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# GENETIC STUDIES OF FOLLICULAR AND MANTLE CELL LYMPHOMA

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Stockholm 2005

**To my family**

O tid av guld, o liv blott tänt
för nöjet och behagen,
då man är ung och är student
och har fullt upp för dagen
och ingen annan sorg försökt
än att mustaschen växer trögt

Fänrik Stål (Runeberg 1848)

# ABSTRACT

Non-Hodgkin lymphoma is a malignancy derived from lymphoid tissues. In Sweden, non-Hodgkin lymphoma constituted 3% of all cancers recorded in 2003. Follicular and mantle cell lymphoma are the two types of non-Hodgkin lymphomas studied in this thesis.

The treatment modalities of these lymphomas vary, and the reasons why some patients have a good response to therapy while other fail to respond are largely unknown. To address this problem we compared the gene expression of follicular lymphomas that had good response to therapy with follicular lymphomas that did not respond. The investigated treatment was combination chemotherapy with CHOP (cyclophosphamide, vincristine, doxorubicin, prednisone). With high-density oligonucleotide arrays we could show that 14 genes involved in the G2/M transition of the cell cycle were up-regulated in the responders compared with the non-responders. Six out of these 14 genes were correlated with survival in a cohort of 57 patients with follicular lymphoma. Furthermore, one of these genes was also investigated with immunohistochemistry and there was a good correlation between mRNA expression and protein expression of this gene (Paper I).

The gene expression measured with high-density oligonucleotide arrays was correlated with survival in two independent cohorts of follicular lymphoma. A total of 21 genes were associated with a prolonged survival in both cohorts. This association was independent of the international prognostic index. Genes of particular interest in this study were *ERCC1*, *PTAFR*, *C4A*, *RPL23A*, *BCLAF* and *ABCC5* (Paper II).

The gene expression in mantle cell lymphoma was also studied with high-density oligonucleotide arrays. Differences in genetic profiles were shown between tumors with a high and a low proliferation rate and also between primary and relapsed tumors (Paper III).

In mantle cell lymphoma the prevalence of mutated $V_H$-genes was investigated. In a cohort of 110, 17% of the tumors had somatic mutations. This implies that a subgroup of mantle cell lymphomas has a post germinal center origin. It was further demonstrated that there was a preferential $V_H$3-21 usage in 19% of the tumors, and that patients with tumor usage of this $V_H$-gene had a longer survival compared with the rest of the patients (Paper IV).

# PAPERS INCLUDED IN THIS THESIS

## PAPER I

Erik Björck, Sara Ek, Ola Landgren, Mats Jerkeman, Mats Ehinger, Magnus Björkholm, Carl A.K. Borrebaeck, Anna Porwit-MacDonald, Magnus Nordenskjöld. High expression of cyclin B1 predicts a favorable outcome of patients with follicular lymphoma. Blood. 2005 Apr 1;105(7):2908-15. Epub 2004 Dec 2.

## PAPER II

Erik Björck, Sara Ek, Ola Landgren, Mats Jerkeman, Magnus Björkholm, Carl A.K. Borrebaeck, Anna Porwit-MacDonald, Magnus Nordenskjöld. Gene expression profile associated with favorable outcome in two independent cohorts of patients with follicular lymphoma. Manuscript

## PAPER III

Sara Ek, Erik Björck, Anna Porwit-MacDonald, Magnus Nordenskjöld, Carl A.K. Borrebaeck. Increased expression of Ki-67 in mantle cell lymphoma is associated with de-regulation of several cell cycle regulatory components, as identified by global gene expression analysis Haematologica. 2004 Jun;89(6):686-95.

## PAPER IV

Sarah H Walsh, Mia Thorselius, Anna Johnson, Ola Söderberg, Mats Jerkeman, Erik Björck, Inger Eriksson, Ulf Thunberg, Ola Landgren, Mats Ehinger, Eva Löfvenberg, Kristina Wallman, Gunilla Enblad, Birgitta Sander, Anna Porwit-MacDonald, Michael Dictor, Tor Olofsson, Christer Sundström, Göran Roos, Richard Rosenquist. Mutated VH genes and preferential VH3-21 use define new subsets of mantle cell lymphoma. Blood. 2003 May 15;101(10):4047-54.

# CONTENTS

# INTRODUCTION

In this thesis, genetic aspects of two types of non-Hodgkin lymphoma (NHL) are studied. These two types are follicular and mantle cell lymphoma. To illustrate lymphoma from the point of view of an affected individual, the imaginary story of a man getting the diagnosis is outlined throughout the introduction.

## CANCER IN SWEDEN

*A 69 year-old man noticed he was swollen on the right side of his neck. The first time he noticed this was three months earlier during a cold. The man started to worry about cancer since the swelling did not spontaneously disappear.*

Every year around 0.5% of the Swedish population will get a cancer diagnosis; about one third of the population will be affected at some time during lifetime. The probability of getting cancer increases with age; two thirds of all patients are above the age of 65 at diagnosis. The recorded number of cancers diagnosed in Sweden 2003 was 48676 (Socialstyrelsen 2004). The ten most common cancers are outlined in table 1. Seen over the past 20 years, the incidence of cancer has increased every year with 1.3 % in men and 1.1 % in women. During this period, the malignancies with an increasing trend were cancers of the skin, lung, breast, prostate, testis and urinary tract and also non-Hodgkin lymphoma, while cancers of the stomach and uterine cervix showed a decreasing trend.

**Table 1** The most common forms of cancer in Sweden 2003

|  | Number diagnosed | Percentage of all cancer |
|---|---|---|
| Prostate | 9035 | 18.6 |
| Breast | 6917 | 14.2 |
| Colon | 3559 | 7.3 |
| Skin (not malignant melanoma) | 3186 | 6.5 |
| Lung * | 3110 | 6.4 |
| Urinart tract (not kidney) | 2256 | 4.6 |
| Rectal | 2036 | 4.2 |
| Malignant melanoma | 1889 | 3.9 |
| Non-Hodgkin-lymphoma | 1404 | 2.9 |
| Uterus | 1324 | 2.7 |
| All other forms | 13960 | 28.7 |

* Primary cancer of trachea, bronchus, lung or pleura

Although the annual increase of non-Hodgkin lymphoma for this period was 0.4% in men and 0.7% in women, the annual incidence during the past decade has leveled out (Socialstyrelsen 2004) (Figure 1). Next to diffuse large B-cell lymphoma, follicular lymphoma is the most common form of non-Hodgkin lymphoma in the western world. It constitutes about 20% of all non-Hodgkin lymphomas while mantle cell lymphoma constitutes 5% (Jaffe et al. 2001).
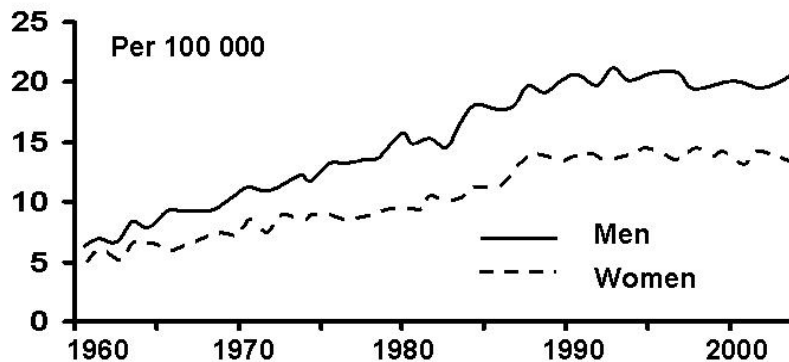


**Figure 1** Incidence of non-Hodgkin lymphoma in Sweden

The probability to survive after a cancer diagnosis has increased dramatically during the past decades (Table 2). There are many explanations for the improved survival in cancer. Two important factors are that treatment modalities have improved and that the diagnosis is often made earlier due to better diagnostic methods (Socialstyrelsen 2001).

**Table 2** Survival after cancer diagnosis

|  | Ten years survival (%) | |
| --- | --- | --- |
| Year of diagnosis | 1960-1962 | 1988-1990 |
| Men | 26 | 44 |
| Women | 42 | 57 |

## LYMPHOMA CLASSIFICATION

*Since summer and vacation came along, the man decided to wait to see his doctor. In the end of August, he visited the doctor, who made a physical examination and asked many questions concerning previous infections. The examination was flawless except an enlarged lymph node on the right side of the neck. A routine laboratory examination was normal. In agreement with the routine at the regional hospital, the patient was sent to have a fine needle aspiration of the enlarged lymph node performed. The preliminary diagnosis was follicular lymphoma, a type of non-Hodgkin lymphoma.*

The classification of lymphomas is complex, and various systems have been used throughout the years. The most recent system is the WHO classification (Jaffe et al. 2001). It is desirable to have a uniform classification of lymphomas that is internationally accepted, since this is the basis for good quality clinical trials. All retrospective studies of lymphomas also rely heavily on correct diagnosis. In 1832 Thomas Hodgkin reported the first thorough description of lymphoma, when he gave a macroscopic description of seven patients with enlarged spleen and lymph nodes. Many years later his name was used to divide the lymphomas into two different groups, Hodgkin lymphoma and non-Hodgkin lymphoma. Between Hodgkin and WHO many classifications have been used; examples of such classifications are Rappaport (Sheehan et al. 1970), Lukes and Collins (Lukes et al. 1974), Kiel, Working formulation (Lennert et al. 1975) and the REAL classification (Anynomous 1982) (Harris et al. 1994). A strength of the WHO classification is that it is based on morphology in combination with current knowledge of immunology and genetics. A good illustration of the complexity of the WHO classification is seen in table 3 in where the major types of lymphoid malignancies are shown. The golden standard of lymphoma classification is lymph node biopsy. Despite new technical advances, the morphologic evaluation of the lymph node is still the most important diagnostic pro-cedure. The combination of morphology and other methods, such as immuno-histochemistry, flow cyto-metry analysis (FACS) or various genetic methods, often facilitates the diagnostic proce-dure.

