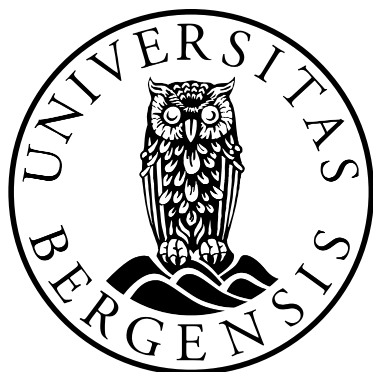
Onconeural antibodies with special reference to anti-Yo

Cecilie Totland



Dissertation for the degree philosophiae doctor (PhD)
at the University of Bergen

Contents

SCIENTIFIC ENVIRONMENT 3

ACKNOWLEDGEMENTS 5

INTRODUCTION 6

ABSTRACT 7

LIST OF PUBLICATIONS 9

ABBREVIATIONS 10

1. THE IMMUNE SYSTEM 11

1.1 Immune system in the central nervous system 13

1.2 Autoimmunity 13

1.2.1 Autoantibodies 15

1.3 Antibody avidity 17

1.4 Tumour immunity 19

2. ATM AND CCDC104 24

3. PARANEOPLASTIC NEUROLOGICAL SYNDROMES 26

3.1 Paraneoplastic cerebellar degeneration 32

3.2 Paraneoplastic antibodies and antigens 33

3.2.1 Yo antibodies 38

4. THE CDR PROTEINS 41

4.1 CDR1 41

4.2 CDR2 42

4.3 CDR2L 45

5. IMMUNOLOGICAL METHODS 46
5.1.1 In vitro transcription/translation and immunoprecipitation assay 46

6. AIMS (PAPERS 1 - 3) 50

RESULTS AND DISCUSSION 51

6.1 Paper I: Hu and Yo antibodies have heterogeneous avidity 51

6.2 Paper II: CDR2 antigen and Yo antibodies 55

6.3 Paper III: Antibody to CCDC104 is associated with a paraneoplastic antibody to CDR2 (anti-Yo)..59

7. SUMMARY AND FUTURE PERSPECTIVES 63

REFERENCES 66

Scientific environment

This work was mainly carried out in the Paraneoplastic research group at the Neurology Research Laboratory, Haukeland University Hospital, Norway and at the Section for Neurology, Department of Clinical Medicine, University of Bergen, Norway. The main funding was from Helse Vest. Additional funding was from the Department of Clinical Medicine, University of Bergen.

Acknowledgements

Many people have contributed to this thesis. First of all I want to thank my main supervisor, Christian Vedeler, for introducing me to the field of paraneoplastic diseases and for his everlasting enthusiasm and encouragement. He has always been open for new solutions and then kindly directed me on the right track. I also want to thank my co-supervisor Bjørn Ivar Haukanes for his invaluable advices in bioinformatics as well as molecular biology. Jan Aarseth deserves huge thanks for all statistical help.

This work would not have been possible without the support of the rest of the Paraneoplastic group. I especially want to thank Mette Haugen for excellent help in the lab, for always being supportive and for listening to both scientific and personal matters. Kibret Mazengia, Jane Kristin Nøstbakken and Emilia Lohndal have given me invaluable technical help. I also want to thank Anette Knudsen, Nina Aarskog, Geir Bredholt, Sissel Monstad, Anette Storstein, Tilo Eichler, Tor Qvale and the rest of the Paraneoplastic group for contributing to the scientific environment. I have received many valuable inputs from them.

I also want to express my gratitude to the other members of the Neuroimmunological Research Laboratory. The warm and welcoming atmosphere has made it easy to become a member of the lab. I have enjoyed many coffee cups and great discussions in Hanne Linda's and Mette's office. Their door is almost always open.

Without the support form my family I would not have made it through. A huge thank to my parents, Grethe and Lars Bjørn, for always being there for me and for invaluable help in daily life matter. I especially want to thank my dear Tommy for believing in me and for pushing me over obstacles I thought impossible. At last I want to thank my two beautiful sons, Tobias and Aksel, for being a part of my life. No one has taught me more than you.

