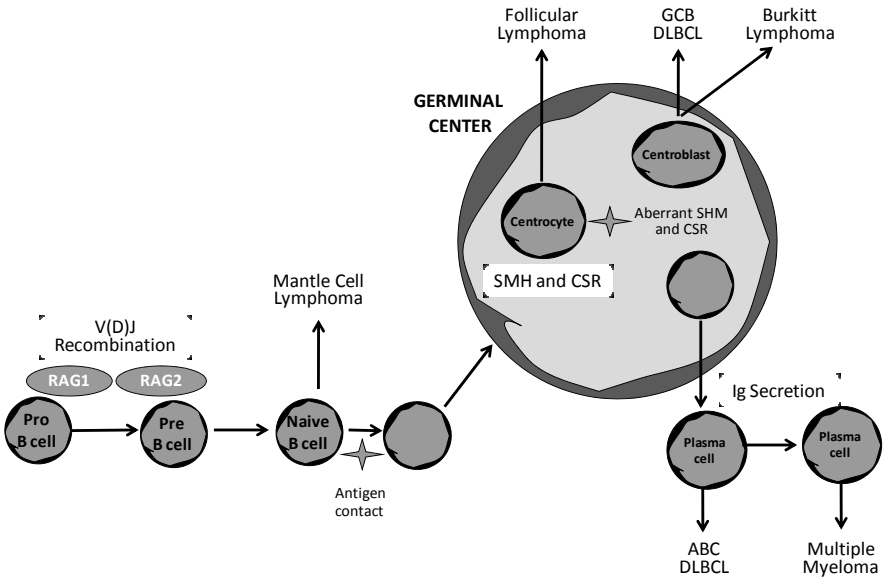

Figure 5. Schematic picture of B-cell development stages that also represent different malignant subtypes. Adapted and modified from [47].

BL, FL and MCL are three B-cell lymphomas that are frequently associated with characteristic translocations. These are t(8;14)(q24;q32), t(14;18)(q31;q21) and t(11;14)(q13;q32) for BL, FL and MCL respectively. The translocations are results from errors in the BCR gene rearrangements during B-cell differentiation but none of them are completely diagnostically specific. For example, up to 10% of mantle cell lymphomas lack t(11;14). The translocations often result in deregulation of oncogenes that control cell proliferation, survival and differentiation but they are often insufficient to cause lymphoma alone. Secondary alterations are required for full malignant transformation [47, 48]

Table 2. Distribution of NHL cases by the consensus diagnosis

Consensus diagnosis	No. Of cases	% of total cases
Diffuse Large B-cell	422	30.6
Follicular	304	22.1
Grade 1	131	9.5
Grade 2	85	6.2
Grade 3	88	6.4
Marginal zone B-cell, MALT	105	7.6
Peripheral T cell	96	7.0
Medium-sized, mixed, and large	51	3.7
Angiocentric, nasal	19	1.4
Angioimmunoblastic	17	1.2
Intestinal	5	<1
Lymphoepithelioid	2	<1
Hepatosplenic	1	<1
Adult T cell leukemia/lymphoma	1	<1
Small B lymphocytic (CLL)	93	6.7
Mantle cell	83	6.0
Primary mediastinal large B-cell	33	2.4
Anaplastic large T/null cell	33	2.4
High grade B-cell, Burkitt-like	29	2.1
Marginal zone B-cell, nodal	25	1.8
Precursor T lymphoblastic	23	1.7
Lymphoplasmacytoid	16	1.2
Marginal zone B-cell, splenic	11	<1
Mycosis fungoides	11	<1
Burkitt	10	<1
All other types	84	6.1

CLL: Chronic Lymphocytic Leukemia
Adapted and modified from [49]

3.2.1 Follicular lymphoma

FL accounts for approximately 20% of all malignant lymphomas. It is the most frequent low-grade NHL and arises from germinal center B-cells [47, 49]. Chromosomal translocation is characteristic of approximately 90% of FL cases, which result in dysregulation of the proto-oncogene BCL2 [50]. The FL cases without the translocation have been shown to have distinct gene expression signatures not similar to cases carrying the translocation [51]. Gene

expression profiling has demonstrated the importance of the microenvironment in the clinical course of FL. Two gene expression signatures of immune cells showing enrichment of either T-cells or macrophages and dendritic cells predicted the length of survival [52]. FL can transform into a more aggressive non Hodgkin's lymphoma, most commonly DLBCL. The transformation is associated with poor outcome and the underlying biology is still not completely understood. The transformation is in general based on histological observations of large cells infiltrating the lymph nodes. The risk of FL transformation is estimated to be 3% per year [53, 54].

3.2.2 Mantle cell lymphoma

MCL accounts for 5-10% of all lymphoma cases in adults and is derived from a naïve pre-germinal center B-cell. There are two main variants, the most common type is the classical subtype and the least common subtype is the blastoid subtype. MCL is an aggressive type of lymphoma with a median survival of 3 to 4 years, but there is also less aggressive behavior in some cases [47, 49, 55]. Cell cycle deregulation is characteristic of MCL and is caused by the translocation t(11;14)(q13;32) leading to deregulation and overexpression of the cell cycle regulator Cyclin D1. Cyclin D1 is important in normal cells taking them from G_1 to S phase of the cell cycle. Other genes that more recently has been shown to be important in MCL are the SOX genes. All SOX genes control cell survival, proliferation and differentiation during embryogenesis. Additional genes directly targeted by SOX protein has also been reported such as DBN1, SETMAR and HIG2 [56, 57].

3.2.3 Burkitt lymphoma

BL is an aggressive type of lymphoma and is most common in children. The disease is characterized by high proliferation of B-cells (>95%). The three clinical variants of BL are associated with different clinical settings: endemic BL, sporadic BL and AIDS-associated BL. The translocation t(8;14)(q24;q32) involving the MYC gene is important in BL development. Infection of EBV is also closely linked to BL, but differ in geographical distribution. Endemic BL is associated with EBV in 95% of cases, mostly in African countries but also where malaria is hyperendemic [58, 59]. The gamma herpes virus EBV is involved in several types of cancer. But, in most cases it causes a harmless latent infection of B-cells. Over 95% of the human population is estimated to carry such infection. It has been shown that EBV infection is an early event and that it precedes the translocation t(8;14)(q24;q32) involving the gene MYC [58, 60]. BL can share common immune phenotypic and genetic features with DLBCL making the clinical diagnosis of BL a challenge in some cases [61].

3.3 Diffuse large B-cell lymphoma

3.3.1 Introduction

DLBCL is the most common type of malignant lymphoma and accounts for approximately 30-40% of all cases in adults. Fifty-five percent of all DLBCL cases are men and the median age is 64 years. The 5 year overall survival is 46% [49]. The biology, morphology

and clinical presentation of DLBCL is highly heterogeneous. There are three major subtypes of DLBCL. The subtypes are thought to arise from different stages of B-cell differentiation. **Germinal center B-cell like DLBCL** (GCB DLBCL) is derived from germinal center B-cells expressing genes characteristic of the germinal center B-lymphocytes. The **activated B-cell like DLBCL** (ABC DLBCL) express genes characteristic of plasma cells and are therefore thought to arise from B-cells activated to differentiate into plasma cells. The third subtype is **primary mediastinal B-cell lymphoma** (PMBL). This subtype seems to originate from rare B-cell populations that reside in the thymus and have a distinct gene expression compared to GCB and ABC DLBCL [62-64].

3.3.2 Classification of DLBCL

The classification of NHL has developed steadily with our increased knowledge of the immune system. The World Health Organization (WHO) published the latest classification system in 2001, which was updated in 2008. It was based on the Revised European American Classification of Lymphoid Neoplasms (REAL) published in 1994. The WHO system recognizes NHL diseases through morphology, immunophenotype, genetic, molecular, and clinical features [65, 66]. The classification system expanded the list of diseases that may facilitate clinical trials and research of rare DLBCL that might need specialized approaches. These include DLBCL entities associated with EBV specified as EBV-positive DLBCL of the elderly and DLBCL associated with chronic inflammation. Gene expression profiling that provided the difference between ABC and GCB DLBCL was not included in the WHO requirements of daily clinical practice due to possible difficulties to distinguish between the subtypes without techniques of gene expression profiling [65]. Therefore, several algorithms based on immunohistochemistry have been developed that can predict the cell of origin of DLBCL. The antibodies used in the algorithms are targeting germinal center or activated specific proteins. Figure 6 shows one of the most commonly used immunohistochemistry algorithms [67]. This algorithm was developed by Hans et al in 2004 and confirmed by Meyer et al in 2011 [68].

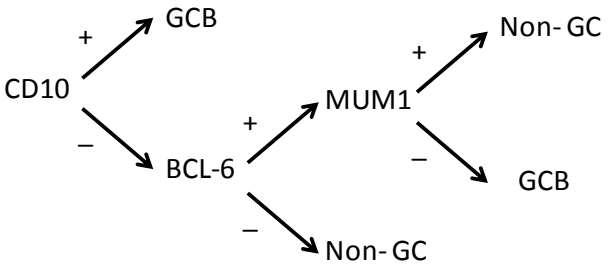

Figure 6. Immunohistochemistry algorithm to define GCB DLBCL and non-GCB DLBCL developed by Hans et al. Adapted and modified from [67].

ABC DLBCL, GCB DLBCL and PMBL differ in their gene expression profiles but also regarding signaling pathways. The t(14;18) translocation associated to the gene BCL2 is found in GCB DLBCL, but not found in ABC DLBCL [63]. Deregulation of the PTEN pathway by different genetic aberrations is also characteristic for GCB DLBCL, but not ABC DLBCL. The gene SPIB was also shown to be critical for the survival of ABC DLBCL, but not for the survival of GCB DLBCL [69].

Several transcription factors are known to regulate the GC reaction in DLBCL. These are BCL6, PRDM1, IRF4, LMO2, E2A and PAX5 [70-72]. Out of these, BCL6 is considered as the master of the regulation, since its deregulation is necessary for differentiation to memory and plasma cells [70, 73]. IRF4 and PRDM1 are key factors for post GC cell differentiation [74, 75] and recent studies suggest IRF4 and PRDM1 to repress BCL6 expression that terminates the GC reaction [76, 77].