**Table 3** WHO Classification of Lymphoid Neoplasms

B-Cell Neoplasms
    Precursor B-cell neoplasm
        Precursor B lymphoblastic leukemia/lymphoma
    Mature B-cell neoplasms
        Chronic lymphocytic leukemia/small lymphocytic lymphoma
        B-cell prolymphocytic leukemia
        Lymphoplasmacytic lymphoma
        Splenic marginal zone lymphoma
        Hairy cell leukemia
        Plasma cell myeloma/plasmacytoma
        Extranodal marginal zone B-cell lymphoma of MALT type
        Nodal marginal zone B-cell lymphoma
        Follicular lymphoma
        Mantle cell lymphoma
        Diffuse large B-cell lymphoma
        Mediastinal (thymic) large B-cell lymphoma
        Primary effusion lymphoma
        Burkitt lymphoma/Burkitt cell leukemia
T and NK-Cell Neoplasms
    Precursor T-cell neoplasms
        Precursor T lymphoblastic leukemia/lymphoma
        Blastic NK cell lymphoma
    Mature (peripheral) T-cell and NK-cell neoplasms
        T-cell prolymphocytic leukemia
        T-cell large granular lymphocytic leukemia
        Aggressive NK-cell leukemia
        Adult T-cell leukemia/lymphoma
        Extranodal NK/T cell lymphoma, nasal type
        Enteropathy-type T-cell lymphoma
        Hepatosplenic T-cell lymphoma
        Subcutaneous panniculitis-like T-cell lymphoma
        Mycosis fungoides/Sézary syndrome
        Primary cutaneous type anaplastic large cell lymphoma
        Peripheral T-cell lymphoma, unspecified
        Angioimmunoblastic T-cell lymphoma
        Anaplastic large cell lymphoma
T-cell proliferation of uncertain malignant potential
        Lymphomatoid papulosis
Hodgkin lymphoma
        Nodular lymphocyte predominant Hodgkin lymphoma
        Classical Hodgkin lymphoma
        Nodular sclerosis Hodgkin lymphoma
        Lymphocyte-rich classical Hodgkin lymphoma
        Mixed cellularity Hodgkin lymphoma
        Lymphocyte-depleted classical Hodgkin lymphoma

## FOLLICULAR AND MANTLE CELL LYMPHOMA

*The lymph node was removed and sent to the department of pathology where it was divided. One part was fixed in formalin, another part was minced for flow cytometry analysis and the remaining part of the lymph node was then frozen at -70ºC. The patient had given his informed consent for this procedure. The microscope examination revealed follicular structures throughout the whole lymph node. Immunohistochemistry stainings of the follicles were positive for bcl-2 and bcl-6. The flow cytometry analysis revealed a monoclonal population positive for CD19, CD20 and CD20, while CD5 was negative. A FISH-analysis was also done on a tumor imprint and demonstrated a translocation t(14;18)(q32;q21) in 90% of the examined cells. The pathology report concluded that the patient had a follicular lymphoma grade 2 with a proliferate fraction (Ki-67) below 10%.*

Histopathologically, a *follicular lymphoma* is composed of malignant cells resembling normal cells of the lymph node germinal center. Two cell types dominate the tumor, the centrocyte and the centroblast. A centrocyte is a small cell with condensed chromatin and few nucleoli while the centroblast has less condensed chromatin and multiple nucleoli. Based on the number of centroblasts per high power field, follicular lymphomas are divided into three different grades in the WHO classification. Grade 1 is dominated by centrocytes, in grade 2 there is an intermediate number of centroblasts (6-15 per high power field), while grade 3 often is dominated by centroblasts (Figure 2). Follicular lymphomas grade 3 can further be divided into grade 3A or grade 3B. In grade 3B, a considerable part of the lymphoma has a diffuse growth pattern besides the follicular appearance (Jaffe et al. 2001).
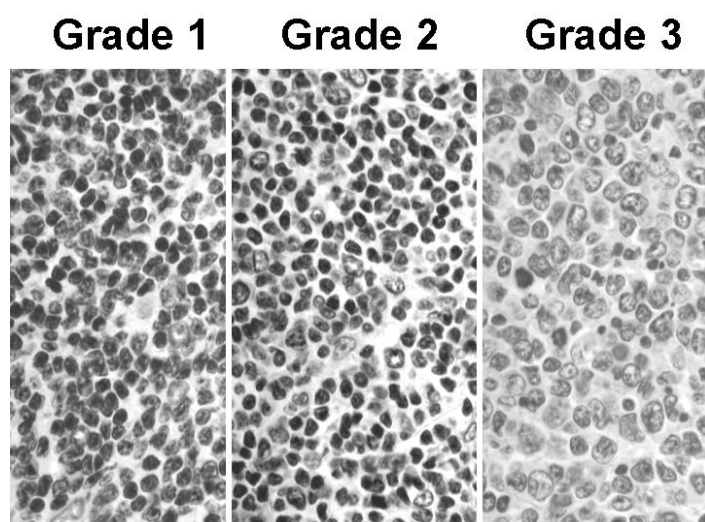


**Figure 2** Follicular lymphoma grade 1-3. Note the difference between the centrocytes with dark condensed chromatin and the larger centroblasts with paler nuclei and prominent nucleoli.

A classical *mantle cell lymphoma* is often characterized by a monomorpheus pattern of small lymphoid cells. The nuclei often have condensed chromatin and generally are angulated nuclei with an indentation on one side. The growth pattern is often diffuse, but 30-50% of all cases have a nodular growth pattern. The blastoid variant of mantle cell lymphoma has a larger proportion of large cells with less condensed chromatin.

Follicular and mantle cell lymphoma can be characterized by different antigens expressed by the tumor cells. This can be visualized by immunohistochemistry of paraffin sections from the tumors. With this technique, antigens of a protein can be detected through staining of specific antibodies directed against the antigen. Bcl-2 is positive in both follicular and mantle cell lymphoma while bcl-6 is positive in follicular but negative in mantle cell lymphoma. This and other important differences between the two types of lymphomas are outlined in table 4. An immunostaining that is commonly used to assess the monoclonality of the tumor population is the light chain (kappa or lambda) of the immunoglobulin. Flow cytometry analysis is an alternative method also often used to characterize tumor-antigens in lymphoma.

**Table 4** Features of follicular and mantle cell lymphoma

|  | Follicular lymphoma | Mantle cell lymphoma |
|---|---|---|
| Clinical course | Indolent | Aggressive |
| Median Survival (years) | 10 | 3 |
| Median age of onset | 59 | 72 |
| Male/female ratio | 1:1 | 3:1 |
| Histopathological appearence | Various sized cells | Monomorpheus small cells |
| Immunophenotype | CD5-, CD10+, CD19+, CD20+,CD22+ | CD5+, CD10-, CD19+ |
| Bcl-6 | + | - |
| Characteristic translocation | t(14;18)(q32;q21) | t(11;14)(q13;q32) |

Genetically, most follicular lymphomas are characterized by the translocation between the long arms of chromosome 14 and chromosome 18 (Rowley 1988). This translocation leads to the up-regulation of bcl-2, an anti-apoptotic protein, which gives the tumor cells a survival advantage (Nunez et al. 1990). In mantle cell lymphoma, there is a translocation between chromosome 11 and chromosome 14 (Williams et al. 1993). The breakpoint of chromosome 14 is the same as in follicular lymphoma and is located at the position of the gene for the heavy chain of the immunoglobulin. In mantle cell lymphoma, the translocation juxtaposes heavy chain immunoglobulin-enhancer sequences on chromosome 14 to the cyclin D1 gene on chromosome 11, leading to over-expression of the cell cycle regulator cyclin D1 (de Boer et al. 1995).

The cells with this translocation thus have a growth advantage over normal cells. Both immunohistochemistry for cyclin D1 and fluorescent *in situ* hybridization (FISH) for the t(11;14)(q32;q13) can thus be used as a diagnostic tool in mantle cell lymphoma (Figure 3) (Björck et al. 2003).
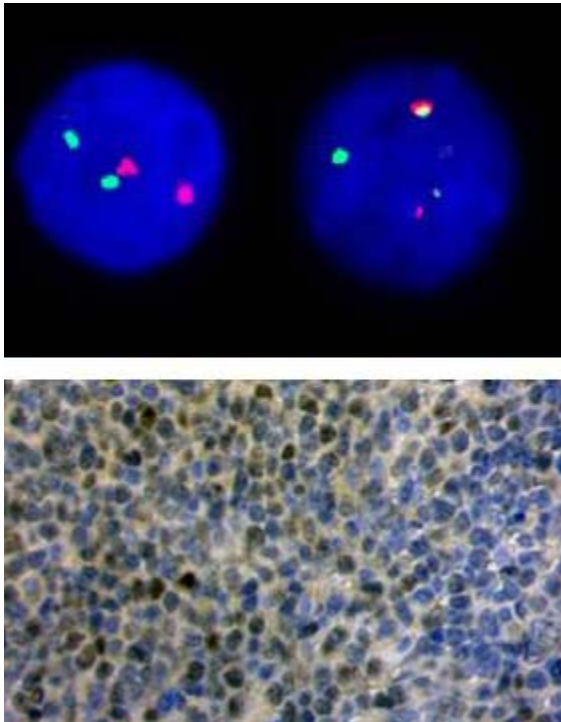


**Figure 3 Top:** FISH-analysis for t(11;14)(q32;q13). Interphase nuclei hybri-dized with LSI® IGH/CCND1 probe. To the left two normal signals from chromosome 11 (red) and 14 (green). One the right: one fusion signal from derivate 14 (large yellow) and one fusion signal from derivate 11 (small yellow). **Bottom:** Cyclin D1 staining of a blastoid mantle cell lymphoma.

## B-CELL DEVELOPMENT AND IMMUNOGLOBULIN REARRANGEMENT

B-cell development is a process in which the B-cell evolves from a precursor B-lymphoblast to a mature B-cell or plasma cell. The mature B-cells express specific surface immunoglobulins, and plasma cells have the ability to secrete antibodies. Throughout the B-cell development, various stimuli promote the maturation. The first part of the development is antigen independent, while the second part which occurs in the germinal center is antigen dependent (Wang et al. 2003).

B-cell lymphomas are believed to derive from various stages of normal B-cell development. Follicular lymphoma has germinal center B-cells as postulated cell of origin, while mantle cells lymphoma is thought to derive from peripheral B-cells of the inner mantle zone of the germinal center (Jaffe et al. 2001).