Introduction

PNS are rare side effects of cancer that occur in less than 1 % of all cancers, most often small-cell lung cancer, ovarian cancer and breast cancer. These tumours express proteins normally only expressed in immuneprivileged tissues like the testis and the central nervous system. The body recognizes these proteins as foreign and an immune response is initiated. The body produces antibodies and activates T cells directed towards these proteins. By a so far unknown mechanism the antibodies and T cells cross the blood-brain barrier, attack the neurones that express these onconeural proteins, and the patients develop neurological symptoms. Often these symptoms are seen early in tumour development, while the tumour is still small and unnoticed. Detection of paraneoplastic antibodies are therefore important diagnostic tools for identifying tumours at an early stage, which again increases the chance of recovery.

The most common paraneoplastic antibodies are Hu, Yo, CRMP5, amphiphysin, Ri and Ma2 antibodies. Yo antibodies are most commonly associated with ovarian or breast cancer and causes paraneoplastic cerebellar degeneration with loss of Purkinje cells followed by ataxia. These antibodies recognize a cytoplasmic protein called CDR2 that is normally expressed in the Purkinje cells in cerebellum and in testis, but the function of this protein is largely unknown. CDR2 is also expressed in more than 60 % of all ovarian tumours and 25 % of all breast cancer tumours. Some of these patients develop antibodies towards this protein, with a prevalence of approximately 2.3 % in ovarian cancer and 1.6 % in breast cancer.

Abstract

The mechanisms behind anti-Yo mediated paraneoplastic cerebellar degeneration is still not understood, and very little is known about the function of CDR2. In our studies we have tried to elucidate the properties of Yo antibodies, potential antibodies that coexist with anti-Yo and whether sequence variants in the CDR2 gene or differences in CDR2 transcription and expression could explain why some patients develop PCD.

The avidity of antibodies is associated with onset of disease and disease severity, and the avidity of paraneoplastic antibodies has never been examined before. We compared differences in antibody avidity among patients with the two most common paraneoplastic antibodies, anti-Hu and anti-Yo. We found that the antibody avidity among patients with these antibodies was heterogeneous, but patients with Yo antibodies generally had antibodies with higher avidity than patients with Hu antibodies. This might reflect differences in the patient's immune response, the severity of the disease or different time points of sampling.

Since antibody avidity increase over time we also did a longitudinal study where we followed patients with Hu or Yo antibodies over time. This study showed that while the avidity indexes increased over time for most patients with Hu antibodies, the avidity indexes for patients with Yo antibodies were fairly constant. This could indicate that Hu antibodies are discovered at an earlier time point in the disease progress, while the Yo antibodies have persisted for a while before the neurological symptoms developed.

More than 60 % of all ovarian tumours express CDR2, but only 2.3 % of these patients develop Yo antibodies and even fewer develop PCD. The reason why some patients develop paraneoplastic antibodies is not known. We wanted to study whether the production of Yo antibodies in some ovarian cancer patients were related to variants in the cDNA sequence or to difference in the CDR2 mRNA or protein level

in tumour tissue from patients with ovarian cancer. We found no differences among the patients that could explain why some of them develop Yo antibodies and PCD. However, we observed that CDR2 was not solely expressed by tumour cells. Also normal ovarian tissue expressed low levels of CDR2. These findings indicate that CDR2 may be more widely distributed than previously reported. Our findings also support the hypothesis that development of PCD is not solely related to CDR2 expression and Yo antibody synthesis, but also to immune dysregulation, such as antigen presentation and cooperation between B and T cells.

Yo antibodies most commonly appear alone. We identified a patient with PCD and Yo antibodies that also harboured antibodies towards a little described protein called CCDC104. We found that this protein was expressed in several tissues, especially brain and testis. We further investigated whether this antibody was a potentially new paraneoplastic marker. CCDC104 antibodies were not related to cancer or PNS. However, 10.5 % of the anti-Yo sera also had CCDC104 antibodies, suggesting there is a significant association between anti-Yo and anti-CCDC104.

List of publications

Totland C, Aarseth J, Vedeler C: Hu and Yo antibodies have heterogeneous avidity, J Neuroimmunol 2007; 185: 162-167

Totland C, Aarskog NK, Eichler TW, Haugen M, Nøstbakken JK, Monstad SE, Salvesen HB, Mørk S, Haukanes BI, Vedeler CA.: CDR2 antigen and Yo antibodies, Cancer Immunol Immunother. 2011; 60:283-289.