3.3.3 Diagnosis of DLBCL

DLBCL, as well as other lymphomas, is diagnosed based on laboratory analysis of tissue specimen excised from the patient. Most DLBCL patients have enlarged lymph nodes that usually are fast growing. Cytology is usually performed and a biopsy is taken from an enlarged lymph node for diagnosis and classification. The possible involvement of the bone marrow is also examined. Staging is based on a CT-scan or a PET/CT scan and blood parameters and virus serology is also examined. If the patient has signs of central nervous system engagement or a generalized disease, the cerebrospinal fluid is examined with cytology [78].

3.3.4 Prognosis of DLBCL

There are two staging systems used for NHL. The Ann Arbor staging system was originally developed for Hodgkin´s lymphoma. It is based on the distribution of nodal disease sites since Hodgkin´s lymphomas often spread to more than one lymph node site [40, 79]. It is used to stage the extent of the patient's disease. The stages are ranged from I-IV. Stage I involves engagement of one lymphoid region or engagement of one external lymphoid organ (Stage IE). Stage II involves at least 2 lymphoid regions on the same side of the diaphragm or localized engagement of external lymphoid organs on the same side of the diaphragm (IIE). Stage III involves engagement of lymphoid regions on both sides of diaphragm. It may also be engagement of the spleen (IIIS) or localized engagement of one external lymphoid organ (IIIE) or both (IIISE). Stage IV involves diffuse or spread engagement of one or several external lymphoid organs or tissues with or without lymph node engagement. If the patient has fever, night sweats or weight loss (B-symptoms) this is indicated with a "B" and if patients have no such B-symptoms, it is indicated with an "A". The International Prognostic Index (IPI) was developed later and collects clinical characteristics to create an informative risk profile at the time of diagnosis. The indicator calculates an IPI score based on the patients age, performance status, level of lactate dehydrogenase and the extent and location of disease (stage) [40, 80].

In a study by Alizadeh et al [62] published in Nature 2000, GCB and ABC DLBCL were shown to have significant differences in overall survival. The average five-year survival was 52% for the entire study population but the five-year survival for GCB

patients was shown to be 76% and by contrast only 16% in ABC patients. The group of patients studied was not previously treated and all patients were de novo DLBCL cases (not arisen from other low grade lymphomas such as FL). The patients were treated after biopsy excision with standard treatment (multi-agent chemotherapy regimens) [62].

3.3.5 Treatment of DLBCL

DLBCL is a curable disease when using the most adequate treatment therapy program. Chemotherapy has been used for many decades for treatment of lymphomas. Among these are drugs based on alkylating agents, anthracyclins or anthracyclin-like drugs and steroids. The activity of chemotherapy induces apoptosis in normal and malignant cells. The chemotherapy agents used for DLBCL are abbreviated CHOP and consists of cyclophosphamide, doxorubicin, vincristine and prednisone. This combination was developed during the 1970s and has, until recently been considered as standard therapy. It has, when compared to other combinations been established as the best chemotherapy combination for NHL [81, 82].

Over the past 20 years, many groups have made attempts to improve the treatment of aggressive lymphomas using more aggressive chemotherapy, dose modifications and additional drugs [83]. Monoclonal antibodies were approved in 1997, in combination with chemotherapy. Rituximab is a chimeric monoclonal antibody targeting the CD20 antigen which all cells of the B-cell lineage express. The combination of rituximab and CHOP (R-CHOP) was evaluated in a clinical trial and demonstrated that patients receiving R-CHOP compared to those given CHOP alone had a significantly better response to treatment [84].

3.3.6 Gene expression studies of DLBCL

Gene expression profiling is a powerful technique to measure the expression of thousands of genes simultaneously. These results provide a molecular profile of RNA in the biopsy specimen showing biologic and clinical diversity. The diagnosis of hematological cancers is a challenge due to the heterogeneity and many subgroups. The most commonly used method for diagnosis of these cancers is morphologic evaluation accompanied by some specific molecular markers. Gene expression profiling is based on the fact that only a small part of genes of the genome is expressed and transcribed into mRNA. The expression of mRNAs of a cell largely represents the protein repertoire making gene expression a great tool to visualize the biology of normal and malignant cells [85]. Since DLBCL subtypes have been traced to a particular stage of B-cell differentiation, these cells express different sets of genes. The first major micro array study of DLBCL was published in 2000. Alizadeh et al [62] examined whether the subgroups of DLBCL could be visualized using gene expression analysis. A genomic view of the full set of genes expressed by the subgroups would provide further understanding of the disease and also give clarity to the previously less clear morphological diagnosis. The study demonstrated that the DLBCL subgroups differed in the expression of hundreds of genes, which were related to separate stages of B-cell differentiation and activation. The study also highlighted that the two groups of DLBCL subtypes also differed in survival following treatment with chemotherapy. The GCB subtype had an overall better survival compared to the ABC subgroup. The study did not show which of the genes that might be responsible for the

chemotherapy responsiveness [62]. Figure 6 shows the gene expression profiles of the DLBCL subgroups. Molecular predictors of survival based on gene expression were later studied. Gene expression revealed that 17 genes might be used to predict overall survival of DLBCL patients following chemotherapy [63].

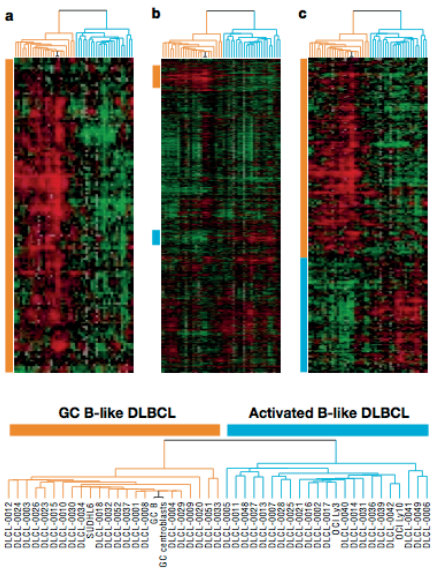

Figure 7. Expression profiling showing specific expression patterns of DLBCL subtypes. Reprinted with permission from Nature Publishing Group [62].

The studies described above all used fresh frozen (FF) biopsies from patients included in the studies. FF biopsies are generally limited in numbers, making gene expression studies difficult to perform. As a routine, tumor biopsies of patients are imbedded in paraffin and fixed with formalin for storage. FFPE tumor blocks can be sliced, put on glass to be analyzed by immunohistochemistry for pathology analysis. The FFPE blocks are available in large numbers making them easier to use for molecular research. During recent years, these FFPE blocks have evolved as a possible alternative to FF biopsies in gene expression. The major limitation of using FFPE material is that the mRNA is partially degraded due to embedding, fixation and storage. The whole genome gene expression analysis by Alizadeh et al using FF tissues was repeated using FFPE tissues. The study shows that the GCB and ABC DLBCL classification can also be verified using gene expression analysis. The study also showed that meaningful gene expression data were generated from RNA taken from FFPE DLBCL tissues [86].

4 STAT: SIGNAL TRANSDUCERS AND ACTIVATORS OF TRANSCRIPTION

4.1 Introduction

STAT are transcription factors that also have the function to transfer signals in cells. Receptors that span over the cell membrane (transmembrane receptors) sense the outside cell environment. When molecules outside the cells, named ligands, bind to receptors with high affinity, the cells can (through signal transduction) alter their metabolism in many different ways. STATs are very important factors in the transfer of signals from the membrane to the nucleus but also in activation of transcription of specific genes. An important part of the signaling pathway also includes the protein family Janus kinases (Jak) that are responsible for the activation of STAT to transfer the signal further to the nucleus. The Jaks are associated to the transmembrane receptor [87]. Since the Jak family and STAT family closely collaborate in signaling transduction, the entire pathway is often named the Jak-STAT pathway in the literature. In recent years, STAT signaling has been revealed to be important in cancer development and it is therefore of interest to understand their pathways and regulatory mechanisms.

4.2 Transcription factors

Gene expression is regulated by many different elements such as enhancers and silencers. Enhancers, together with associated transcription factors (TFs) are the most important players in the initiation of gene expression. TFs recognize DNA sequences with typically clusters of different TF binding sites leading to controlled and precise patterns of transcriptional activity. Since the discovery of miRNAs, these together with TFs are the largest family of gene regulatory elements sharing common regulatory properties [88, 89].

4.3 Family of signal transducers and activators of transcription

STAT proteins belong to a family of seven members: STAT1, 2, 3, 4, 5A, 5B and 6. Extracellular signaling peptides binding to specific receptors activate STATs that are latent in the cytoplasm prior to their activation. They become activated through phosphorylation by Jak proteins. They dimerize and enter the nucleus to regulate transcription of target genes. The STATs length range from 750-850 nucleotides where STAT2 and 6 are the longest and STAT1, 3, 4, 5A and 5B are the shortest ones. STATs get activated through more than 35 different polypeptide ligands and are therefore involved in a wide variety of biological events [90].

4.4 Structure and function

The general structure of the STAT proteins includes a tyrosine phosphorylation site, a phosphotyrosine-binding domain, a sequence specific DNA-binding domain, a C-terminal transcriptional activation domain (TAD) and several regions responsible for protein-

protein interactions. These sites are crucial for activation and dimerization, receptor recognition, DNA binding and protein interactions [91]. Figure 7 shows the general structure of a STAT protein. Among transcription factors, Stat proteins are unique containing a src-homology 2 (SH2) phosphotyrosine-binding domain. The domain has the ability to interact with tyrosine phosphorylation sites resulting in the recruitment of STATs to receptor complexes. After tyrosine phosphorylation, the dimerization of the STATs takes place between the SH2 domains and the phosphotyrosine-containing domain. The phosphorylation of STATs is essential for the dimerization and the nuclear translocation of the dimer. However, the STATs do not contain any obvious sequence dedicated to the nuclear translocation, and this process is largely unknown. Some has speculated that additional serine phosphorylation is important in the process but without any conclusive results [92].