A major characteristic of B-cells is that they express immunoglobulines. The immunoglobulin molecule consists of four polypeptide chains, two heavy chains and two light chains (Figure 4). Each chain has a constant region and a variable region. Different combinations of gene segments code for the variable regions of the immunoglobin. There are four types of gene segments coding for the heavy chain, variable ($V_H$-genes), diversity
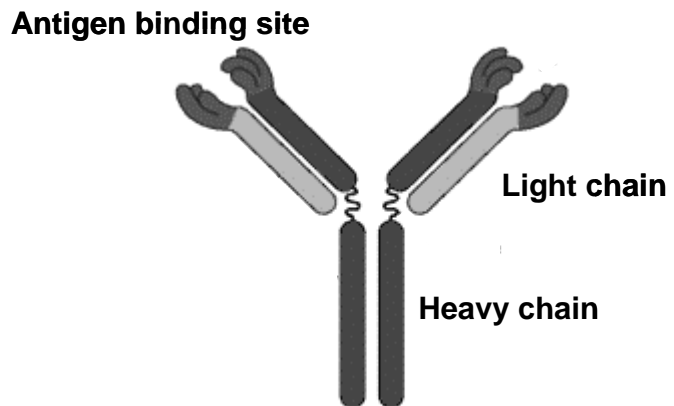


**Figure 4** Schematic outline of an immunoglobulin.

(D), joining ($J_H$-genes) and constant ($C_\mu$). All these gene segments are located at the locus for the heavy chain on chromosome 14q32. There are 51 functional $V_H$-genes, 30 D segments, 6 $J_H$-genes and various constant genes. By sequence similarity, the $V_H$-genes can be divided into different families ($V_H1$- $V_H7$) (Cook et al. 1995). There are three types of gene segments coding for the light chain: variable ($V_L$-genes), joining ($J_L$-genes) and constant ($C_L$-genes) (Siminovitch et al. 1990).

Since there are many different gene segments to choose from, a vast amount of combinations exists for the immunoglobulin molecule. This is important since different immunoglobulins bind different antigens. Another way to alter the affinity for antigens in the variable part of the immunoglobulin is through somatic hypermutations. These usually occur after the B-cell has encountered an antigen in the germinal center. A tumor with somatic hypermutations can thus be considered to have a post germinal center origin. The somatic hypermutations are acquired sequence alterations, mainly located in specific regions of the variable gene segments. These regions are called the complementarity-determining regions (CDR) (Tonegawa 1983).

The presence of somatic hypermutations has also been shown to have a clinical impact. This was first shown in chronic lymphocytic leukemia (CLL) by Hamblin 1999 et al who found that patients with somatic hypermutations in their malignant cells had a better prognosis then patients without hypermutations (Hamblin et al. 1999).

## CLINICAL STAGING

*The man was then further investigated. Serology was negative for HIV and EBV. A bone marrow aspiration and biopsy showed normal morphology. A computerized tomography scan of the chest, abdomen and pelvis was also normal. After all examinations the man returned to the oncologist and was informed that he had follicular lymphoma grade 2. Since there was no sign of the disease other then the lymph node, the patient was considered to have lymphoma of stage I according to theAnn Arbor staging system.*

At diagnosis lymphoma is generally classified according to the Ann Arbor classification system, which originally was developed for Hodgkin lymphoma (Table 5)(Carbone et al. 1971) (Lister et al. 1989).

**Table 5** Ann Arbor staging classification

| | |
|---|---|
| Stage I | Involvement of a single lymph node |
| Stage II | Involvement of two or more lymph nodes on the same of the diaphragm |
| Stage III | Involvement of two or more lymph nodes on both sides of the diaphragm |
| Stage IV | Involvement of one or more extranodal site(s) |

Each stage can be further divided into A or B
A: No B criteria fulfilled
B: One or more of the following: night sweats, Unexplained fever over 38 C, weight loss >10% of the body weight during the preceeding 6 months

Nearly 80% of patients with follicular and mantle cell lymphoma have stage III or IV at diagnosis (Solal-Celigny et al. 2004) (Svenska lymfomregistret 2002). Mantle cell lymphoma is often widespread at diagnosis with extra-nodal infiltration of the bone marrow. The gastrointestinal tract, liver and spleen are other commonly infiltrated organs at diagnosis.

## TREATMENT

*Different treatment strategies were discussed with the patient, who demanded an immediate treatment with best available methods. Since the patient had localized disease, radiotherapy was addressed as the major alternative. The patient was also informed about the risk of relapse. Chemotherapy was also discussed but decided against, since it could later be useful as a second line therapy. The patients received involved field radiotherapy (30 Gray) targeted at the affected area of the neck.*

Follicular lymphoma grade 1 and 2 is considered an indolent lymphoma with slow progression of the disease. Despite this, follicular lymphoma is generally considered an incurable disease, since it usually eventually progresses and will cause the death of the patient. Treatment of follicular lymphomas depends on grade and stage of the disease. There are many different treatment possibilities available for patients with follicular lymphoma, but follicular lymphoma is usually not curable with standard treatment approaches. Some patients with a localized disease might actually be cured with local radiation therapy. Since most patients have a stage III or stage IV at diagnosis, radiotherapy is not the treatment of choice for the majority of patients. It is important to individualize the treatment for each patient and carefully evaluate indications for treatment. Indications for treating follicular lymphoma grade 1 and 2 are B symptoms (Table 5), obstructive adenopathy or progression. Otherwise, a watchful waiting strategy is a good alternative. Examples of lymphoma treatments are listed in table 6. Follicular lymphoma type 3 with a localized tumor is often treated with radiation and combination chemotherapy while a generalized disease often is treated with more intense combination chemotherapy (Armitage 2002).

**Table 6** Examples of treatment of follicular and mantle lymphoma

| Type of therapy | Substance | Comment |
|---|---|---|
| Chemotherapy | 2-chlorodeoxyadenosine (CdA) | |
| | Chlorambucil + Prednisone | KNOSPE |
| | Cytarabine | |
| | Fludarabine | |
| | Cyclophosphamide | |
| | Vincristine | CHOP |
| | Doxorubicine | |
| | Prednisone | |
| | Mitoxantrone | |
| Other drugs | Interferon-alpha | |
| | Rituximab | Anti CD20-antibody |
| | 131[I]-tositumomab | Radio-nucleotide linked anti CD20-antibody |
| Radiation | | |

Mantle cell lymphoma is an aggressive type of lymphoma, and most patients experience a rapid progress of the disease, although some patients may have a more indolent course of the disease. The vast majority of patients with mantle cell lymphoma cannot be cured, although lately primary high-dose chemotherapy with the addition of antibodies directed against a B-cell antigen (e.g. Rituximab) and autologous stem cell transplantation have shown promising results (Khouri et al. 1998) with prolonged survival of the patients (Brugger et al. 2004) (Forstpointner et al. 2004).

# PROGNOSTIC FACTORS

*The man tolerated the treatment well and returned for clinical evaluation. There was still no sign of the disease. During treatment the man had considered the question whether the prognosis of follicular lymphoma could be predictable. He had also spent considerable time to find out the reason why he developed lymphoma. He did not know of any relative who also had cancer. He also asked if he had any known risk factors for developing lymphoma.*

A prognostic factor can either be tumor-related or patient-related (Specht et al. 1999). Two examples of tumor-related factors are the type of cancer and the genetic aberrations of the tumor. Typical patient-related factors are age of the patient, performance status, and co-morbidity with other diseases. The division between tumor-related and patient-related factors might sometimes be artificial, since it is often difficult to distinguish between these two types of factors. How widespread the disease is at diagnosis and how it responds to therapy are examples of such factors. Both these factors depend on the tumor-specific properties as well as on the general status and constitution of the affected patient.

Prognostic indexes have been developed for lymphomas (Anonymous, 1993) (Perea et al. 2003). The international prognostic index (IPI) was developed in 1993 for aggressive lymphomas, but is often used also for indolent lymphomas. The factors of the IPI are age, stage, performance status, number of extra-nodal sites and serum level of lactate-dehydrogenase ( Anonymous, 1993).

Over a decade later, the follicular international prognostic index was published (Solal-Celigny et al. 2004). The aim of this study of 4167 patients was to develop a prognostic index that was more specific for follicular lymphoma. The clinical factors were first included in a univariate analysis to evaluate what factors had a prognostic relevance (Table 7). All such factors except four where then included in a multivariate analysis. The reasons for exclusion were: sedimentation rate (only assessed in European patients), performance status (too few had a poor performance status), serum $\beta_2$-microglobulin and serum albumin (lack of patient data).

In the multivariate analysis, eight parameters retained their prognostic importance, and five of them were selected to constitute the FLIPI. This index has also been shown to be a useful prognostic marker at the relapse of follicular lymphoma (Montoto et al. 2004). A separate index for mantle cell lymphoma does not exist; and hence IPI is often used instead. It is important to consider that all indexes predict survival in groups of patients and not for single individuals.

**Table 7** Prognostic factors in follicular lymphoma

|  | Univariate | Multivariate † | FLIPI |
|---|---|:---:|:---:|
| Age | <60 | x | x |
| Stage | I or II | x | x |
| Number of nodal sites | 0-4 | x | x |
| Serum lactate-dehydrogenase | ≤Upper level of normal | x | x |
| Hemoglobin level | ≥120g/l | x | x |
| Sex | Female | x |  |
| Bone marrow involvement | Absence | x |  |
| Lymphocyte count | ≥ $1 \times 10^9$/L | x |  |
| B symptoms | Absence |  |  |
| Performance status * | Not bed ridden during day-time |  |  |
| Number of nodal sites other than bone marrow | None |  |  |
| Spleen involvement | Absence |  |  |
| Serum $\beta_2$ microglobulin * | ≤Upper level of normal |  |  |
| Sedimentation rate * | ≤ 40 mm/h |  |  |
| Thrombocyte count | ≥ 150 x$10^9$/L |  |  |
| Serum albumin level * | ≥35 g/L |  |  |

\* Not included in the multivariate analysis
† Significant in multivariate analysis, each factor has an independent prognostic value

As mentioned above, follicular lymphomas can be divided into grade 1-3 based on the number of centroblasts. The relation between the grades of follicular lymphoma and survival remains controversial. Two studies have shown that patients with grade 3 had an inferior survival rate than grade 1 and 2 (Martin et al. 1995) (Miller et al. 1994), while a recent study showed that there were no difference between the groups (Chau et al. 2003). The difference between the grades has been suggested to be treatment-related. (Jaffe et al. 2001).