Totland C, Bredholt G, Haugen M, Haukanes BI, Vedeler, C: Antibody to CCDC104 is associated with a paraneoplastic antibody to CDR2 (anti-Yo). Cancer Immunol., Immunother 2010; 59: 231-237

Abbreviations

AChR	Acetylcholine receptor
ATM	Ataxia telangiectasia mutated
β_2-GPI	β_2–glycoprotein
CCDC104	Coiled-coil domain containing 104 protein
CDR	Cerebellar degeneration related
cpm	counts per minute
CSF	Cerebrospinal fluid
CRMP5	Collapsin response mediator protein 5
EBV	Epstein-Barr virus
FIGO	International federation of gynaecology and obstetrics
GABA	Gamma aminobutyric acid
HLA	Human leukocyte antigen
HPV	Human papilloma virus
IgG/ IgM	Immunoglobulin G/M
IL-1/6	Interleukin 1/6
IFN	Interferon
ITT	In vitro transcription/translation and immunoprecipitation
MHC	Major histocompatibility complex
NMDAR	N-methyl-D-aspartate receptor
PNS	Paraneoplastic neurological syndromes
SCLC	Small cell lung cancer
SD	Standard deviation
SOX	Sex determining region Y related HMG box
Tag	SV40 T antigen
T_{reg}	T regulating cells
VGCC	Voltage-gated calcium channels
VGKC	Voltage-gated potassium channels

1. The immune system

The immune system is divided into the innate and the adaptive immune system. The innate immune system is regarded as the primitive, first-line defence towards pathogens. It is not specified towards specific pathogens, but rather recognizes intruders in a general manner. The innate immune system is characterized by a rapid response to danger that activates mechanisms like fever and the complement cascade. Phagocytic cells, such as macrophages, play an important role in innate immunity. The macrophages digest microbes and presents antigenic fragments of the microbes to members of the adaptive immune system.

The adaptive immune system can recognize and selectively eliminate specific foreign microorganisms and antigens. B lymphocytes (B cells) and T lymphocytes (T cells) are the two major cell types in the adaptive system. They both arise from hematopoietic stem cells of the bone marrow, but while the B cells continue their maturation in the bone marrow, the T cells migrate to the thymus gland to mature.

B cells are exposed to self antigens in the bone marrow. Those who recognize self antigens are eliminated, while those that do not, leave the bone marrow displaying a wide variety of antigen-binding receptors (bound antibodies) that can identify a huge variety of foreign antigens. When a B cell binds to a matching antigen, the B cell divides rapidly and differentiates into memory B cells and antibody secreting plasma cells that secrete huge amounts of circulating antibodies.

The T cells undergo a similar selection as the B cells. In the thymus those that recognize self antigens are destroyed, while the others leave the thymus. Mature T cells express a unique antigen-binding receptor called the T cell receptor. While the B cell receptors can recognize antigens alone, T cells only recognize antigens when bound to MHC molecules on cells. MHC I is expressed on nearly all nucleated cells, while MHC II is expressed by antigen-presenting cells. When a T cell encounters an antigen bound to MHC on a cell, it differentiates into memory T cells, T helper

(CD4+) and T cytotoxic (CD8+) cells. Activated T helper cells become effector cells that secrete cytokines that can activate B cells, T cytotoxic cells, macrophages and other cells that are involved in the immune response. Cytokines also differentiate T cells that recognise antigen-MHC I complexes into effector cells called cytotoxic T cells. Cytotoxic T cells eliminate virus-infected cells, tumour cells and cells of a foreign tissue graft. Figure 1 shows a schematic illustration of the different processes in an immune response.

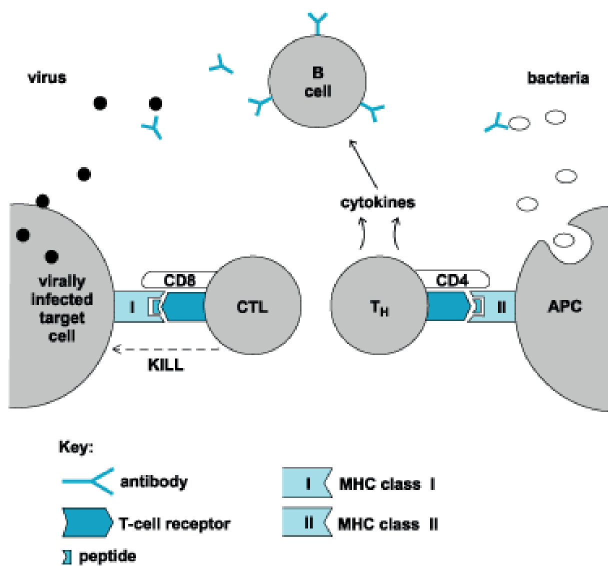


Figure 1: **Key processes in an immune response.**
APC= antigen presenting cell; TH= T helper cell; CTL= Cytotoxic T lymphocyte (http://www.answers.com/topic/cellular-immunology)