Interferon (IFN)α, β and γ activate STAT1 which is important for the activation of macrophages in the defense response to pathogenic agents. STAT1 deficient mice are much more sensitive towards viral and microbial infections [93]. STAT2 is activated through IFNs and Interleukin (IL) 6 (IL-6) and does not bind DNA as homodimers as all the other STATs do. STAT2 is thought to have an important role during normal development [94]. STAT3 is activated by IL-6, IL-10, IL-11, IL-21 and IL-23 and other chemokines such as CNTF. STAT3 induces expression of a variety of genes [92, 95] and is very important during early embryogenesis and development [96]. STAT4 is activated through IL-12 and IFNs. Mice lacking STAT4 are defective during T-helper responses and in the production of IFNγ, but little is generally known about the transcriptional activity of STAT4 [97]. STAT5A and B are activated by several cytokines including prolactin, growth hormone, erythropoietin, thrombopoietin, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, IL-3, IL-5, IL-7, IL-9 and IL-15 and are involved in functions of cell growth regulation. STAT6 is activated through IL-4 and IL-13 and is functionally involved in the differentiation of T-helper cells and induces transcription of IgEs. Thus, STAT6 and STAT4 both have functions during T-helper responses [94-96]. In general, the STATs can be divided into two groups according to their function. STAT2, STAT4 and STAT6 are grouped together being activated by a small number of cytokines and their role in the development of T-cells and IFN signaling. STAT1, STAT3 and STAT5 on the other hand play important roles controlling cell-cycle progression and apoptosis. This group is therefore also associated to the development of cancer [94]. Three chromosomal regions are the locations for all STAT genes. STAT1 and STAT4 are localized on human chromosome 2, STAT2 and STAT6 are localized on human chromosome 12 and STAT3, STAT5A and STAT5B are localized on chromosome 17 (*www.genecards.org*) [92]. Even though STAT5A and STAT5B share >90% homology, they are encoded by two separate genes.

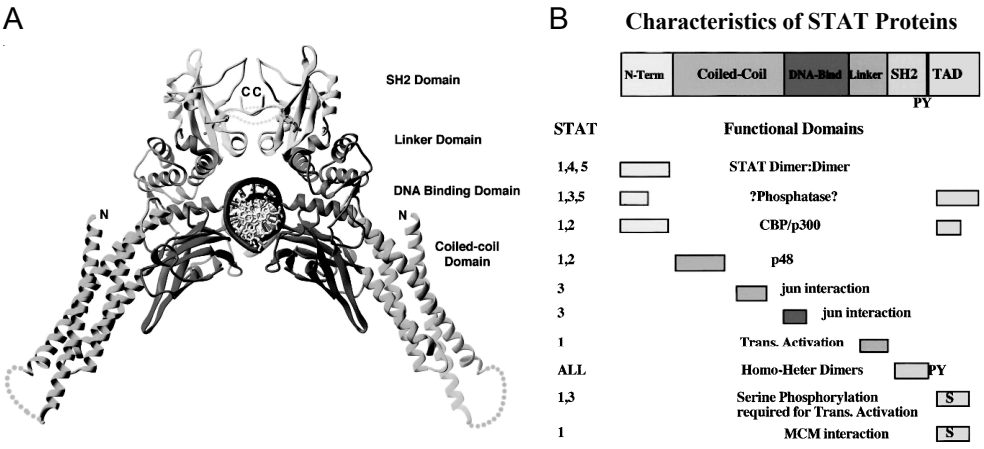

Figure 8. A. Crystal structure of STAT1 bound to DNA. B. Functional domains of STAT proteins. Reprinted picture with permission of Nature publishing group [98].

4.5 The role of stats in cancer

Activation of several STATs has been reported in a variety of human cell lines and primary tumors. These include malignancies of the blood (leukemia, multiple myeloma and lymphoma) and solid tumors of the neck, brain, breast, lung, prostate and the pancreas [99-103]. Tumorigenesis is a multistep process in which cells loose their ability to sense and repair DNA damage, regulate cell cycle progression and to regulate apoptosis. STATs play an important role in normal cells regulating differentiation, proliferation, apoptosis and angiogenesis. STATs are not known to be directly associated to cell cycle checkpoints and DNA repair, but abnormal activation of STAT signaling may give rise to oncogenesis through deregulation of signaling pathways associated to tumorigenesis such as growth factor signaling, apoptosis and angiogenesis [94, 99]. Table 3 shows the constitutive activation of STAT1, STAT3 and STAT5 in different tumors and cancer derived cell lines [99].

Inflammation associated with tumor initiation has during the recent years been in focus. Inflammatory cells mediated by cytokines and chemokines characterize cancer progression related to inflammation. The STAT family proteins have been discussed as factors maintaining a procarcinogenic inflammatory microenvironment, both at the initial stage of malignant transformation and during cancer progression. There is evidence for an association between inflammation, cancer progression and STAT3 [95, 104, 105].

Table 3. Activated STATs in cancer

Solid tumors	Activated STAT	Liquid tumors	Activated STAT
Breast cancer	1,3	Chronic myelogenous leukemia	5
Head and neck cancer	1,3	Acute myeloid leukemia	1,3,5
Prostate cancer	3	Chronic lymphocytic leukemia	1,3
Melanoma	3	Mycosis fungoides	3
Ovarian cancer	3	Acute lymphoblastic leukemia	1,5
Lung cancer	1,3	Erythroleukemia	1,5
Brain tumors	1,3	Burkitt lymphoma	3
Pancreatic cancer	3	Large granular lymphocyte leukemia	3
Renal carcinoma	3	Myeloma	3
		Hodgkin´s lymphoma	3
		Anaplastic large cell lymphoma	3

Adapted and modified from [99].

4.5.1 The role of STATs in DLBCL

Aberrant activation of the JAK/STAT pathway has been linked to Hodgkin's lymphoma and NHL, more specifically DLBCL. BCL-6 is a negative regulator of STAT3 in germinal center B-cells and constitutive activation of STAT3 represents an oncogenic pathway in ABC DLBCL [106]. STAT3 is also associated with the ABC subtype of DLBCL. Cases with higher expression of STAT3 have higher activity of the Nf-κB pathway. These observations have been confirmed by others [107]. All studies conclude that STAT3 plays an important role in proliferation and survival of the disease and that targeting STAT3 may lead to anti-lymphoma effects [106-109]. Another oncogenic pathway discussed for ABC DLBCL is associated to MYD88 mutations promoting Nf-κB and Jak-STAT3 signaling that mediates cell survival of ABC DLBCL cells [110].

4.6 STAT3

The functions of STAT3 have been difficult to define. Knockout studies in mice of STAT3 have shown that embryos die early in embryogenesis prior to gastrulation [96]. After ligand stimulation of a transmembrane receptor, STAT3 is phosphorylated on the tyrosine residue followed by dimerization and translocation to the nucleus to activate gene transcription [98]. More recently, unphosphorylated STAT3 (U-STAT3) has also been shown to be capable of controlling gene transcription [111]. Accumulating evidence suggest that STAT3 is activated in several tumors. The role of STAT3 in tumor formation is based on the link between the activated STAT3 and transformation. The STAT3 molecule by itself, when highly expressed, is able to transform cells [36]. STAT3 is thought to be associated to several genes. Examples of genes upregulated by STAT3 are BCL-X_L, MYC, BIRC5 (encoding survivin), MMP9, MMP2, CCND1, HIF1α, ICAM1, TWIST1, VIM (encoding vimentin), MCL1, HSP70, HSP90, IL-10, VEGF, FGF2, COX2, CXCL12, IL-11, Il23, IL-21, IL-17 and IL-6 [95].

4.6.1 STAT3 mediating inflammation associated cancer

Liver and gastric cancers arise through infections by HBV and HCV causing chronic hepatitis and Helicobacter pylori causing chronic gastritis respectively. The inflammatory bowel diseases Chron´s disease and Ulcerative colitis are also associated with an increased incidence of cancer. However, cancer types that do not have an inflammatory history do also involve hematopoietic-derived cells (cells that arise from hematopoietic stem cells with the multipotency to develop into any kind of blood cell) in different states of activation. This suggests that cancer and inflammation are linked together by environmental (extrinsic), epigenetic and genetic (intrinsic) events [31]. Since STAT3 is frequently activated in malignant cells and are capable of inducing a large number of genes that are crucial for inflammation, STAT3 signaling is a major intrinsic pathway for cancer inflammation [95].

4.7 STAT5

Deletion of STAT5A results in lack of prolactin-dependent mammary gland development. Deletion of both STAT5A and STAT5B in mice results in loss of other prolactin functions and the development of female fertility. The deletion of STAT5B alone and growth hormone receptor deficient mice share the same phenotype. These mice have reduced levels of insulin-like growth factor and reduced growth of males and females. These facts suggest that STAT5A and STAT5B are important for physiological functions mediated by growth hormone [92]. STAT5 is commonly found to be constitutively activated in leukemia and lymphoma [99]. Both persistently activated STAT5 and STAT6 have been shown to upregulate genes important for hematopoietic tumor survival and proliferation [95].

4.8 Negative regulators of STATs

STAT activation is a temporary process, which is terminated within hours after the activating signal. When not activated, STATs are latent in the cytoplasm awaiting activation. Some important negative regulators of STATs have been recognized. These include members of the suppressor of cytokine signaling (SOCS) family, phosphatases and enzymes [112]. Phosphatases in the nucleus dephosphorylate the STATs, which is an important signal for transportation out from the nucleus. Protein inhibitors of activated STATs (PIAS) family are nuclear proteins regulating tyrosine-phosphorylated STATs [113]. SOCS are the major regulators of STAT activity in the cytoplasm acting on JAK kinases [114].

4.9 Anticancer treatment targeting STATs

Many studies have recently discussed the opportunity to target STAT and STAT signaling in new anticancer treatments. STAT signaling inhibitors do not only block STAT function but may also increase the tumoral response to chemotherapy and radiotherapy. Failure of commonly used treatments may be because constitutive activated STATs inhibiting apoptosis [94].