Various cytogenetic aberrations have been shown to be associated with prognosis in follicular lymphoma. In a study of 165 follicular lymphomas, +X, 1p-, +1q, +12, 17p-, and 17q- were associated with poor outcome. This was also demonstrated for structural abnormalities of 1p21-22, 6q23-26 and 17p in another study including 66 patients (Höglund et al. 2004) (Tilly et al. 1994). With comparative genomic hybridization (CGH) of tumors from 82 patients, Viardot showed that deletion of 6q25 to 6q27 was associated with shorter survival (Viardot et al. 2002).

In a small study of 27 patients, Zhao et al showed that an over-expression of *BCL-XL* measured with RT-PCR was associated with a shorter survival. *BCL-XL* is a member of the bcl-2 family and acts as an anti-apoptotic factor (Zhao et al. 2004). A high expression of the protein mdm2, an oncoprotein involved in the regulation of p53, has also been associated with a shorter survival in follicular lymphoma (Møller et al. 1999). A high protein expression of bcl-6 and CD10 has been associated with longer survival in follicular lymphomas (Bilalovic et al. 2004).

## ETIOLOGY AND RISK FACTORS

*The patient was told by the doctor that the reason why he got a lymphoma was unknown and that he did not have any known risk factor for developing lymphoma.*

The etiology of follicular and mantle lymphoma is largely unknown, but a number of risk factors and agents contributing to the development of non-Hodgkin lymphoma have been identified. In a single patient, it is almost impossible to determine what exactly causes the disease. In this context, it is important to consider that the malignant transformation to lymphoma most likely is a multi-step process. In follicular and mantle cell lymphoma this is supported by the fact that the two translocations t(11;14) and t(14;18) are not sufficient *per se* to cause lymphoma in mouse models (Bodrug et al. 1994) (Lovec et al. 1994). Other observations supporting the multi-step concept are the facts that t(14;18) has also been demonstrated in a considerable share of healthy individuals (Schuler et al. 2003) and that lymphomas incidence increases with age (Socialstyrelsen 2004)

Immunodeficiency is a strong risk factor for non-Hodgkin lymphoma. The WHO classification recognizes four clinical settings with increased risk of lymphoma, namely primary immunodeficiency syndromes, infection with human immunodeficiency virus (HIV), post-transplant of a solid organ or bone marrow and immunodepression caused by metotrexate treatment (Jaffe et al. 2001)

Both bacterial and viral agents have been shown to contribute to the development of lymphomas. Antigens from the bacteria *Helicobacter pylori*, which lately has received much attention as a causative agent of gastric ulcers and chronic gastritis, have been shown to activate T-cells and through this mechanism contribute to a continued proliferation of gastric MALT lymphoma cells (Hussell et al. 1993). Treatment with antibiotics has induced remission for some of these patients (Wotherspoon et al. 1993) (Nakamura et al. 2003). A lymphoma that previously sometimes required surgery and removal of the stomach can now at times be treated with antibiotics (Yoon et al. 2004)! Viral infection with Epstein-Barr Virus (EBV) is essential for the development of endemic Burkitt's lymphoma (Facer et al. 1989), which is the most common malignancy in childhood in some parts of Africa. Prior to developing lymphoma, all these children with Burkitt's lymphoma have been infected by EBV (Prevot et al. 1992). It is well known that EBV has the ability to infect and immortalize B-lymphocytes both *in vivo* and *in vitro*, and infection with EBV has thus been proposed to contribute to the multi-step development of lymphoma (van den Bosch 2004). Another example of a virus that is causally linked to lymphoma is human T-cell leukemia virus type 1

(HTLV-1). This virus is important in the pathogenesis of adult T-cell lymphoma (Franchini 1995).

A few mendelian inherited disorders are also associated with an increased risk of lymphoma. These might either be linked to the immunosystem as in X-linked lymphoproliferative disease (Oertel et al. 2002) or through other mechanisms contribute to the development of lymphoma as do ataxia-teliangiectasia (Olsen et al. 2001), Nijmegen breakage syndrome (Seidemann et al. 2000) and Li-Fraumeni syndrome (Kleihues et al. 1997).

In epidemiological studies, autoimmune diseases have been shown to be associated with an increased risk for lymphoma. Patients with a severe rheumatoid arthritis have a higher risk to develop lymphoma compared with controls (Gridley et al. 1993). The reason for this increased risk is unknown but is most likely linked to the severity of the disease and to the treatment with immunosuppressive drugs (Baecklund et al. 2004). Another possible risk factor for non-Hodgkin lymphoma is a high intake of dairy products and fried red meat (Chang et al. 2005). In the same study, a high consumption of fruit and vegetables were associated with a lower risk. Interestingly, ultraviolet exposure has lately also been shown to be associated with a lower risk of developing non-Hodgkin lymphoma (Smedby et al. 2005).

## MICROARRAY STUDIES OF LYMPHOMAS

*Five years later at his annual control the patient was still in complete remission, and by this time the tumor sample from the patient was included in a retrospective study of follicular lymphoma with high-density oligonucleotide arrays.*

As mentioned above a prognostic factor can be tumor-related. The study of the gene expression profile of a tumor is one way of determining the underlying genetic mechanisms behind tumor-related factors. The rationale behind this is that tumor cells express different genes reflecting basic cellular mechanisms of importance for tumorigenesis. In 1995, new technical developments allowed global gene expression analysis by microarray (Schena et al. 1995).

The first microarray study of a larger series of lymphoma was published in 2000 (Alizadeh et al. 2000). In this study, three types of lymphomas (diffuse large cell lymphoma, chronic lymphocytic leukemia and follicular lymphoma), were compared with lymphocyte subpopulations and leukemia cell lines. The most important result of this study was the fact that diffuse large cell lymphoma could be divided into two distinct groups based on the gene expression. One group expressed genes characteristic of germinal center B-cells and the other

group expressed genes that were induced during *in vitro* activation of lymphocytes. Patients in the first group had a significantly better overall survival than those in the second group.

The difference in gene expression between diffuse cell large and follicular lymphoma was investigated by Shipp et al 2002 using a supervised learning prediction method. With this method, a 30-gene predictor set managed to classify 71 of 77 samples to the right group. The same method was then successfully applied to distinguish between diffuse large cell lymphomas with a good and poor outcome (Shipp et al. 2002). Several microarray studies have evaluated which genes are important for the transformation of a follicular lymphoma into a diffuse large cell lymphoma (Lossos et al. 2002) (Elenitoba-Johnson et al. 2003) (de Vos et al. 2003).

In a microarray analysis of 24 patients with follicular lymphoma, the response to rituximab was predicted by gene expression. The expression pattern in tumors with a poor response to rituximab appeared to be more similar to the pattern of normal lymphoid tissues than to that of the rituximab responders (Bohen et al. 2003). Also in a follicular lymphoma study by Glas et al the genetic signatures were based on clinical parameters (Glas et al. 2005).

The largest prognostic study of follicular lymphoma with microarray technique was the study by Dave et al 2004 (Dave et al. 2004). In this study, the authors divided the studied 191 patients into a training set and a validation set. The training set was used to select 3299 genes correlated with survival with a P-value below 0.1. These genes where then clustered into genetic signatures according to their similarity of gene expression. Ten signatures that could predict survival were found when the expression level of the genes within each signature was averaged. To consider a genetic signature relevant, it had to be correlated to survival in both the training set and the validation set. When the ten groups were further studied in a multivariate analysis, two genetic signatures, designed as immunoresponse 1 and immunoresponse 2, had the strongest correlation to survival. The genes of these signatures were then shown not to be primarily expressed by the malignant B-cells, but rather by other cells present in the tumor. The conclusion of this study was that the T-cells, macrophages and dendritic cells in follicular lymphoma contribute to the pathogenesis of the tumor and are not innocent by-standers.

In 2003, Rosenwald published the largest prognostic microarray study of mantle cell lymphoma (Rosenwald et al. 2003). In this study of 101 patients, a gene-expression-based predictor of survival was used to select genes associated with prognosis. Also here the patients were also divided into a training set and a validation set. A total of 20 prognostic genes were selected. They all had a function linked to proliferation. For each sample, the gene expression of the 20 genes was averaged. This averaged value significantly predicted survival in both the training set and in the validation set.

# AIMS OF THE STUDY

The general purpose of this thesis was to investigate various aspects of gene expression in follicular and mantle cell lymphoma. The focus of the study was the assessment of clinical variables, such as survival, and tumor associated variables, such as proliferation and variable immunoglobulin gene segments. The more specific aims were to

- examine the gene expression difference between tumors from patients with a good response to combination chemotherapy and tumors from patients with a poor response to the therapy.
- identify genes that had previously not been shown to be expressed in follicular and mantle cell lymphoma.
- identify genes associated with longer survival in follicular lymphoma.
- compare the gene expression between mantle cell lymphomas with a high and a low protein expression of Ki-67.
- compare the gene expression of primary and of relapsed mantle cell lymphomas.
- evaluate the extent of restricted usage of $V_H$ gene in mantle cell lymphoma.

# PATIENTS AND TUMORS

## PAPERS I AND II

Lymph node biopsies from patients diagnosed with follicular lymphoma at Karolinska Hospital 1994-1999 (44 patients) and at Lund University Hospital 1996-2001 (13 patients) were used in papers I and II. To be included in the study the lymph node had to yield good quality RNA and successful oligonucleotide array hybridization. Clinical records were obtained for all patients. All cases were carefully reviewed and classified as a follicular lymphoma according to the WHO classification (Jaffe et al. 2001). Details about the selection of patients are given in paper I.

The patients and oligonucleotide data included in paper I were also used in paper II, where in addition data from another cohort were used for comparison. All data from this cohort were published by Dave et al in November 2004 (Dave et al. 2004) and are publicly available from the web site of "Lymphoma and Leukemia molecular Profiling Project"
 at http://llmpp.nih.gov/FL/.