1.1 Immune system in the central nervous system

Protecting the brain is a difficult immunological challenge. Effective and rapid clearance of pathogens is essential to maintain the structural integrity of the brain. Even small lesions in critical neuronal networks can have devastating effects. Since any cellular immune reaction leads to damage followed by scarring, the intensity of the immune reaction must be tightly regulated to avoid unnecessary damage. The central nervous system has many complex functions and a large number of tissue specific proteins. It is therefore unlikely that there is complete immunological tolerance to all of these antigens (Wucherpfennig 1994). To avoid unnecessary immune activation, access to the brain is restricted to activated lymphocytes that scan the central nervous system. If there is no danger, the T cells leave the brain through the blood-brain barrier (Kleine and Benes 2006). The MHC I and MHC II expressions are very low in normal central nervous tissue, and the low MHC expression probably protects the brain against autoimmunity. In normal microglia cells, MHC II is not detected, but in early stages of experimental allergic encephalitis MHC II is upregulated in microglia cells (Wucherpfennig 1994). Some nerve cells, like Purkinje cells and hippocampal neurons, express high levels of MHC I (Darnell 1998), which renders them particularly susceptible to autoimmune diseases.

1.2 Autoimmunity

Autoimmune diseases occur when the body's immune system attacks its own cells and tissues. Common for the diseases are the presence of circulating autoantibodies and autoreactive lymphocytes in affected tissues. In many autoimmune disorders there are extensive interactions between the brain and the immune system. Several proteins that have important functions in the brain also have a crucial role in the immune system. For instance Toll-like receptors are very important in the innate immune system and flies that lack the Toll gene do not develop dorsal ventral polarity. Several cytokines stimulate lymphocytes, but can also affect important brain

regulated functions like sleep, temperature and appetite. The principal inhibitory synaptic transmitter in the nervous system, GABA, can also be secreted by immune cells and participate in immune protection of the brain [reviewed in (Bhat and Steinman 2009)]. MHC I proteins are involved in activity-dependent signals in developing and mature neurones (Darnell 1998). Common autoimmune diseases are multiple sclerosis, diabetes mellitus, systemic lupus erythematosus and rheumatoid arthritis. Approximately 5 % of the population have an autoimmune disease, and 80 % of these are women [reviewed in (Libert, Dejager et al. 2010)].

Breakdown of self tolerance occurs in autoimmune diseases. The mechanisms that lead to autoimmunity are not well understood, but both genetic and environmental factors play a role. Only a few gene defects have been linked to specific autoimmune diseases. Breakdown of self tolerance can be caused by hormones, viral or bacterial infections, immunological challenges during pregnancy, fetal microchimerism, or it can be X chromosome related (Libert, Dejager et al. 2010). Some autoimmune diseases can be initiated by bacterial or viral infections. In these cases the pathogens share similar structures with the autoantigens. This is called molecular mimicry. For instance, it has been shown that *Campylobacter jejuni* shares a structural homology with the lipo-oligo-saccaride of the peripheral nerve, GM1 ganglioside, and this infection can cause Guillian-Barré syndrome (Ang, Jacobs et al. 2004). Another theory is that incomplete phagocytosis of apoptotic cells leads to release of self antigens and development of autoimmune diseases (Nagata 2010). There is also the possibility that posttranslational modification of proteins has an impact. All 20 primary amino acids have the potential to undergo posttranslational modification. The most common modifications are acetylation, glycosylation, hydroxylation, methylation, phosphorylation, deamidation and citrullination. In some cases such modifications alter the protein so that it is no longer recognized as self. For instance, some patients with rheumatoid arthritis have antibodies towards citrullinated fillagrin, while citrullination and acetylation of myelin basic protein has been associated with multiple sclerosis [reviewed in (Doyle and Mamula 2005)].