When selecting targets for new therapeutic development, several requirements must be met. First, the selected target must be expressed or regulated differently comparing malignant to normal cells. Secondly, there must be an opportunity to manipulate the target reversing the abnormality. Thirdly, the modulation must not cause unnecessary toxicity in normal cells. STATs seem to fulfill each of the above criteria [115]. The discussed therapy may be targeted against different levels of the STAT pathway.

Targeting of receptors: Targets may be antagonists of cytokines or growth factors that activate STAT signaling. The molecules are similar in structure with the ligand but with a stronger affinity to the receptor. These would then not bring out any STAT activation. Sant7 is an antagonist of IL-6, which has the ability to inhibit tumor growth by blocking STAT3 in myeloma cells [116]. Recent studies show that STAT3 also may be activated through a G-protein-coupled receptor called sphingosine-1-phosphate receptor 1 (S1PR1) through JAK2 kinase activity [117]. The ligand to the receptor S1P has an analog FTY720 that binds the receptor with higher affinity than S1P [118]. Using TFY720, STAT3 expression may be inhibited leading to tumor cell growth arrest and apoptosis in ABC DLBCL [119].

Targeting of kinases: STAT activation is dependent on kinases for their activity. Tyrosine-kinases such as JAKs and serine-kinases would therefore be possible targets for disrupting STAT functions. AG490, a JAK2 inhibitor has been shown to induce cellular growth arrest and apoptosis in leukemias and in different tumor cells by blocking the STAT3 signal [120]. Serine phosphorylation does not activate STATs, it may influence the growth and survival characteristics of a cell. This has been shown to be important for the biology of CLL [121, 122].

Targeting dimerization of STATs: The dimerization of STATs is necessary for nuclear transportation and transcriptional activity (the DNA binding). The SH2 domain of one STAT molecule can form dimers through interaction with a phosphorylated tyrosine on the other STAT molecule. Inhibition of the SH2 domain would interrupt the dimerization process. Artificial compounds with high affinity for STAT monomers could inhibit the dimerization. This approach has been used for STAT3 inhibiting the phosphorylation, dimerization and DNA binding activity [122].

Targeting DNA binding: Inhibition of STATs binding to a target sequence in a promoter region would be a practical approach inhibiting the transcriptional activity of STATs. The binding sites of the STAT molecule could be targeted with small molecules. It would also be possible to introduce short stretches of double stranded DNA that mimics the target sequence. Molecular excess of these stretches would make activated STATs to bind to them instead of the real target sequence.

Targeting dominant inhibitory STATs: The use of nonfunctional STATs that still have the ability to form dimers with endogenous STATs. The dimer would then be functionally inactive suppressing the overall STAT activity.

Other processes have also been discussed as possible targets to inhibit STAT functions. These are the nuclear translocation process that we know very little about. The identification of molecules that imitate the functions of proteins naturally inhibiting the Jak-STAT pathway as SOCS and PIAS proteins is also a possible process to target as well as development of therapeutic antibodies [94, 121]. The use of short hairpin RNA (shRNA) has also been used to directly target STAT3 in ABC DLBCL patients. Small interfering RNA (siRNA)-based gene therapy is a feasible approach compared to targeting upstream STAT3 signaling [107]. This is an important area of research since STATs are shown to be involved in many types of malignant processes.

5 MicroRNAs

5.1 Introduction

RNA was long thought to simply be a molecule mediating the process of protein generation from genes, a process known to be a part of The Central Dogma: DNA makes RNA makes protein. This hypothesis has been challenged by the discovery of several classes of noncoding RNAs (ncRNA) regulating the most important levels of genome functions as transcription, translation, RNA processing and RNA stability [123]. The first miRNA discovered was called lin-4 which negatively regulated lin-14 by binding to the 3´untranslated region (3´UTR) of the lin-14 gene. These discoveries were made in *Caernorhabditis elegans* (*C. elegans*) [124]. Following lin-4 discovery, another miRNA in *C. elegans* was discovered called let-7. It was shown to negatively regulate lin-41 in the same regulatory manors as for lin-4 [125]. Lin-4 and Let-7 were initially called short temporal RNAs (stRNAs) but was renamed miRNA – a collective name for all small RNAs with similar features, but with unknown functions [126]. Since the original discovery, approximately 940 human miRNA have been identified and miRNAs have evolved to be the major gene regulatory molecules in animals [127].

5.2 Biogenesis of miRNAs

miRNA are derived from hairpin structures and are ~22 nucleotides (nt) long endogenous RNAs with important gene regulatory activity. They bind their complementary target sequence regulating transcription [126, 128]. The binding to the target (usually in the 3´UTR region) leads to regulation such as repression and destabilization, the most common types of regulation [129]. However, miRNA also has the ability to activate translational and heterochromatin formation [130]. Targets of miRNA can be predicted through searching for conserved parts of the miRNA called the seed region. This region is found in the 5´region of the miRNA and is complementary to the 3´region of the target mRNA [131]. miRNAs may be located in the non coding regions between protein coding genes (intergenic) or in the non coding sequence of specific genes (intronic). In general, transcription of genomic protein coding DNA generates precursor mRNA (pre-mRNA) with a 5´UTR region, a protein coding exon, a non-coding intron and a 3`UTR region. The introns were long believed to be genetic waste until the discovery of intronic miRNAs [132].

The complex process of miRNA biogenesis involves several steps. Initially, it was believed that miRNAs were transcribed by RNA Polymerase III, but it is now known that the majority of miRNA genes are transcribed by Polymerase II (Pol II) while some require Polymerase III (Pol III) [133, 134]. In the nucleus, miRNAs are transcribed as long primary transcripts (pri-miRNA) by the Pol II/III. The transcripts are typically several kilo bases long with stem loop structures that serve as substrates for cleavage. The RNase III endonuclease Drosha with cofactor DGRC8 cleaves the pri-miRNA into a miRNA precursor (pre-miRNA). The pri-miRNA 5´ and 3´ arms are cleaved leaving the stem-loop intermediate pre-miRNA [135, 136]. The pre-miRNA processed in the nucleus is exported to the cytoplasm by a nuclear transport receptor exportin-5 binding to double-stranded RNA (dsRNA) [137]. The RNAse III endonuclease Dicer cleaves the pre-miRNA near the loop

resulting in ~22 nt miRNA duplexes [138]. The miRNA duplexes are loaded into the miRNA-induced silencing complex (miRISC) where one of the strands becomes the mature miRNA while the other is degraded [139] (Figure 8).

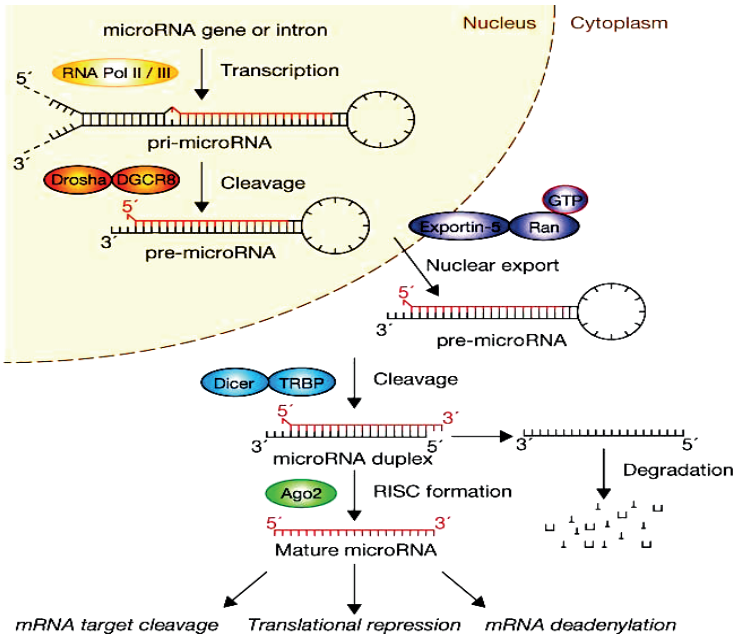

Figure 9. miRNA biogenesis from nuclear processing to mature miRNA in the cytoplasm regulating mRNA. Reprinted with permission from Nature Publishing Group [140].

5.3 Function of miRNAs

The mature miRNA, still bound to the RISC complex, is guided to the target gene by sequence complementarity between the miRNA and its target. The complex binds to the target sites, usually within the 3´UTR region of the target gene [127, 141]. Perfect interaction between the seed region of the miRNA and the mRNA is the basic requirement for pairing. The seed region is counted between the nt 2-7 to the 5´end. Pairing of the 3´end of the miRNA might enhance the interaction and may also compensate for mismatches in the seed region [126]. The functional outcome of the miRNA-mRNA interaction seems to be dependent on the degree of complementarity. Imperfect pairing leads to repression of translation and destabilization of the target while perfect pairing allows for cleavage of the mRNA target. miRNA dependent cleavage is rarely observed [126, 142]. Some miRNAs have also been shown to bind to the 5´UTR region of the target mRNA. These interactions also results in repression of the mRNA, making 5´UTR biding as sufficient as 3´UTR binding [143]. It was also shown that some miRNA binding to the 5´UTR region enhanced the translation of the target gene [144].

5.4 miRNAs in cancer

Since the discovery of miRNAs, extensive research worldwide has tried to reveal their role in cancer. miRNAs are involved in cancer by targeting oncogenes and tumor suppressors having important roles in regulation of cancer stem cell biology, angiogenesis, metastasis and drug resistance. We are still in the beginning of understanding the complex miRNA mechanisms in cancer and improved understanding would give us invaluable information about key pathways, diagnostics and prognosis [145]. The first report of miRNA involvement in cancer in 2002, showed that the gene cluster with miR-15 and miR-16 was deleted in patients with CLL [146]. Further studies of miR-15 and miR-16 have suggested these to acts as tumor suppressors [147].