## PAPER III

Lymph node or spleen biopsies from patients diagnosed with mantle cell lymphoma at Karolinska Hospital 1998-2002 (14 biopsies) and at Lund University Hospital 1997-2001 (7 biopsies) were used. To be included in the study, the lymph node had to yield good quality RNA and successful oligonucleotide array hybridization. Clinical records were obtained for all patients. A further inclusion criterion were to have either a positive immunohistochemistry staining for cyclin D1 or a positive fluorescent *in situ* hybridization (FISH) for the characteristic t(11;14)(q13;q32).

## PAPER IV

Tumor samples from 110 cases of mantle cell lymphoma diagnosed at Karolinska Hospital, Huddinge Hospital, Lund University Hospital, Uppsala University Hospital and Umeå University Hospital were used. Clinical records were gathered for all patients. The inclusion criteria were the same as in paper III.

# METHODS

## EXPRESSION ANALYSIS WITH HIGH-DENSITY OLIGONUCLEOTIDE ARRAYS (PAPERS I-III)

As mentioned above, the development of microarrays made a global assessment of gene expression in tumors possible. This was the reason we choose this methodology to investigate follicular and mantle cell lymphoma. There are two main microarray technologies to study gene expression, cDNA arrays and oligonucleotide arrays (Schena et al. 1995) (Lockhart et al. 1996). After an initial evaluation of the two technologies, the Affymetrix high-density oligonucleotide array u95Av2 was used as main technology in paper I-III.

High-density oligonucleotide arrays were obtained from Affymetrix Inc. (Santa Clara, CA, USA). This gave the possibility to assess over 10 000 genes with U95Av2 (Figure 5) in one single experiment Each gene is represented by 16 different probe pairs which together constitute a probe set (Table 8). The individual probe pair consists of two oligonucleotides that each has a length of 25 bases. One of the oligonucleotides in a probe pair has perfect complementary match (perfect match) with the transcript of a specific gene, while the other oligonucleotide has a complementary match for all nucleotide except one (mismatch). More than 300000 oligonucleotides are printed on the surface of one half square inch of the U95Av2 chip.



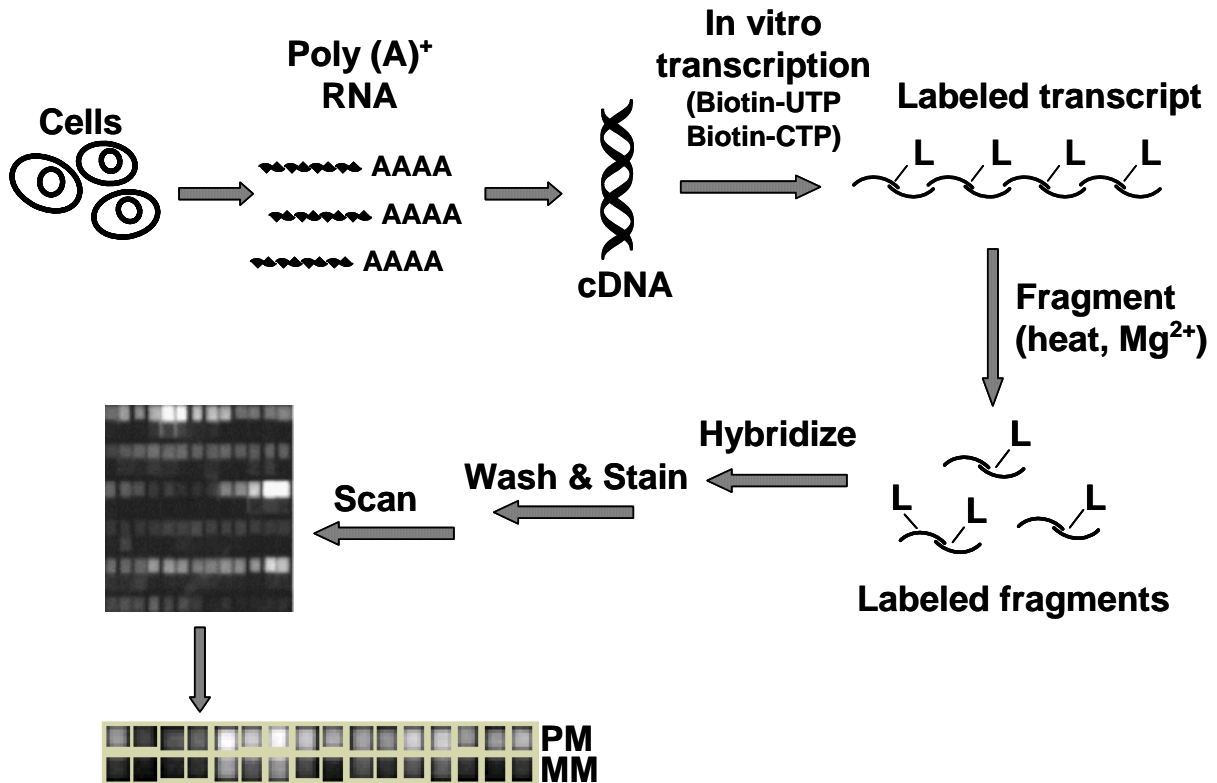**Figure 5** High-density oligonucleotide array U95Av2

**Table 8** A brief Affymetrix glossary

| | |
|---|---|
| Oligonucleotide | 25 base pair long probe |
| Perfect match | Oligonucleotide with a perfect complementarity to targeted RNA sequence |
| Mismatch | Oligonucleotide with one basepair changed compared with targeted RNA sequence |
| Probe pair | Perfect match + mismatch |
| Probe set | 16 probe pairs representing different sequences of a gene |

Before a sample can be hybridized to the array, good-quality RNA has to be extracted from the tumor that is to be investigated. This RNA is then first reversely transcribed to cDNA and *in vitro* transcribed to biotin-labeled cRNA, fragmented and hybridized to the array. After staining and a series of washings, the array is scanned to produce a digital image where each oligonucleotide is represented (Figure 6).



**Figure 6.** Procedure from cell to scanned image of an Affymetrix high-density oligonucleotide array.

The signal intensities for each probe pair represents the number of cRNA copies hybridized to that specific probe pair. The signal algorithm is used to calculate the final probe set value. In this algorithm, each probe pair is first assigned two values, one for the perfect match probe and one for the mismatch probe (Figure 7). The probe pair value is calculated by subtracting the mismatch value from the perfect match value. The probe pair values are then weighted according to their vicinity to the median of all probe pairs. The mean value of these weighted probe pair values is the final value of the whole probe set. The statistical method used to weight the probe pairs is the One-Step Tukey's Biweight Estimate (Affymetrix 2002).
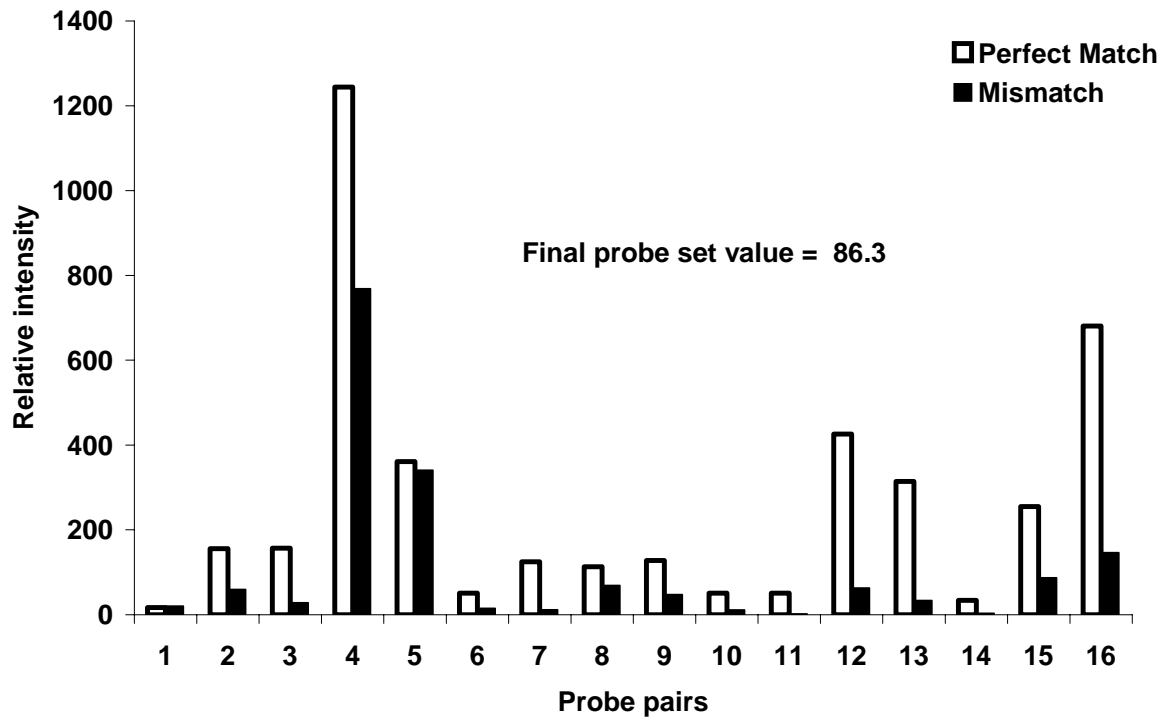
**Figure 7** Detection values of the 16 probe pairs for 1945_at (*CCNB1*).

With the detection algorithm, each probe set is assigned a Present, a Marginal or an Absent call. To decide which call the probe set should be assigned, the target-specific intensity of each probe pair is calculated by dividing the perfect match signal (with the mismatch value subtracted) with the total intensities of the perfect match signal and the mismatch signal. A strong perfect match signal and a weak mismatch signal thus give a value close to 1. If the perfect match signal is weak compared with the mismatch signal, a low value results. If all probe pairs have values close to 1, the probe set will be assigned a Present call, whereas a low value will give an Absent call. Details of these algorithms can be found at www.affymetrix.com (Affymetrix 2002).

When a comparison analysis is made, the results of two hybridizations (experiment array versus baseline array) are compared using two algorithms, the change algorithm and the signal log ratio algorithm (paper I and paper III).

In the change algorithm, the differences between the perfect match signal and mismatch signal and also the differences between the perfect match signal and the background in both experiments are examined using a non-parametric Wilcoxon's rank test. The final output of these comparisons is given as an Increase, Marginal Increase, No Change, Marginal Decrease or Decrease call for each probe set. A *P*-value is also presented for the final output.