Irregularities in the innate immune system are the cause of some very rare autoimmune disorders affecting the brain, but it has also been implicated in Alzheimer's disease (Bhat and Steinman 2009). Autoimmune diseases that implicate the adaptive immune system involve both secretion of specific antibodies and activation of T cells that target the antigens. Multiple sclerosis, neuromyelitis optica and paraneoplastic neurological syndromes are examples of diseases where the adaptive immune system is involved. In T cell mediated autoimmune diseases the proportion of autoaggressive T cells is crucial for whether or not an individual who is predisposed to autoimmunity develops an autoimmune disease. It has also been postulated that if the body develops low-avidity autoaggressive T cells during an initial infection, secondary infections later in life might result in accumulation of high-avidity autoreactive T cells and development of autoimmune disease (Christen, Hintermann et al. 2009).

1.2.1 Autoantibodies

Evolutionary, immunoglobulins have probably been developed for clearance of body waste in the first animals with three germ layers. Some antibodies can react with self-antigens, and these are referred to as autoantibodies. Autoantibodies can be directed towards cell surface membranes or receptors, cytoplasmic proteins or nuclear proteins. Naturally occurring antibodies exist in all vertebrates and have important functions in maintaining tissue homeostasis. They are mainly of the IgG or IgM type, have low affinity and circulate in plasma. Some of them are directed against intracellular and cytoskeletal proteins like anti-tubulin, anti-actin and anti-spectrin [reviewed in (Lutz 2007)].

Not all autoantibodies are beneficial. In many cases upregulation of autoantibodies is associated with autoimmune diseases. In systemic autoimmune disorders antibodies are deposited in affected tissues and cause injury. Many autoantibodies can bind to the surface membranes causing cell destruction. Cell membrane antibodies like thyroid antibodies, phospholipid antibodies and aquaporin-4 antibodies bind to the

cell membrane and activate the complement cascade, with subsequent cell lysis (Lazarus, Parkes et al. 2002; Di Simone, Luigi et al. 2007). Other autoantibodies, like antibodies towards the AChR in myasthenia gravis can bind cell surface receptors, following aggregation and redistribution of the receptors in the membrane. The antibodies block the ligands from binding to the receptors, and the receptors are internalized. This leads to impaired neuromuscular function (Drachman 1994). NMDAR antibodies act in a similar way. They decrease the surface density and synaptic localization of NMDAR clusters via antibody-mediated capping and internalization (Hughes, Peng et al. 2010). Some antibodies, like thyroid stimulating hormone antibodies, bind to receptors and cause constant activation leading to hormone overproduction (Chistiakov 2003). Other autoantibodies form large immune complexes that activate the complement system and cause tissue damage, e.g. in glomerulonephritis (Nangaku and Couser 2005).

In many autoimmune diseases patients harbour antibodies towards intracellular proteins. They serve as important diagnostic tools, but their role in disease mechanisms is unresolved. It has been suggested that autoantibodies to intracellular proteins may bind to cell surface membranes. In some cases this can be caused by cross-reactions between intracellular and membrane antigens as is the case for anti-ribosomal P protein antibodies (Caponi, Anzilotti et al. 2007). Other studies suggest that injury, activation or apoptosis of the cell can translocate a normally intracellular antigen to a site were circulating antibodies could bind to it. It has been reported that nuclear autoantigen translocation can lead to autoantibody opsonisation (marker for phagocytosis), increased dendritic cell phagocytosis and presentation of nuclear antigens (Frisoni, McPhie et al. 2005).

The theory that antibodies are able to penetrate living cells has long been debated, but several autoantibodies are able to penetrate cells (Alarcon-Segovia 2001). Recently, it was demonstrated that Purkinje cells are able to take up IgG and IgM antibodies independent of the immunoglobulin’s reactivity with Purkinje cell antigens, and that uptake of Yo antibodies causes Purkinje cell death in a non-apoptotic way (Hill,

Clawson et al. 2009; Greenlee, Clawson et al. 2010). Further, amphiphysin antibodies can be internalized at the nerve terminals and bind strongly to amphiphysin on the presynaptic side of the nerve terminals (Geis, Weishaupt et al. 2010).

1.3 Antibody avidity

An antibody's ability to bind effectively to its ligand is an important feature of antibodies. The antibody-antigen interaction is a combination of several weak physical forces: van der Waals forces, hydrogen bonding and electrostatic forces. The energy of this antibody-antigen binding is called affinity or avidity. *Affinity* is defined as the force of binding when one single paratop of an antibody binds to its corresponding epitope on the antigen molecule. *Avidity* is the binding force between a multivalent antibody and a multivalent antigen. The measured binding energy between antibodies and their antigens reflect the avidity of antibodies. In many cases the two terms are used interchangeably.