The current research of miRNAs associated to cancer is mainly based on gene expression profiling comparing cancer cells to normal cells. Up- and/or downregulation of miRNA of interest is a useful approach to study miRNA functions. There are several approaches to conduct these studies. One example is blocking the expression of the target miRNA using antisence inhibitors that bind to the miRNA blocking its activity. Another method introduces point mutations to the seed sequence decreasing the gene regulation function [148].

5.4.1 miRNAs as oncogenes and tumor suppressors

As mentioned above, miR-15 and 16 were the first miRNAs to be classified to act as tumor suppressors [147]. miR-21 on the other hand is an example of an oncogenic miRNA (oncomir) being overexpressed in many types of cancers causing tumor growth, maintenance and survival in vivo [149-152]. miR-21 target tumor suppressor genes such as PTEN leading to increase of proliferation and to decrease of apoptosis [153, 154]. Additionally, tumor suppressor proteins and oncoproteins are thought to modify miRNA expression. The proto-oncogene MYC activates transcription of the miR-17-19 cluster leading to inhibition of PTEN, which results in increased cancer cell survival [155, 156]. The expression of the Let-7 family is often reduced in many cancers, which is correlated with poor survival. The MYC gene is one of the suppressing genes of the Let-7 family targeting the enzyme RAS involved in cell growth, differentiation and survival [148, 157] (Figure 9).

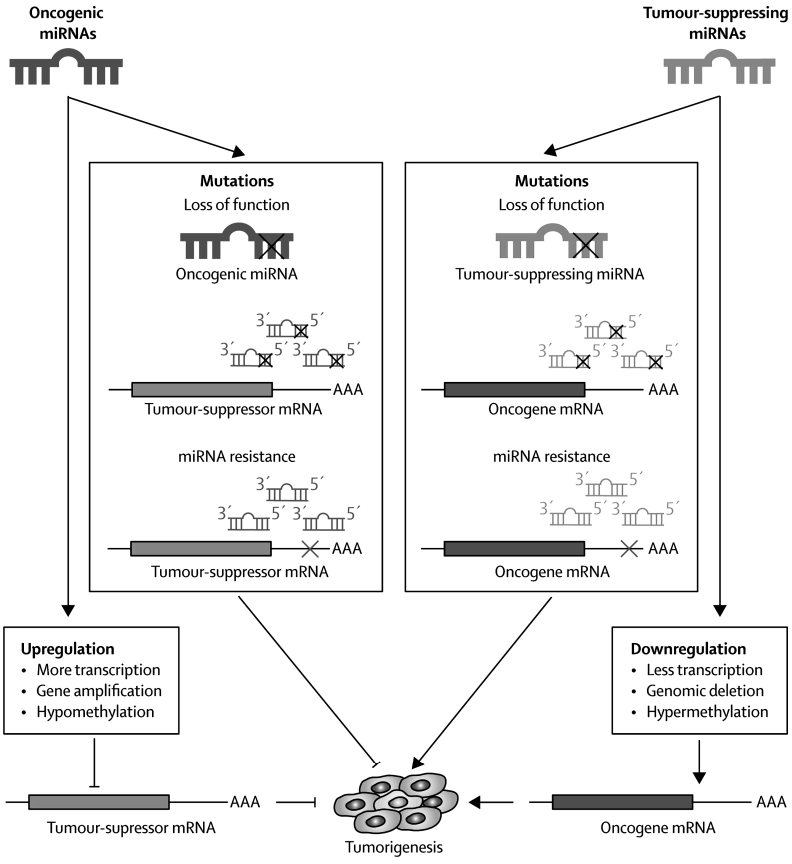

Figure 10. Figure describing how oncogenic and tumor suppressive miRNAs regulate cancer development. Reprinted picture with permission from Elsevier [145].

5.4.2 miRNAs in B-cell lymphoma

The recent identification of specific miRNAs suggests that they are possible to use to refine the current B-cell lymphoma classification, prognosis and diagnosis. Since Calin et al [146] published data on the association between miRNA expression and lymphoid malignancies in 2002, many studies have explored their potential role as diagnostic, prognostic and predictor markers [158]. Some miRNAs have been shown to play an important role in different steps of B-cell differentiation. For example, the cluster of miR-23a (miR-23a, miR-27a, miR-24) and miR-123b reduce B-cell lymphocyte lineage to favor myeloid differentiation. miR-150 seems to introduce blocking of the maturation process at the pro-B-cell stage and the miR-17-92 cluster has been shown to regulate survival of early B-cell progenitors. Finally, miR34a regulates B-cell differentiation from pro-B-cell to pre-B-cell and miR-155 and miR-181b controls germinal center reaction. miR-155 more specifically negatively regulates CD10, a marker for germinal center cells [159-166].

In DLBCL, a significantly higher level of miR-155 has been shown for the non-germinal center subtype compared to the germinal center subtype and other miRNAs have been added to the list that distinguishes between the DLBCL subtypes [167]. It has been demonstrated that miR-125b plays an important part in the germinal center reaction and post-germinal center differentiation downregulating IRF4 and PRDM1/BLIMP1. The miR-125b expression was higher in germinal center centroblasts than in memory B-cells. The 3´UTR region of IRF4 and PRDM1 contains complementary binding sites for miR-125b and may therefore repress their expression. The study also identified 9 miRNAs whose expression was different between the DLBCL subtypes, in cell lines. In DLBCL patients, a higher expression of miR-222 correlated with shorter overall survival (OS) and progressive free survival (PFS) noticed in ABC- like subtype [168]. Other studies have also demonstrated that miR-222, miR-18a and miR-181a can predict OS and PFS in R-CHOP treated DLBCL patients [169]. Other miRNA signatures have also been recognized in other types of B-cell lymphomas. For example, in MCL, miR-16-1, oncomiR-1 and miR-29 target genes controlling the cell cycle such as Cyclin D and CDK6 [170-172].

5.5 miRNAs and STATs

STATs are involved in many cellular processes and it is therefore assumed that STAT pathways are under great regulatory pressure. miR-155 has been associated to the expression of STAT1 [173, 174]. Expression of miR-155 in human hepatocellular carcinoma cells (Hep G2 cells) results in increased phosphorylation of STAT1 and STAT3 but also to suppression of SOCS1 [174]. Conversely, STAT1 may also regulate miR-155 creating a positive feedback loop of miR-155 and STAT1 [173]. miR-221, miR-222 and miR-145 interacts with both STAT1 and STAT2 but are differently expressed in tumors. miR-221 and miR-222 have been shown to be upregulated in cancer types as glioblastoma and human thyroid papillary carcinomas while miR-145 has been demonstrated to be downregulated in prostate cancer, colon cancer, bladder cancer, ovarian cancer and B-cell malignancies [175-177]. STAT3 is regulated by the miR-17 cluster family members consisting of miR-17-5p, miR-17-3p, miR18a, miR-19a, miR-20a, miR-19b and miR92-1 as well as 2 paralog clusters (miR-106b-25 and miR-106a-363). The miR-17-92 cluster is also known as OncomiR-1 for its ability to promote tumorigenesis [166, 178-183]. The most studied miR/STAT interaction is the miR-21/STAT3 pathway. STAT3 directly activates miR-21, which is upregulated by IL-6 and therefore STAT3 dependent. Downregulation of STAT3 prevents IL-6 mediated upregulation of miR-21. IL-6 activation of STAT3 also promotes miR-181b-1 expression. miR-21 and miR-181b-1 target tumor suppressor genes making them act as oncomiRs[184, 185]. Let-7a also directly targets STAT3 in Hep G2 cells and it has been shown that the Hepatitis B virus X protein (HBx) downregulates let-7a leading to increase in STAT3 and further proliferation of HBV infected cells. Therefore, let-7 may be used to target STAT3 in cancer to reduce tumor cell proliferation [186]. miR-222 has also been reported to target STAT5a [187].

6 AIMS

6.1 General structure of this thesis

This thesis is based on four studies. The first part was a methodology study describing methods for gene expression profiling using FFPE tissues. The proceeding three studies were based on these methods addressing the questions if ABC DLBCL could be associated to environmental factors. Two patient populations were selected from two different areas of the world with a various environmental factor exposure, Sweden and Egypt.

6.2 General aim of this thesis

The general aim of the thesis was to analyze molecular processes in ABC DLBCL and if environmental factors with a focus on microbial agents may be associated to the dysregulated processes.

6.3 Study I

The aim of Study I was to evaluate the use of FFPE tumor material for gene expression analysis as microarray and qPCR.

6.4 Study II

The aim of Study II was to compare gene expression in two groups of ABC DLBCL patients exposed to different environmental factors that may be associated to the development of DLBCL.

6.5 Study III

The aim of Study III was to analyze miRNA expression patterns of ABC DLBCL patients exposed to different environmental factors, in which miRNA may be important molecular regulators.

6.6 Study IV

The aim of Study IV was to analyze microbial agents in ABC DLBCL patients exposed to different environments, which may be associated to the development of ABC DLBCL.

7 MATERIAL AND METHODS

7.1 Patient material

All patients included in the thesis were collected from National Cancer Institute, Cairo, Egypt and Karolinska University Hospital, Stockholm, Sweden. In Study I, NSCLC patient tissue samples were collected from the Department of Pathology at Karolinska University Hospital. For Study II, III and IV the patient materials from Egypt and Sweden was collected and characterized at the Department of Pathology at Karolinska University Hospital and at the National Cancer Institute, Cairo, Egypt. The patient material was evaluated using standard criteria. The International Prognostic Index (IPI) was applied [188]. In general, R-CHOP treatment regimens were given as first-line therapy, as previously described [189]. All control material used in the studies was reactive lymph nodes received from the National Cancer Institute, Cairo, Egypt and Karolinska University Hospital, Stockholm, Sweden. These were surgically removed from patients in whom the histopathological diagnosis was a non-malignant disease. The primary selection of patient samples were done with eosin/hematoxylin staining as well as immunohistochemistry using antibodies against CD20, CD3, Ki67, bcl-2, bcl-6, CD10 and MUM1 as described earlier [67].