In the signal log ratio algorithm, each individual probe pair in the experiment array is compared with the corresponding probe pair in the baseline array. As with the signal algorithm, these differences are weighed with the one-step Tukey's biweight method. The final value will then be a mean of the weighted probe pair log ratios of intensities across the two arrays. The output of this algorithm is presented in a log scale with base 2.

## IMMUNOHISTOCHEMISTRY

A widely used way to confirm the data of a microarray experiment is to perform real-time quantitative PCR. With this method it is possible to confirm the presence of mRNA for the assessed gene. Another method to confirm microarray data is to use immunohistochemistry, which was the method we chose in papers I and III. Immunohistochemistry is used to visualize antigen expression in tissues. An advantage of this method compared with real-time quantitative PCR is that the gene expression can be evaluated on the protein level.

In this thesis, all immunohistochemistry stainings were made on tumor paraffin sections. In immunohistochemistry, a primary antibody against a specific antigen of the protein of interest is used. A developing system with a secondary antibody with an attached reporter molecule is then used for staining. The result can be evaluated in a light microscope. The pattern of the protein expression is usually, but not always, restricted either to the nucleus or to the cytoplasm. The percentage of positive cells can be determined by manual counting, but in clinical practice a protein is often evaluated as present or absent or semi-quantified as absent, low, medium or high. In the evaluation of an immunohistochemical staining, it is always important to consider the fact that only a section of the tumor is stained, and that this section might not always be representative for the whole tumor.

## PCR AMPLIFICATION AND DNA SEQUENCE ANALYSIS (PAPER IV)

DNA sequence analysis can be used to identify mutations in tumor cells. With this method isolated tumor cells can be subject to PCR amplification. In paper IV, PCR fragments were used as templates in the sequencing reaction in order to determine which specific gene segments of the heavy and the light chain were used in the lymphomas. This technique was also used to investigate whether somatic hypermutations were present.

DNA was prepared from fresh-frozen material or paraffin-embedded tissue using standard protocols. The PCR amplification of the $V_H$ and $V_L$ gene was carried out using family-specific primers, with platinum Taq DNA polymerase (Life Technologies, Paisley, UK). Activation of the enzyme (95ºC), denaturation (94ºC), annealing (61-64ºC) and elongation (72ºC) were done with a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA,

USA). The PCR fragments were then analyzed in an automated sequencer (ABI 377 or ABI 3700, Applied Biosystems). Details of the PCR amplification and sequence analysis can be found in paper IV. The obtained sequences were compared with publicly available databases, and sequences with less then 98% homology were considered unmutated.

## FLUORESCENT *IN SITU* HYBRIDIZATION (PAPERS I AND IV)

FISH was used to confirm the diagnosis of mantle cell lymphoma by showing the presence of t(11;14)(q32;q13) in paper IV, and to investigate if the expression of *TOP2A* were gene dose dependent in paper I. In a FISH experiment, a fluorescent-labeled DNA probe is hybridized against a target DNA. (Figure 8).
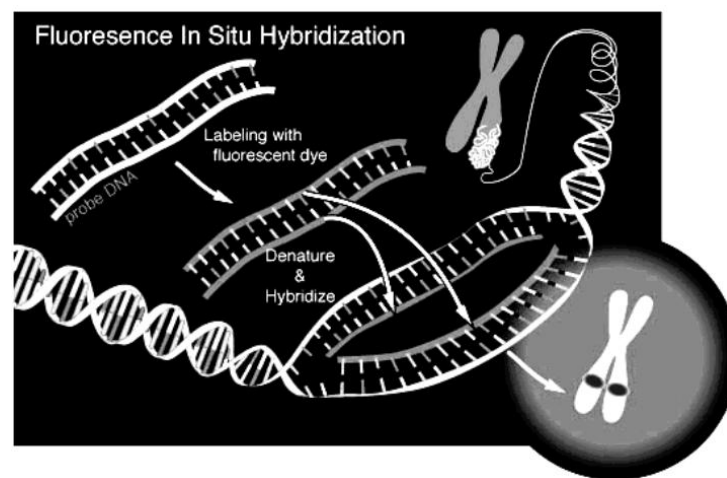


**Figure 8** Fluorescent *in situ* hybridization

The sequence of the FISH-probe is complementary to the targeted DNA. The signal is detected with a fluorescence microscope. Hybridization can be done to metaphase chromosomes or to interphase nuclei. FISH allows the detection of deletions (absent signal), amplification (extra signals) or rearrangement of a specific genetic locus (fusion of signals). For hematological malignancies, a large number of probes targeted at specific regions or specific translocations are commercially available. Locus specific probes can also be made in-house using specific BAC or PAC clones.

## SURVIVAL ANALYSIS (PAPERS I, II AND IV)

There are various ways to measure outcome in a prognostic study. Survival is the widely most used variable; overall survival (paper I, II and IV) is defined as the time from diagnosis to death irrespective of cause. The advantage of using death as outcome is that the time of death is impeccable. A disadvantage is that the studied patient might have another cause of death

than the studied disease. Since many of the studies of lymphoma enroll elderly patients this can be a problem. In larger epidemiological studies this factor is often compensated for, but in smaller retrospective clinical studies, this information is seldom considered. As an example, the probability for a 70-year-old man in Sweden to die during the following 5 years is 0.18; in other words, there is an 82% chance that a Swedish 70-year-old man will celebrate his 75th birthday (Statistiska central byrån 2005). One way to surpass this problem is to measure cause-specific survival (paper I), which can be defined as the time from diagnosis to the death in the studied disease, or death judged to be related to the treatment of the disease. Cause-specific survival can be difficult to judge in a uniform way, since information or documentation of what actually ended the life of a patient may be missing.

Survival analysis was performed using both a log-rank test and Cox proportional hazard regression (Cox 1972). The log-rank test was used to compare survival in two groups. Kaplan-Meyer curves were used to illustrate the survival curves of various groups (Kaplan et al. 1958). Cox proportional hazard regression was used to compare the relative risk of one group with that of another group and was also used when a continuous parameter was evaluated. With the same method the variables were correlated to survival in a univariate or in a multivariate mode. The purpose of the multivariate analysis was to determine which variables have independent significance.

When a survival analysis is performed, it is also of importance to consider what variable is studied and related to survival. In this thesis, the gene expression level was measured with high-density oligonucleotide arrays. In papers I and II, we retrospectively correlated the concentration of mRNA of a certain gene in the diagnostic tumors to the survival of the patients. A key issue is how big influence the tumor properties have on the clinical course of a patient. There are of course many other factors with a crucial influence on the clinical course, i.e. morbidity in other diseases, socio-economic factors and the treatment of the patient.

In this thesis, the studies were done retrospectively. When evaluating variables in a retrospective way there is always a risk of hindsight bias (Hoffrage et al. 2003). The ideal study of a prognostic marker in follicular lymphoma would be to study a certain variable in a prospective manner. Even better would be to do this study in two separate cohorts. A drawback of studying follicular lymphoma is that a long follow-up time for the patient is needed to evaluate survival properly.

# RESULTS AND DISCUSSION

## DOES THE GENE EXPRESSION OF FOLLICULAR LYMPHOMA CORRELATE TO TREATMENT AND OUTCOME? (PAPER I)

In paper I, a cohort of 57 patients with follicular lymphoma was studied. The hypothesis behind this paper was that the gene expression of tumors from patients with a poor response to chemotherapy differed from the gene expression of tumors from patients with a good response to the same therapy. There are several treatment modalities of follicular lymphoma, but a considerable portion of the patients receive combination chemotherapy with CHOP (cyclophosphamide, vincristine, doxorubicin and prednisone). The microarray data of eight non-responders to CHOP was compared with the data of 13 patients with a good response. A total of 14 genes were expressed significantly higher in the good responders than in the non-responders (Table 9). With the same selection criteria, no gene was found to have a high expression in the poor responders and a low expression in the responders.

**Table 9** Genes up-regulated in tumors with a good response to CHOP

| | | |
|---|---|---|
| Cell-cycle related | | |
| * | *CCNB1* | cyclin B1 |
| * | *CDC2* | cell division cycle 2, G1 to S and G2 to M |
| * | *CKS1B* | CDC28 protein kinase regulatory subunit 1B |
| * | *CDKN3* | cyclin-dependent kinase inhibitor 3 |
| * | *ANP32E* | acidic (leucine-rich) nuclear phosphoprotein 32 family, member E |
| | *CCNB2* | cyclin B2 |
| Mitosis | | |
| | *KNTC2* | kinetochore associated 2 (highly expressed in cancer - HEC) |
| | *BUB1B* | BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast) |
| | *HMMR* | hyaluronan-mediated motility receptor (RHAMM) |
| DNA-modulation | | |
| | *TOP2A* | topoisomerase (DNA) II alpha 170kDa |
| | *HMGB2* | high-mobility group box 2 |
| Other/Unknown | | |
| * | *KIAA0101* | KIAA0101 gene product |
| | *GMDS* | GDP-mannose 4,6-dehydratase |
| | *PAICS* | phosphoribosylaminoimidazole carboxylase † |

* Genes with a *P*-value below 0.05 (Log-rank test cause-specific survival)

† Other name: phosphoribosylaminoimidazole succinocarboxamide synthetase

Eleven of the fourteen genes were involved in the cell cycle, in mitosis or in DNA modulation. When the gene expression of these 14 genes was divided by the median, high expression of six genes were correlated with a longer survival. The genes that correlated with a longer survival were *CCNB1*, *CDC2*, *CKS1B*, *CDKN3*, *ANP32E* and *KIAA0101*. Each one of these genes retained its prognostic impact when it was tested together with the factors of

the follicular lymphoma international prognostic index. The Kaplan Meier curve in figure 9 shows that a high expression of *CCNB1* was associated with a better survival rate.
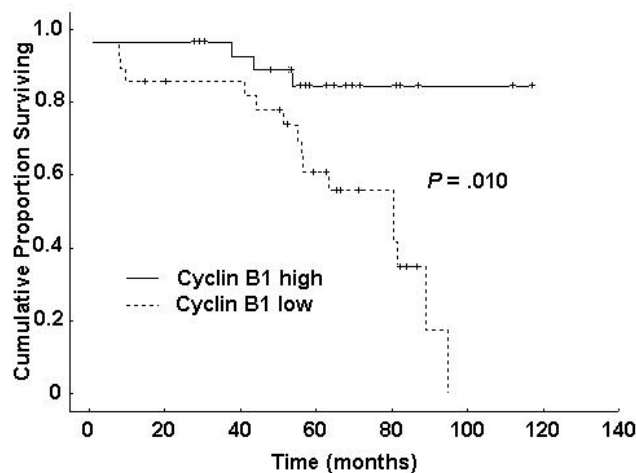


**Figure 9** Survival of patients with follicular lymphoma divided by the median expression of cyclin B1 probe set 34736_at.