The specificity of antibodies is defined by its relative affinity. Antibodies with high affinity to a specific epitope can bind to similar epitopes with lower affinity. High antigen density is one of the crucial requirements for binding to such epitopes. High antigen density is also important for binding of low affinity antibodies, depending on enhanced avidity provided by bivalent attachment to the antigen (Zuckier, Berkowitz et al. 2000). Antibody avidity is independent of antibody concentration, and an individual's avidity response is partly genetically controlled (Kim and Siskind 1978). The MHC composition, the influence of several genes and the immunoglobulin subclass are all factors that affect the antibody avidity (Steward, Reinhardt et al. 1979; Persson, Brown et al. 1988; Devey, Bleasdale-Barr et al. 1990; Achenbach, Koczwara et al. 2004).

IgG avidity is low in primary infections or early in the disease course. The avidity increases with time as the disease progresses or after secondary infections. Avidity determination can therefore give important knowledge of the nature and phase of the infection. In this way avidity measurements can give an indication of whether the

infection is new or has persisted for a while. Such information is especially valuable for distinguishing recent from old toxoplasmosis infection or rubella infection in pregnant women (Hedman and Seppala 1988; Hedman, Lappalainen et al. 1989). Some naturally occurring autoantibodies have high affinity. Some of these antibodies are directed against IL-1, IL-6 and interferon alpha. The high affinity is a prerequisite to prevent the antibodies from binding to other plasma components and high avidity cytokine binding only occurs when the cytokines are oligomerized (Lutz 2007). In autoimmune diseases the avidity of anti-β2-GPI autoantibodies increased during the course of systemic lupus erythematosus (Cucnik, Kveder et al. 2004). High-avidity antibodies have been associated with disease onset and have been shown to impair nerve fibre regeneration in Guillain-Barré syndrome (Comin, Yuki et al. 2006; Lopez, Zhang et al. 2010). Rabbits immunized with GM1 develop antibodies with lower affinity than what is seen in humans, and this is believed to be the reason why these antibodies do not elicit disease in rabbits (Lopez, Villa et al. 2002). Patients with aquaporin-4 antibodies have heterogeneous affinity, but whether this has any clinical relevance remains to be elucidated (Crane, Lam et al. 2011). Affinity maturation is believed to be clinically less relevant in diseases where avidity of antibodies is not relevant for pathogenicity, e.g. AChR antibodies in myasthenia gravis (De Baets and Stassen 2002).

The most used methods for avidity measurements involve use of chaotrops like NaCl, thiocyanate (e.g. KSCN) or urea (Hedman, Lappalainen et al. 1989; Saalman, Dahlgren et al. 2003). Chaotrops can be added to a solution to break weak, existing antibody-antigen complexes, or the chaotrope can be added to the solution to prevent the formation of low-avidity complexes. The signal ratio between two wells, one with and one without chaotrope, gives the avidity index. Another way of measuring antibody avidity can be surface plasmon resonance. Surface plasmon resonance measures macromolecular interactions, like antigen-antibody interactions, in real time. It detects alterations in the refractive index of the medium surrounding the receptor immobilized on a solid support at the moment of ligand binding, and can be employed to determine kinetic parameters, equilibrium binding constants and

concentration (Laffer, Lupinek et al. 2008; Li, Chen et al. 2008). The benefit of this approach is that the protein is in its native form. When using chaotrophs for avidity measurements, one risks partially denaturing the protein in such a way that the conformational epitopes are altered. With surface plasmon resonance this problem is avoided. However, this technique requires advanced equipment and in many cases chaotrop-avidity assays might be easier, more accessible and cheaper to perform.

1.4 Tumour immunity

The immune system plays an important role in suppressing cancer cells. It protects the host against viral infections, thereby suppressing virus-induced tumours. It also eliminates pathogen infections which can create an inflammatory environment that facilitates tumour development. In addition, the immune system can eliminate tumour cells. Nascent tumour cells often co-express both ligands that activate receptors on innate immune cells and tumour antigens, thereby activating an immune response [reviewed in (Schreiber, Old et al. 2011)]. How important the immune system is for cancer surveillance is illustrated by the high cancer incident ratio in patients with immune deficiency like AIDS (Simard and Engels 2010) and the high levels of skin cancer seen in immunosupressed individuals (Schulz 2009). Common for patients with various forms of immune deficiencies is that they develop cancers that are triggered by previous viral infections. An increase in non-infectious lung, colon, pancreas, kidney, and endocrine system cancers have also been observed in immunosupressed individuals, which supports the idea that the immune system is important for monitoring many forms of malignancies [reviewed in (Vesely, Kershaw et al. 2011)].