7.2 Quantitative PCR

Polymerase chain reaction (PCR) was originally developed in 1983 by Kari Mullis [190] and was rewarded with the Nobel Prize in chemistry 1993. The method qPCR was used to quantify gene expression in real time during the PCR reaction. mRNA is reversed transcribed into cDNA, amplified in the presence of primers specific for the gene of interest, a DNA-dependent DNA polymerase (Taq Polymerase), dNTPs and a DNA-binding reporter dye. The technique of qPCR is based on improvements of the original PCR technology [191]. A gene specific probe was labeled in each end with flourecsent dyes-one reporter and one quencher. When intact, the quencher inhibits florescence of the reporter. The probe binds DNA and gets degraded by the polymerase machinery during amplification resulting in increased fluorescence by the reporter detected by the instrument used. There were two different methods: absolute quantification and relative quantification. Absolute quantification determines the input copy number of a transcript using a standard curve while relative quantification describes the change in the expression relative to a reference. In the studies of this thesis, TaqMan chemistry (www.appliedbiosystems.com) was used and the data was analyzed using relative expression analysis based on the $2^{-\Delta\Delta C}{}_T$ method [192]. The analysis was based on threshold cycle C_T, which is a measure of the PCR cycle when the fluorescence passes a specific threshold. In Study III, poly-A tailing was used on total mRNA samples followed by cDNA synthesis and qPCR analysis with miRNA specific primers.

7.3 Microarray

Microarray techniques are based on the ability to measure expression of many genes simultaneously. The use of expression profiling was published as early as 1995 [193] and has been developed since then to be used for several applications. One of the most commonly used microarray is the complementary DNA (cDNA) array, based on cDNA clones or oligonucleotides (probes) spotted on glass slides (chip). Each probe is representative for a certain gene or transcript. There are several probes for one gene as well as mismatch probes attached to a small area of the chip. The expression is measured based on signal intensities of the probes and the mismatch probes to determine the specificity of the measured signal. For the studies in this thesis, Affymetrix Human Gene Chip U133 Plus 2.0 and GeneChip miRNA array was used (www.affymetrix.com).

7.4 Microbial detection array

The Lawrence Livermore Microbial Detection Array (LLMDA) used in Study IV was developed by a group in at the Lawrence Livermore National Laboratory [194]. The MDA is an oligonucleotide array with high probe density. The array contains target probes for all sequenced bacteria and viruses. The probe lengths are 50-60nt long. There are two categories of probes; census and discovery probes. Census probes target regions unique to an individual species or strain used to specify an organism as precisely as possible. Discovery probes are optimized for detection of novel species within a known family [195]. The array has been used for clinical samples detecting both RNA and DNA viruses [196].

7.5 Ingenuity pathway analysis

Ingenuity pathway analysis (IPA) (www.ingenuity.com) is a software for analysis of complex gene expression data putting findings into a biological context. It may be used to identify signaling pathways, molecular networks and biological processes but also to build custom networks of up- and downregulated genes in the data set. The Ingenuity Knowledge Base is a library based on published data of functional relationships between genes, cells, tissues, drugs and diseases. The library is the basis of the IPA analysis tool. IPA was used in Study II in this thesis analyzing the microarray expression data.

7.6 Confocal microscopy

Confocal microscopy initially developed by Marvin Minsky [197] allows for imaging deeper inside biological tissues compared to fluorescent microscopy. A main advantage of confocal microscopy is the ability to view three-dimensional structures. Confocal microscopy was used in Study II looking for the protein expression of Stat3 and Stat5b in tissue sections of ABC DLBCL patients.

7.7 Western blot

Western blot is a method to analyze proteins using gel electrophoresis to separate proteins followed by transfer to a membrane and antibody staining with specific antibodies (blotting). Western blot was used in Study III to detect the difference in Stat3 expression after treating cells with miRNA mimic.

7.8 Serology and antigen detection

In serum of patients, antibodies against a specific microbial antigenic epitope may be detected. After an infection by a virus, the body may respond with an immune reaction by producing antibodies. Prior to antibody production, the antigens may also be detectable in serum. Antigen detection reflects an active infection while antibody detection reflects that an immune response has been developed. We used antigen detection in Study IV to detect HBV antigens against HBV in Egyptian ABC DLBCL patients.

8 RESULTS AND DISCUSSION

8.1 Study I

Gene expression analysis using long-term preserved formalin fixed and paraffin-embedded tissue of non-small cell lung cancer

The molecular understanding of diseases has advanced rapidly due to the use of molecular profiling techniques as qPCR and microarrays. qPCR is known to be specific generating valuable data from clinical specimens. The major limitation of qPCR is that it cannot be used to measure many genes simultaneously. Microarray technology, on the other hand may measure a great amount of genes simultaneously but with less specificity compared to qPCR [198]. Until recently, the gene expression methods were limited to frozen samples that are difficult to get in sufficient amounts for research purposes. However, FFPE specimens are available in great numbers making them an invaluable resource for molecular studies of diseases. The major disadvantage of using FFPE specimens is chemical modifications of RNA as cross-linking and partial degradation but technological developments during recent years have made it possible to use RNA extracted from FFPE specimens [199, 200].

There is a rising demand of biologic materials from large patient populations. Biobanks are established collections of biological material as tissues, blood or other body fluids from patients and healthy individuals. Tissue based banks contains a large number of FFPE tissues whereas FF tissues are limited in quantity. The recent development of high-throughput technologies such as gene expression using microarrays makes national and international biobanks invaluable sources of tissues. The use of FFPE tissues in such technologies has been underestimated. There is accumulating evidence that FFPE tissues may be used for high-throughput technologies enabling large retrospective and follow-up studies of various diseases [201].

In Study I we evaluated the use of FFPE tissues in RNA extraction, qPCR and microarray analysis. High quality RNA and cDNA were obtained from all FFPE and FF RNA extraction kits used. The input amount (20ng vs. 100ng) for qPCR was analyzed using endogenous control genes. A significant difference in C_T values was found for all FFPE kits comparing 20 and 100ng as input cDNA. Using 100ng of input cDNA was shown to give lower C_T values. The amount of input cDNA was also analyzed between the FFPE kits, which showed a significant difference in C_T values of 100ng of cDNA input comparing Ambion and Roche (33.07±3.04 vs. 34.76±3.46, $p<0.005$) and Ambion and Qiagen (33.07±3.04 vs. 34.3±2.9, $p<0.005$). There was no significant difference using 20ng of cDNA. The results show that the Ambion FFPE RNA extraction kit (using 100ng input cDNA) gave the most reliable qPCR results and it was therefore the most suitable kit for RNA extraction of long time stored FFPE tissues.

RNA extracted using the Ambion FFPE RNA extraction kit was further used in microarray analysis to compare gene expression between FF and FFPE tissue. The gene expression analysis showed a higher variation of intensity values using FFPE tissue compared to using FF tissue. This was expected due to the higher grade of RNA degradation in the FFPE tissue. FFPE tissue presented a higher frequency of 2-, 5- and 10-

fold up- or downregulated genes compared to FF tissue. Of all genes identified using FFPE tissue, 55% were at least 2-fold up or downregulated compared to FF tissue. The higher frequency of up-or downregulated genes using FFPE tissue may be due to a lower specificity compared to using FF tissue.

To compare specific gene expression data between FFPE and FF tissues, 16 endogenous control genes were selected. A high correlation for the endogenous control genes was noted comparing FF with FFPE tissue. The 16 endogenous genes were also compared using qPCR revealing that fold expression of 14 out of 16 genes were comparable in qPCR and microarray, yielding an 88% confirmation rate. We demonstrate a good concordance comparing the global gene profiles using FF and FFPE tissues. Correlation of expression comparing FFPE and FF tissues also showed a high degree of specificity, i.e. the expression of genes detected in FFPE tissue could also be detected in FF tissue. However, the overall sensitivity of gene expression was lower in FFPE tissue. We also wanted to verify our gene expression signatures in concordance with earlier publications describing gene expression of NSCLC. We used data from two previous publications [202, 203]. Fifteen common genes in the two publications were selected. All 15 genes could be identified in our data set with similar expression levels except for two genes.

In summary, Study I suggest that FFPE tissues could be used for molecular analysis as qPCR and microarray. The Ambion FFPE RNA extraction kit was shown to give the best quality RNA. Using FFPE specimens in a larger extent opens up new opportunities for genetic research making it possible to perform large retrospective studies. Based on the results from Study I, the methods presented was used throughout the studies of the thesis.

8.2 Study II

Patients with activated B-cell like diffuse large B-cell lymphoma in high and low infectious disease areas have different inflammatory gene signatures

Different regions of the world have varying prevalence of endemic microbial infections [9]. It should therefore be of interest to study tumors associated with microbial infections originating from regions with different infectious prevalence. In 2002, it was estimated that 18% of neoplasms are associated with infections, ranging from less that 10% in western countries to 25% in African countries [3, 9]. It is also estimated that our lifestyle and the environment accounts for 90-95% of most chronic illnesses. Environmental factors that play an important role in the development of cancer are tobacco, diet, radiation and infectious organisms [3]. These factors have also been shown to be involved in lymphomagenesis [204].

In Study II, two groups of ABC DLBCL patients originating from geographical regions that differ in exposure to environmental factors were studied on a molecular level using gene expression analysis. The two patient groups were from Sweden and Egypt. Global gene expression microarray analysis was done to compare the gene expression profiles. Three thousand and forty-seven genes were differentially expressed between the groups: 1827 genes were upregulated and 1420 were downregulated (>2-fold up- or downregulation). The IPA analysis grouped the genes into biological functions as cell cycle control, cell morphology, cellular development, cellular growth and proliferation that are all associated to cancer development and maintenance of tumor growth. The analysis also showed the genes to be involved in 20 canonical pathways. The majority of these were involved in cytokine and growth factor signaling. STAT family members were involved in 13 of the 20 pathways. More specifically, this analysis revealed that STAT3 and STAT5b were highly differently expressed between the patient groups. STAT3 was 16 fold upregulated in Swedish patients compared to Egyptian patients while STAT5b was 30 fold upregulated in Egyptian patients compared to Swedish patients. The difference in STAT3 and STAT5b gene expression was verified using qPCR. The expression of STAT3 and STAT5b were also evaluated in comparison to control material indicating that the expression pattern of STAT3 in patients and controls were more distinct compared to the expression patterns of STAT5b.