*CCNB1* and *CDC2* form a complex that is essential for the G2/M transition of the cell cycle. The expression of these two genes has a peak at the G2/M phase and must be degraded before completion of cytokinesis. Both *CKS1B* and *CDKN3* are interaction partners of *CDC2,* and *ANP32E* interacts indirectly with *CDC2*.

The role of cell cycle related genes as prognostic markers in follicular lymphoma remains controversial. For the most used proliferation marker in clinic practice, Ki-67, we could not show any correlation to survival. Earlier studies of this the prognostic impact of this marker have given contradictory results (Czader et al. 1996) (Miller et al. 1997) (Martin et al. 1995) (Llanos et al. 2001). One explanation of these differences could be that the compared groups were not treated uniformly. It may be true that untreated patients with high proliferative tumors have a poor survival compared with  patients with low proliferative tumors, but that the situation is reversed for patients treated with combination chemotherapy.

No previous study has shown that cyclin B1 in lymphoma is associated with a prolonged survival. In the study of Jin et al, cyclin B1 did not have a prognostic significance in non-Hodgkin lymphoma measured by immunohistochemistry (Jin et al. 2002).

The function of *KIAA0101* is unknown. *KIAA0101* is located on chromosome 15q22.31 and has four exons and codes for a protein of 111 amino acid residues (Ensemble 2005).Since the function of *KIAA0101* is unknown, we investigated what genes were co-regulated with this gene. For this analysis, the 36 samples of follicular lymphoma not treated with CHOP, the 21 samples of mantle cell lymphoma used in paper III, and 11 samples representing different stages of B-cells development were used. A total of 21 genes had a correlation coefficient above 0.8, and 18 of these genes were cell cycle related, involved in mitosis or had a DNA modulating function. *CCNB2* was the gene with the strongest correlation to *KIAA0101*. The correlation between these two genes in the patients with follicular lymphoma is seen in figure 10.These results strongly suggests that *KIAA0101* and cyclin B2 have related functions in mitosis or in cell cycle regulation.
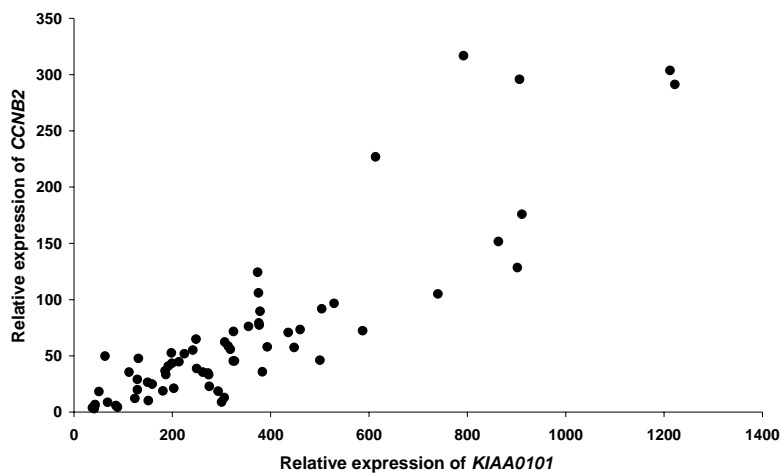


**Figure 10** Correlation between of gene expression of cyclin B2 (*CCNB2*) probe set 32263_at and of *KIAA0101* probe set 38116_at.

## GENE EXPRESSION AND CLINICAL OUTCOME (PAPER II)

The aim of this study was to identify genes with a prognostic significance in follicular lymphoma. To investigate this, survival was correlated with the gene expression of the tumors included in paper I. In a recently published independent study, high-density oligonucleotide arrays were used to assess survival in follicular lymphoma (Dave et al. 2004). The data from this study gave us the possibility to increase the power of our survival analysis. In paper II, a gene could then be considered significant if it was correlated to better survival in *both* cohorts independently of the international prognostic index. The method used to screen for these genes was the Cox proportional hazard regression. With this method, the gene expression could be evaluated as a continuous parameter.
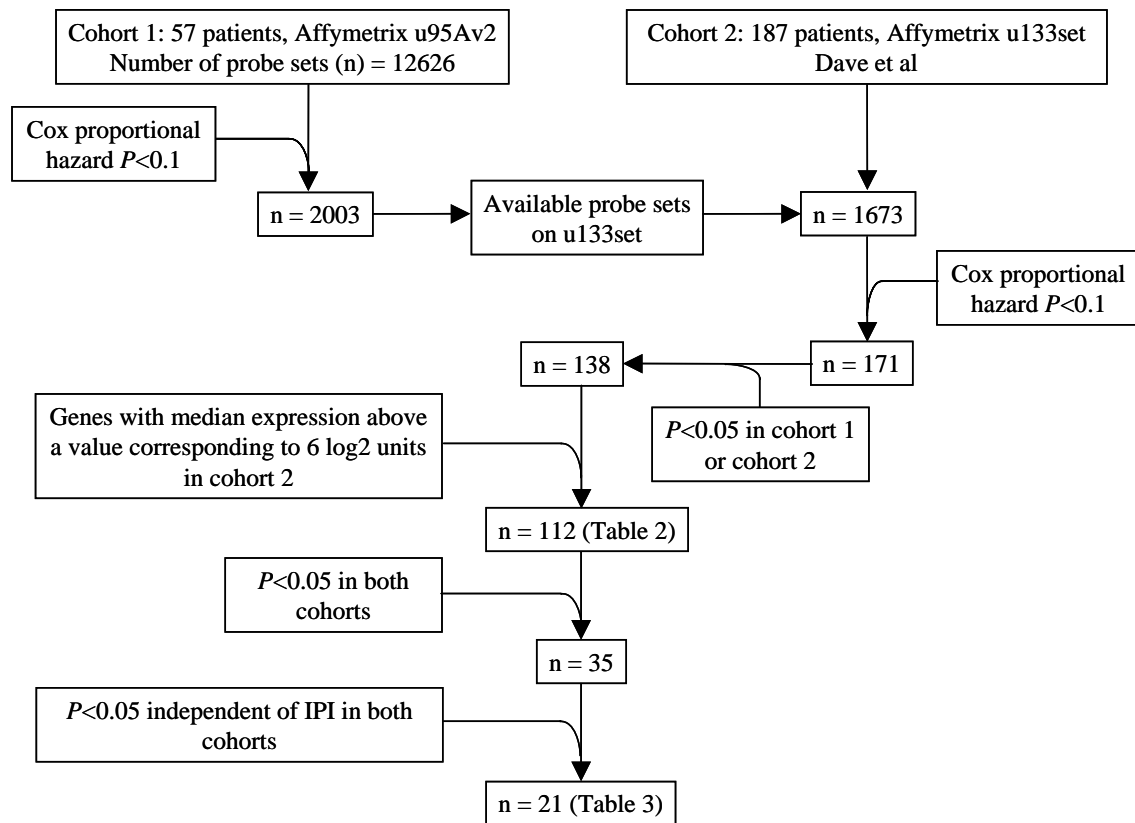
**Figure 11** Algorithm used to select genes in paper II

A total of 21 genes had an independent prognostic value in both cohorts (Figure 11). When the gene expression was dichotomized by the median expression, four genes retained their prognostic significance; a high expression of *ERCC1* and *PTAFR* was correlated with longer survival, and a low expression *RPL23A* and *C4A* was a good prognostic marker.

*ERCC1* is a structure-specific DNA repair endonuclease responsible for the 5-prime incision during DNA repair. In a recent study of mouse cell-lines, *ERCC1* together with *ERCC4 (XPF)* contributed to the stability of telomeres through the removal of the 3' overhang from uncapped telomeres (Zhu et al. 2003). It is therefore reasonable to speculate that a high expression of *ERCC1* may contribute to the maintenance of the chromosome integrity, and through this mechanism *ERCC1* may slow down the progression of lymphoma.

A high expression of the platelet-activating activating receptor (*PTAFR*) was also associated with a prolonged survival. This receptor is present on B-lymphocytes and belongs to the superfamily of G protein-coupled receptors. In malignant cells, *PTAFR* has been showed to be expressed on the membrane of leukemic cells of patients with chronic lymphocytic leukemia and acute lymphoid leukemia (Donnard et al. 2002; Denizot et al. 2004). Its ligand, the platelet-activating factor (*PAF*) has well-documented *in vitro* effect to modulate the

immunoglobulin production in B-cells (Mazer et al. 1990; Smith et al. 1994). *PAF* has also been shown to rescue B-cells from apoptosis following B-cell receptor ligation (Toledano et al. 1997). No previous study has linked the expression of *PTAFR* to survival in human malignancies.

A low expression of *RPL23A* was associated with a better prognosis. *RPL23A* codes for one of the 80 structural proteins of the ribosomes. *RPL23A* has been suggested to be a molecular target for the growth inhibition of beta-interferon since it has been demonstrated that mRNA levels of *RPL23A* were diminished in human cell tumor cell lines (Jiang et al. 1997). A decrease of *RPL23A* mRNA has also been demonstrated in a human melanoma cell line after treatment with alpha-interferon (Jiang et al. 1997). *RPL23A* might thus have other functions than its structural one. This may explain its role as potential prognostic indicator in follicular lymphoma.