Patients with rheumatoid arthritis and other autoimmune diseases have an increased risk of cancer. This may be due to the use of immunesuppressive medications and chronic immune activation, but many polymorphisms that lead to autoimmunity also predispose for cancer (van de Schans, van Spronsen et al. 2010). Recently, it was shown that CD4+ T cells from patients with rheumatoid arthritis have decreased levels of the DNA repair kinase ATM, a protein that is associated with both immune

deficiency and increased risk of cancer (Shao, Fujii et al. 2009). Viral-associated cancers make up for a large proportion of new cancers also in immunocompetent individuals. The most common being EBV-associated lymphomas, HPV-associated cervical cancer and Hepatitis B and C-associated hepatocellular carcinoma (Schulz 2009).

Changes in the cell that may ultimately lead to the development of cancer cells happen all the time in the body. Thus, the immune system's ability to recognize cancer cells as foreign is crucial. The adaptive immune response is important in cancer surveillance. When a healthy cell transforms into a cancer cell, multiple genetic changes occur. This results in different protein expression patterns in the cancer cells. The proteins can be mutated, differently folded, degraded, or in some cases the cells express proteins normally only expressed in other tissues. More than 2000 tumour-associated antigens have been recognized by patient sera, but only a few of these have been associated with cancer in general, which suggests that many immunogenic mutations may be unique for each individual cancer (Anderson and LaBaer 2005). These altered proteins are expressed on the cell surface as tumour antigens that can activate the immune system. In many cases the patients develop antibodies towards these tumour antigens, and these antibodies can be used as tumour biomarkers. Among the antibodies that are common for various forms of cancers are antibodies towards testis-cancer antigens like NY-ESO-1 and antibodies towards mutated forms of tumour suppressor p53 (Vesely, Kershaw et al. 2011). It is not known if the antibodies reflect the underlying immunosurveillance of cancer, or if they have an impact on the clinical outcome of the disease.

Upregulation of fetal proteins or overexpression of proteins normally only expressed in immune privileged sites like the central nervous system can occur. An example of this is small cell lung cancer (SCLC). SCLC is a severe form of cancer that originates from primitive neuroendocrine cells in the lung. SCLC cells express several antigens normally only expressed in the nervous system. All SCLC cells express the neuronal protein HuD (Dalmau, Furneaux et al. 1992; Dalmau, Graus et al. 1995). In addition,

a large proportion of the SCLC cells express other neuronal proteins like VGCC, SOX or recoverin (Kazarian and Laird-Offringa 2011). Some of the patients with SCLC develop antibodies towards these proteins. Such antibody production is linked to a group of autoimmune diseases called paraneoplastic neurological syndromes (PNS), which will be discussed later. The role of these antibodies is not yet clear. There have been reports stating that patients with low titres of Hu antibodies do not develop PNS, but the cancers appear to be smaller, and the patients have a more favourable outcome than Hu-negative patients (Winter, Sekido et al. 1993; Graus, Dalmau et al. 1997; Mason, Graus et al. 1997). Some cases where the SCLC has spontaneously regressed in anti-Hu positive patients have also been reported (Darnell and DeAngelis 1993). This suggests that these antibodies have a role in tumour control.

In recent years a new theory has evolved for the role of the immune system in cancer. It was discovered that tumours formed in immunodeficient mice were more immunogenic than similar tumours that developed in mice with a normal immune system. The immune system not only protects the host against malignancies, but can also facilitate tumour growth. This concept is called immunoediting. Normally, transforming cancer cells will start to express Type I IFNs. These cytokines activate dendritic cells which elicit an anti-tumour response. Other proteins such as stress ligands (e.g. RAE-1 and MICA/B) probably also facilitate activation of the immune system. Activation of T cells is required to effectively eliminate cancer cells (Schreiber, Old et al. 2011).

In some cases the cancer cells can survive the elimination process and enter equilibrium where the adaptive immune system keeps the tumour cells from developing further. In this phase the immunogenicity of the cancer cells can be moulded. In patients with a previous cured cancer, not all cancer cells are necessarily eradicated. Occult cancers can lie dormant for decades. For example, 20-45 % of patients with breast or prostate cancer will have a relapse several years after their

initial disease. Use of immunosuppressants can increase the risk of relapse (Vesely, Kershaw et al. 2011).