In addition to STAT3 and STAT5b, we also analyzed the ROR1 expression in the patient groups. ROR1 was expressed in 61% of the Egyptian patients and in 81% of the Swedish patients ($p<0.05$). ROR1 was also upregulated two-fold in Swedish compared to the Egyptian patients. The receptor tyrosine kinase ROR1 is a known survival factor in malignant cells and has been shown to be upregulated in malignancies as CLL [205, 206]. ROR1 expression has been shown to be upregulated by STAT3 binding to the ROR promoter region [207] linking ROR1 and STAT3 expression. Our results are in line with these results as ROR1 was upregulated and more frequently expressed among Swedish compared to Egyptian patients.

To visualize the STAT3 and STAT5b protein expression, tissue sections were analyzed using confocal microscopy. A higher STAT3 staining intensity was estimated both in the

cytoplasm and in the nucleus of Swedish patients compared to Swedish controls and Egyptian patients. STAT5b staining intensity was higher in Egyptian patients compared to Egyptian controls and Swedish patients. The overall intensity was estimated to be higher for STAT3 when compared to STAT5b. These results show that STAT3 and STAT5b also differed on the protein expression level.

The seven members of the STAT family of transcription factors are important players of signaling pathways activating differentiation, proliferation, apoptosis, angiogenesis and inflammation [90]. STAT1, STAT3 and STAT5 are generally grouped together based on functions as controlling cell cycle progression and apoptosis. These three STATs are thought to be associated to oncogenesis [94]. Several studies have shown overexpression of STAT3 and STAT5 in different cancer types as prostate cancer and cervical carcinoma [208-210]. However, no study has shown differences in STAT expression among patients originating from different geographical regions. The results of Study II might be of importance for anti-cancer therapy targeting the activity of STATs [115]. Indirect targeting of upstream signaling components as well as direct targeting of STATs has been discussed. Recent studies have suggested that inhibition of STAT3 through shRNA may be an effective therapeutic strategy in ABC DLBCL [107].

In summary, Study II demonstrates a difference in the expression profiles of genes that may be important for the development of ABC DLBCL. The difference of STAT expression between the patient groups might be associated to different environmental factors as microbial agents.

8.3 Study III

Expression of miRNA-1234 related STAT3 in patients with Diffuse Large B-cell Lymphoma (DLBCL) from high and low infectious disease areas

The difference in STAT expression revealed in Study II comparing Swedish and Egyptian ABC DLBCL patients may be due to the difference in environmental backgrounds. Furthermore, miRNAs may be important regulators in a wide variety of biological processes including cell proliferation, differentiation, apoptosis and signal transduction [211] and also in tumorigenesis. In Study III, we wanted to investigate the difference in miRNA expression of patients originating from different environmental backgrounds and the regulation of STAT3 by miRNAs.

It is estimated that approximately one third of human mRNAs are regulated by miRNAs and each one may have multiple targets and each target may be regulated by multiple miRNAs [212]. Accumulating evidence suggest that disruption of miRNA expression correlates with human malignancies including breast cancer [150], ovarian cancer [213], cervical cancer [214], prostate cancer [215], colorectal cancer [216], gastric cancer [217], nasopharyngeal carcinoma [218], lung cancer [219], renal cancer [220] and hematopoietic malignancies [221]. Studies have shown that miRNAs are deleted as well as having altered expression in hematological malignancies such as CLL and DLBCL [146, 222-224]. miRNAs have also been associated to viral infections and inflammation [127, 225]. miRNAs have been identified in almost all studied multicellular eukaryotes in the plant and animal kingdoms and recent data suggest that miRNAs can be encoded by viruses [226]. Since miRNAs seems to play an important regulatory role in many cellular processes, it would not be surprising to find viruses to interact with these pathways, modulating the host environment. HBV, Kaposi´s sarcoma herpes virus (KSHV) and HIV infections have been shown to be associated to various miRNAs. Viruses such as herpes, encode their own miRNAs, which regulate the expression of cellular proteins or viral proteins might lead to a facilitated infection cycle [212].

The miRNA expression patterns of the Swedish and Egyptian ABC DLBCL patients were analyzed using miRNA microarray and revealed 848 human miRNAs. A difference in miRNA expression patterns was shown between the two groups. Twenty-one miRNAs (at least 2 fold up- or down regulated) were differently expressed in the two patient groups. Nine miRNAs were downregulated and 12 upregulated in the Swedish DLBCL samples by comparison to Egyptian samples. To analyze the possible target interactions of these miRNAs, an In Silico target analysis using the database miRWalk was used. Based on our previous finding of differences in STAT3 activation between the two groups, the target analysis was limited to genes involved in the STAT3 pathway. Six out of the 17 miRNAs were predicted to have STAT3 as a specific target and were further analyzed based on the level of intensity, significance and detection. miR-1234 was selected for further analysis using qPCR and Western blot to detect its possible regulatory function of STAT3 expression.

The expression levels of miR-1234 were analyzed using qPCR. The relative expression of miR-1234 was significantly higher in Egyptian patients compared to Swedish patients

($p<0.03$). The ratio of miR-1234 and STAT3 expression was also calculated showing that the samples from the Swedish patients had a high expression of STAT3 but low of miR-1234. The opposite was true for Egyptian patients. The results indicated a relation between the STAT3 and miR-1234 expression. Using Western blot, we investigated downregulation of the Stat3 protein in STAT3 expressing cells transfected with a miR-1234 mimic. Downregulation of Stat3 protein could be visualized indicating that miR-1234 targets STAT3 inducing a reduced level of Stat3 protein expression.

miR-1234 is located on chromosome 8, is 22 nucleotides long and is located in the CPSF1 gene sequence. miR-1234 may regulate the transcription factor Six1 involved in the progression and metastasis of breast cancer [227].

In summary, Study III showed a different miRNA expression profile in ABC DLBCL patients with a different environmental background exposure. miR-1234 was shown to be upregulated in Egyptian patients and predicted to specifically target STAT3. The novel finding of miR-1234 in DLBCL may indicate that this miRNA regulates STAT3. Both miRNA and STAT signaling might be associated to the tumorigenesis and the different environmental background exposure.

8.4 Study IV

Differential expression of viral agents in lymphoma tissues of patients with ABC Diffuse Large B-cell Lymphoma from high and low endemic infectious disease regions

Environmental factors as infectious organisms play an important role in the development of cancer [3]. Several viruses have been classified as infectious agents causing cancer as EBV, HTLV-1, herpes virus-8 and hepatitis [9]. Since the introduction of vaccines against HPV and HBV, the incidence of cervical cancer and HCC respectively has decreased [13]. However, not all infections by an oncogenic virus lead to cancer development. The viral infection might be necessary but not sufficient. The involvement of other factors for the transformation is important for the accumulated genetic and epigenetic changes. Transformation may be stimulated by viral infections in combination with cytokines and growth factors. Co-infections of other pathogens may also play a role in certain virus-related cancers [13].

In Study IV, we analyzed the detection of viral and microbial agents in the lymphoma tissues of ABC DLBCL patients from Sweden and Egypt. The LLMDA technique was used to identify viruses in the tissues and standard techniques for antigen detection in serum were also used. The expression of STAT3 and miR-1234 was related to the LLMDA results.

The LLMDA results were analyzed using the maximum likelihood analysis method [194] generating a log-odd score for each target. Different viral expression patterns in lymphoma tissues were noted comparing Swedish and Egyptian patients. HBV had the highest log-odd scores including the complete genome and was only found in Egyptian patients. JCV was found both in Egyptian and Swedish patients but with a higher log-odd score in the Egyptian patients while not detected in Swedish and Egyptian controls. Generally, the highest log-odd scores were found in Egyptian patients compared to Swedish patients and controls from both countries. Few viruses were found in the control groups. HBV antigens were detected in the serum of 38% of the Egyptian patients but not in any of the Swedish patients. According to previous studies, the prevalence of the common HBV antigen (HBsAg) in the Egyptian population is 4% [228] and in Swedish <1% [229]. All Swedish patients were HBsAg negative. Based on literature data, we calculated that the difference between the Egyptian DLBCL patients and the general Egyptian population (with regard to the presence of HBV antigen) was statistically significant ($p < 0.05$).

Based on serum HBV PCR analyses, Egyptian patients were divided into a HBV antigen positive (HBV+) and HBV negative (HBV-) group. The relative expression of STAT3 did not differ between HBV+ and HBV- Egyptian patients but was significantly higher in the Swedish patients compared to the total Egyptian patient population. Since we showed in Study III that miR-1234 might regulate STAT3, we also related the relative miR-1234 expression to HBV status. There was no difference in the relative expression of miR-1234 between HBV+ and HBV- Egyptian patients, while Swedish patients (HBV negative) showed a significantly lower relative expression of miR-1234, compared to the

total Egyptian patient population. miR-1234 related inversely to the expression patterns of STAT3. However, we could not detect a relation of STAT3 expression to HBV.

The results indicate that Egyptian patients have a higher viral load and that HBV was the most common virus. Since none of the Swedish patients were HBV positive, the virus may not be associated to the development of ABC DLBCL originating in Sweden. However, our results may be of importance for therapy of Egyptian patients since HBV reactivation in patients from high endemic infectious area has been noted during Rituximab treatment [230].

The relation of JCV to human malignancies is controversial but several studies have indicated JCV to be related to human cancers as colorectal-, gastric-, lung- and brain cancers [22-26]. BK and JCV were recently classified as "possibly carcinogenic to humans" by a WHO international cancer research working committee [21]. Study IV showed the presence of JCV in both Swedish and Egyptian patients, but not in Swedish and Egyptian control groups. These data might be interesting as it may suggest a role for JCV in DLBCL.

In summary, the results of Study IV indicated that Egyptian patients have a higher viral load compared to the Swedish patients and that viral agents as JCV and HBV may be involved in the tumorigenesis of ABC DLBCL. We could however not detect a relation between STAT3 and the expression of HBV.