A low expression of *C4A* was also a significant prognostic marker. *C4A* and *C4B* are both products of the cleavage of the complement C4 precursor, a major histocompatibility complex class-III protein. The protein C4b plays a central role in the activation of the classical complement system, while *C4A* codes for the protein C4a anaphylatoxin. C4a anaphylatoxin is a mediator of the histamine release from mast cells and basophilic granulocytes, and thus important for the local inflammatory response. *C4A* was one of the 24 genes included in the prognostic immunoresponse 2 signature in the study by Dave et al (cohort 2) (Dave et al. 2004). Thus, the inflammatory function of this gene might be the biological link between *C4A* and lymphoma.


Two other genes of particular interest of the selected genes were *ABCC5*  and *BCLAF*. *ABBC5* is a member of the multi-drug resistance protein MRP/ABCC subfamily. The association between a low expression of *ABBC5* and better prognosis is of interest since this gene confers resistance to nucleosides and purine analogs (Dean et al. 2001). The purine analog fludarabine is one of the drugs used in the treatment of patients with follicular lymphoma (Czuczman et al. 2005). The full name of *BCLAF* is BCL2-associated transcription factor. *BCL2* is up-regulated in follicular lymphoma through the translocation t(14;18)(q32;q21) which is present in most tumors. The exact function of *BCLAF* remains to be elucidated but this gene has been suggested to play a role in the promotion of apoptosis or cell cycle arrest (Kasof et al. 1999). Interestingly, deletions of the long arm of chromosome 6 harboring *BCLAF1* (6q23.3) have been reported as the second most common cytogenetic aberration in follicular lymphoma next to the t(14;18)(q32;q21) (Höglund et al. 2004). Patients with follicular lymphoma showing a deletion of the long arm of chromosome 6 have a shorter survival then those with tumors without this deletion (Tilly et al. 1994; Viardot et al. 2002). This is in accordance with our observation that a high expression of the *BCLAF1* gene is associated with better survival. This suggests that the expression of *BCLAF1* is gene dose-dependent.

## GENE EXPRESSION IN TUMORS WITH HIGH AND LOW EXPRESSION OF THE PROLIFERATIVE MARKER KI-67 (PAPER III)

The aim of this study was to establish a genetic signature distinguishing tumors with a high protein expression of Ki-67 from those with a low expression, and also to investigate the gene expression differences between primary and relapsed tumors.

A common marker of proliferation in the clinical setting is Ki-67 which is the common name for the gene "Antigen identified by monoclonal antibody Ki-67" with the official abbreviation *MKI*-67. Expression of this antigen occurs during late G1, S, G2 and M phases of the cell cycle. In this paper, the gene expression in two tumors with high protein expression of Ki-67 determined by immunohistochemistry was compared with the gene expression in seven tumors with low expression. Twelve genes were up-regulated and 20 genes were down-regulated in the tumors with a high expression of Ki-67. Using the expression level of these 32 genes, a hierarchical clustering was performed with 12 additional tumors and the original nine tumors. In this clustering, two tumors with high Ki-67 expression clustered together with the highly proliferate tumors, four tumors out of five with Ki-67 expression also clustered among the highly proliferate tumors, while the tumors with a Ki-67 expression below 20% clustered into four different groups. The genes that were up-regulated are involved in mitosis (*HEC1 - KNTC2*, *BUB1B*, *C20ORF1*) or in the cell cycle (*MKI67*, *CCND1*), interacts with DNA/chromatin (*TYMS*, *HMGA1*, *HMGB2*, *TREX2*) or have another function (*HSPA6* and *HCK)*. Down-regulated genes of particular interest were *ZNF148* and *PPM1A* which interact with p53 and in this way may influence the growth of the cell. In the comparison between relapsed and primary tumors, 11 genes were up-regulated and 15 genes were down-regulated. A gene of particular interest was *TFRC* (transferring receptor) that was higher expressed in the relapsing tumors. An increased expression of the transferring receptor might thus confer a growth advantage for the cells of the relapsed tumor.

## MUTATED V$_H$-GENES AND V$_H$3-21 USAGE IN MANTLE CELL LYMPHOMA (PAPER IV)

The aim of this study was to evaluate the prognostic impact of the group of patients that had mutated V$_H$-gene and a preferential V$_H$3-21 usage. In this study, DNA from 110 patients with mantle cell lymphoma was studied. The V$_H$-gene was analyzed and 18 patients had mutated V$_H$-genes with a frequency of mutations of 2.2-6.7%. A V$_H$-gene was considered mutated if it had more than 2% sequence alteration. The mutations represented true somatic mutations, since the germline sequence was determined in patients with mutated V$_H$-genes and found to be equal to the published sequence. The fact that a subset of mantle cell lymphomas had mutated V$_H$-genes suggests that they have been exposed to antigen in the germinal center.

There was no difference in survival between the patients with mutated $V_H$-genes and the patients without mutations (Figure 12). This result is in agreement with previous studies (Camacho et al. 2003; Kienle et al. 2003; Bertoni et al. 2004). The most commonly used $V_H$-gene was $V_H$3-21 which was present among 21 patients. A bias with preferential usage of $V_H$-3-21 was also shown in the previously mentioned studies (Camacho et al. 2003; Kienle et al. 2003; Bertoni et al. 2004). In paper IV we showed that the group of patients using the $V_H$3-21 gene had a longer survival than the patients using other $V_H$-genes ($P$=0.030) (Figure 12). The median survival for the 21 patients with $V_H$3-21 was 53 months compared with 34 months for the patients with using other $V_H$-genes. In the study by Kienly there was a trend towards better survival for the patients using $V_H$3-21 ($P$=0.08) (Kienle et al. 2003). These twelve patients had a median survival of 103 months compared with 36 months for the remaining 93 patients. In the study of Bertoni which included 42 tumors there was also a tendency for better survival in the patients using $V_H$3-21 ($P$=0.1) (Bertoni et al. 2004). In the study of Camacho which included 96 patients, the 12 patients with $V_H$3-21 also had a trend towards a better survival ($P$=0.061) (Camacho et al. 2003). The results of paper IV thus still need to be confirmed, but it is of interest that all three series show a trend towards a better survival. A biological explanation for the prolonged survival of the $V_H$3-21 group of patients, could be that this group had a lower number of genomic aberrations compared with the group using other $V_H$-genes (Flordal Thelander et al. 2005). An additional result of the study in paper IV was that 17 out of the 21 patients with $V_H$3-21 usage also had used the same type of light chain, namely the $V\lambda$3-19. This is of particular interest since these results suggest a possible role for a common etiological antigen for these lymphomas.
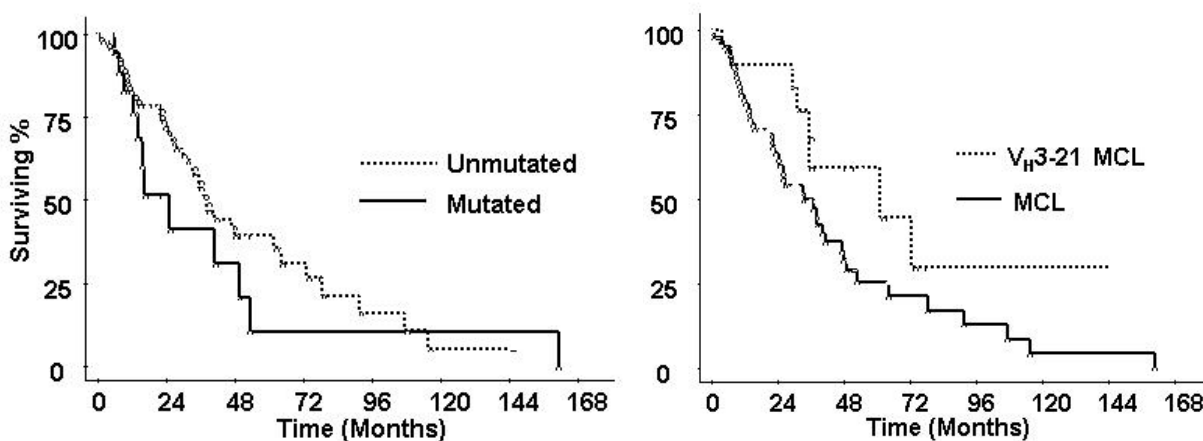


**Figure 12** Mantle cell lymphoma **Left:** Survival of patients with mutated tumor $V_H$-genes versus unmutated $V_H$-genes. **Right:** Survival of patients with tumor $V_H$-3 21 usage compared with all other VH genes

# CONCLUSION

The general aim of this thesis was to investigate various aspects of gene expression in follicular and mantle cell lymphoma. To achieve this aim, the response to chemotherapy of the patients with follicular lymphoma was compared, the correlation of gene expression with survival of patients was investigated and the difference between tumors with high and low protein expression of Ki-67 was assessed. We also investigated the $V_H$ genes in mantle cell lymphoma.

We conclude that
- genes involved in G2/M transition of the cell cycle, mitosis or DNA-modulation are higher expressed in patients with follicular lymphoma with a good response to CHOP combination chemotherapy.
- high expressions of several genes interacting with cyclin B1 and its partner *CDC2* correlate with survival in follicular lymphoma in the studied cohort.
- *KIAA0101*, a gene with unknown function, is likely to be involved in either mitosis or cell cycle regulation.
- a total of 21 genes were associated with longer survival in follicular lymphoma in two independent cohorts and that *ERCC1*, *PTAFR*, *RPL23A*, *C4A*, *BCLAF* and *ABCC5* were of special interest among these genes.
- a genetic signature of 32 genes could be defined to differentiate between mantle cell lymphoma tumors with high and low expression of Ki-67.
- a subset of mantle cell lymphomas has mutations in the $V_H$-gene, and that the survival rates do not differ between patients with and without mutations.
- the most common used $V_H$-gene is $V_H$3-21 and that patients with this $V_H$-gene have a better prognosis in our study.
- the patients using $V_H$3-21 as heavy chain of the immunoglobulin often use $V\lambda$3-19 as light chain.

# ACKNOWLEDGMENTS

Björn Hammar for scrutinizing my knowledge in everything from linkage analysis to the positions of the 01-19 and 08-26 at Arlanda. Keep calling!

All co-authors not previously mentioned, especially Richard Rosenquist Brandell, Sarah Walsh, Mia Thorsélius, Anna Laurell, Mats Jerkeman and Mats Ehinger.

Ingrid Svensson for patiently answering any big or small question concerning statistics.

To my family for all the love and support I was granted during this work.

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