9 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The most important findings of this thesis were:

I. FFPE tissues could be used for molecular analysis using qPCR and microarray.

II. Swedish and Egyptian patients show diverse gene expression patterns. STAT3 and STAT5b were differently expressed between Swedish and Egyptian patients.

III. Swedish and Egyptian patients show diverse miRNA expression patterns. STAT3 may be directly regulated by miR-1234.

IV. Swedish and Egyptian patients had a different pattern of viral detection but both patient groups showed detection of JCV not detected in controls.

The use of FFPE tissues for RNA extraction and gene expression analysis is feasible and comparable to data obtained using FF tissues. Extended use of FFPE tissues should enable large retrospective studies and long-term follow up of e.g. treatment effects of various diseases.

Comparing the gene expression profile of two groups of patient populations with ABC DLBCL showed differentially expressed genes that might be related to environmental factors. Both STAT3 and STAT5b were differentially expressed comparing Swedish and Egyptian patients. As STAT3 is discussed as a therapeutic target, STAT3 expression might be a biomarker for STAT3 targeting therapy. The STAT3 expression pattern might also be of interest for other cancer types.

STAT3 might be regulated by miR-1234. miRNAs are major regulatory factors in cancer. Their modes of action as well as their targets are important to understand. miRNA based therapy has been predicted to have potential for cancer treatment. miR-1234 might be of importance in ABC DLBCL expressing STAT3. The difference in STAT and miRNA expression may be associated to the difference in environmental exposures. Analyzing STAT3 and miR-1234 in additional patient groups with a different geographical background as well as in patients with other hematological malignancies should be of interest.

Other factors may also be associated to the expression of STAT. STAT3 has previously been shown to be associated to the expression of ROR1 [207]. We could show that ROR1 expression was more frequent in Swedish patients expressing STAT3 compared to Egyptian patients. The possible regulatory association between ROR1, STAT3 and miR-1234 is of interest as ROR1, has been suggested to be an important survival factor for various malignancies.

Egyptian patients had a higher viral load compared to Swedish patients. HBV was detected in the Egyptian patients but not detected in Swedish patients or in controls. In addition, both Egyptian and Swedish patient groups expressed JCV. These viruses may be associated to the biology of ABC DLBCL but it remains to be explained in what way and at

what stage of the disease. STAT3 and miR-1234 did not seem to be dependent on the HBV status but other genes or miRNAs might be associated to viral infections. The association between ABC DLBCL and JCV is of special interest to further investigate as well as how HBV may be associated to the disease in Egyptian patients as none of the Swedish patients had HBV. The viral involvement in lymphoma should be studied in more depth since prevention of viral infections may reduce the number of individuals at risk for lymphoma development. Other environmental factors as life style and ethnicity may also be factors contributing to the observed differences and is of interest to study further.

10 POPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer är ett samlingsnamn för över 200 olika former av cancersjukdomar där okontrollerad celldelning är den främsta gemensamma egenskapen. Vissa cancertyper utvecklas långsamt medan andra former är mer aggressiva och har snabb tillväxt. Cancerceller skiljer sig från friska celler bl.a. genom att de undgår celldöd, har en okontrollerbar celldelning, egen stimulering av tillväxt samt invasion av närliggande vävnad. Det finns flera orsaker till hur en cancercell uppkommer. Det kan t.ex. vara genetiska mutationer (skador) eller yttre miljöpåverkan från strålning, rökning eller virusinfektioner. Även övervikt och hög alkoholkonsumtion har visats vara associerade till ökad risk för cancer. Virusinfektioner såsom Epstein Barr virus (EBV), Papilloma virus (HPV) och Hepatit virus (HBV och HCV) har kopplats till lymfcancer (lymfom), livmoderhalscancer respektive levercancer.

Lymfsystemets primära uppgifter är att transportera och filtrera lymfa samt att delta i kroppens immunförsvar. I lymfan transporteras de vita blodkropparna (bl.a. lymfocyter). Det finns tre olika typer av lymfocyter: B-celler, T-celler och NK-celler. B-celler bär på receptorer som känner igen främmande partiklar (antigen). Receptorn kallas B-cell receptor när den är bunden till cellen och antikropp då den frisätts från cellen. För att B-celler skall kunna känna igen så många antigen som möjligt krävs att alla receptorer ser olika ut och att de har olika specificitet.

Lymfom är ett samlingsnamn för cancer som utvecklats i lymfocyter och den vanligaste är B-cells lymfom. Ca 2 200 patienter diagnostiseras med lymfom i Sverige varje år. Den vanligaste typen av lymfom är Diffust Storcelligt B-cells lymfom (DLBCL) vilken är indelad i tre subtyper: Aktiverad B-cells typ (ABC), Germinal center B-cells typ (GCB) och primär mediastinal B-cells typ (PMBL). Subtyperna kan man skilja från varandra genom att studera olika geners uttryck (genexpression) med hjälp av genchip (microarray). Dessa metoder möjliggör undersökning av uttrycket av tiotusentals gener samtidigt.

I celler pågår ständiga metaboliska processer. Signalöverföring (signal transduction) är en kemisk process där signaler skickas från ytan av cellen in till cellkärnan. Signal Transducers and Activators of Transcription (STAT) är proteiner vilka är en del av signalöverföringen och som har förmågan att aktivera uttrycket av specifika gener. Nyligen publicerade studier har visat att STAT kan vara viktiga gener i utvecklingen av cancer. De har visats vara aktiverade (uppreglerade) i flera former av cancer. Gener som är viktiga för cancerutveckling kan vara reglerade av flera olika faktorer såsom t.ex. microRNA (miRNA), som har förmågan att nedreglera (blockera) geners uttryck. miRNA finns i det genetiska materialet i cellerna och har som uppgift att reglera deras sedan tidigare bestämda målgener. Som nämnts ovan kan även virusinfektioner och yttre miljöfaktorer påverka tillväxt och uppkomst av cancer. Lymfom har specifikt kopplats till infektioner orsakade av EBV, HBV eller HCV.

Studierna i denna avhandling är uppdelade i fyra separata delarbeten. Det övergripande syftet med avhandlingen var att öka förståelsen för de molekylära processer som är involverade i uppkomst av lymfom och att öka kunskapen om miljöfaktorer som t.ex. virus kan vara kopplade till ABC DLBCL. Det första delarbetet är en metodologisk studie i

vilken vi undersökte om vävnadsmaterial, som enligt standardmetoder har formalinfixerats för lagring under längre tid, kan användas för att studera genuttryck. Formalinfixerat vävnadsmaterial, till skillnad från frusen tumörvävnad, finns i större kvantiteter och det vore därför värdefullt att kunna använda dessa för molekylära genexpressionsstudier. I **Study I** jämförde vi genexpressionen i celler från formalinfixerat material med fruset material från lungcancer-vävnad. Studien visade att genexpressionen är jämförbar mellan fruset och fixerat material. Vi föreslår därför att fixerat material kan användas i framtida genexpressionsstudier vilket möjliggör studier av större patientgrupper. Metoderna i studie I användes därefter i Study II, III och IV i vilka vi undersökte hur patienter från Sverige och Egypten med diagnosen ABC DLBCL skiljer sig på gennivå. Detta är intressant eftersom patienterna kommer från olika regioner med olika yttre miljöpåverkan. I **Study II** fann vi att genuttrycket i de två patientgrupperna skiljde sig signifikant. Mer specifikt fann vi att STAT gener (STAT3 och STAT5b) skiljde sig i genuttryck och att STAT3 var uppreglerat hos de svenska patienterna medan STAT5b var uppreglerat i tumörceller från de egyptiska patienterna. Dessa fynd konfirmerades även på proteinnivå. Vi fann även att genen ROR1, som tidigare visats bli aktiverad av STAT3, hade ett högre uttryck i de svenska patienterna samt att fler svenska patienter uttryckte ROR1 jämfört med de egyptiska patienterna. I **Study III** undersökte vi om uttrycket av miRNA skiljde sig mellan patientgrupperna och om miRNA reglerar uttrycket av STAT3. Vi fann att uttrycket av miRNA skiljde sig mellan patienter från Sverige och Egypten och att en specifik miRNA (miR-1234) kan reglera STAT3. Skillnaderna i gen- och miRNA-uttryck som vi observerade i Study II och III kan eventuellt vara associerade till de olika patientgruppernas yttre miljöpåverkan. STAT3 har föreslagits vara en möjlig behandlingskandidat för cancer. Fler studier bör göras av STAT3 uttrycket i celler från olika patientgrupper för att utröna möjligheten att optimera och precisera framtida behandling.

I **Study IV** undersökte vi förekomst av virusinfektioner i de båda patientgrupperna. Enligt WHO är Sverige och Egypten låg- respektive högriskområden för endemiska virusinfektioner. Vi fann att de egyptiska patienterna hade en högre frekvens av virusinfektioner. Vi detekterade Polyoma virus (JCV) i båda patientgrupperna som inte kunde detekteras i friska kontroller från respektive land. Förekomst av JCV är därför av intresse att studera hos patienter med ABC DLBCL. JCV har tidigare diskuterats vara en viktig faktor för uppkomst av cancer men ännu har inga säkra evidens presenterats. Vi påvisade HBV hos de egyptiska patienterna men inte hos de svenska patienterna eller i bägge kontrollgrupperna. Dessutom fann vi att 38% av de egyptiska patienterna hade HBV-antigen i serum.

Sammanfattningsvis visar resultaten i denna avhandling att formalinfixerat vävnadsmaterial kan användas för genexpressionsstudier samt att olika patientgrupper med samma tumördiagnos från olika regioner i världen kan ha olika uttryck av gener förknippade med cancerutveckling. STAT3 och STAT5b uttrycks olika i svenska och egyptiska patienter. Vi fann även att STAT3 kan vara reglerat av miR-1234. De egyptiska patienterna hade en högre frekvens av virusinfektioner, såsom t.ex. HBV till skillnad från de svenska patienterna. I båda patientgrupperna påvisades JCV som kan vara associerat till cancerutveckling och som därför är viktigt att studera vidare.

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