CD8+ T cells destroy antigen-presenting cells, leaving behind less immunogenic cells. The tumour cells can remain in the dormant state for the rest of the life, or they can escape and evolve into growing tumours. Alterations of the tumour cell, such as loss of antigens, can lead to reduced immune recognition. Increased resistance to the cytotoxic effects of immunity can lead to tumour growth. Loss of tumour antigen expression can happen if the tumour cells stop expressing tumour antigens, through loss of MHC I or if the tumour cells loose their antigen processing function which abolishes the cell's MHC I-antigen presentation. This generates tumour cells with low immunogenicity, i.e. the tumour cells become "invisible" to the immune system and the cells can grow uncontrolled. The tumour cells can also facilitate their escape from the immune system by producing immunosuppressive cytokines, like the growth factors VEGF and TGF-β or by recruiting immune suppressing cells like T regulating cells (T_{reg}) (Schreiber, Old et al. 2011). In some patients with SCLC the tumour secretes interleukins that skews HuD-specific T cells towards a noncytolytic subtype (Roberts, Deluca et al. 2009). It has also been shown that chronic inflammation can contribute to tumour genesis by generating genotoxic stress, to cancer promotion by inducing cellular proliferation and to cancer progression by enhancing angiogenesis and tissue invasion (Schreiber, Old et al. 2011). Figure 2 gives an illustration of the different steps of immunoediting.

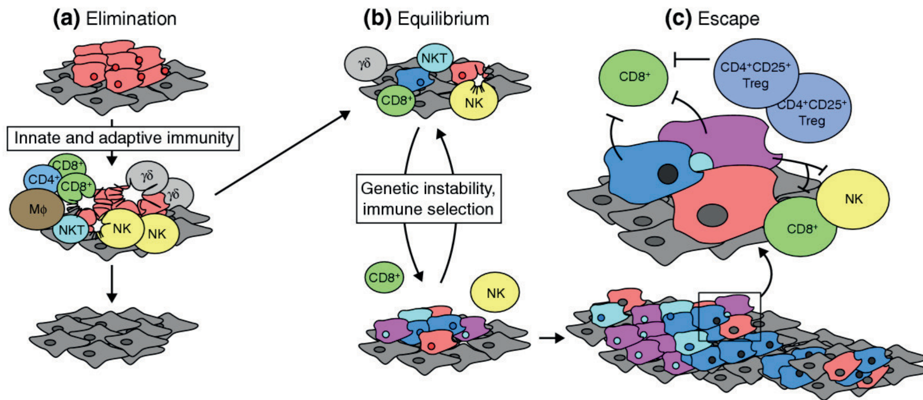


Figure 2: **Immunoediting.** The three stages of cancer immunoediting: elimination, equilibrium, and escape. **(a)** After transformation of normal cells (grey) into cancer cells (red), the cancer cells are attacked by different immune cells (round cells). This may lead to *elimination* of the cancer cells. **(b)** If elimination is unsuccessful, the immune system and the cancer can reach an *equilibrium* in which immune cells keep the cancer in check but cannot remove it completely. The genome of the cancer cells is unstable, and during the elimination phase there is selection of the cancer cells. This can lead to *escape* **(c)**, in which mutated cancer cells can inhibit the immune system and thereby grow without restrictions. $CD4^+$, $CD8^+$, $CD4^+CD25^+$ T_{reg}, γδ and NKT cells are all types of T cells; Mφ cells are macrophages and NK cells are natural killer cells. [From: (Strausberg 2005)].

The tumour promoting inflammation and the protective tumour immunity can probably coexist. Even though pro-inflammatory cytokines, like IL-1β, IL-23 and MyD88, are recruited during tumour induction, other immune components, like IFN-γ, IFN-α/β, IL-12 and T cells, are recruited later in the tumour development. It has also been shown that IL-1β and MyD88 can facilitate recognition of tumour cells undergoing immunogenic death at later stages of the tumour genesis (Vesely, Kershaw et al. 2011). In humans it has been found that cancer patients with tumour infiltrating lymphocytes have improved prognosis (Sato, Olson et al. 2005; Galon, Costes et al. 2006; van Houdt, Sluijter et al. 2008). It has been suggested that even though these T cells have not been able to prevent tumour growth, they may be active in keeping the tumour from spreading to the lymph nodes.

Several forms of immunotherapy are being explored to try to evoke the body's own mechanisms for controlling cancer. One approach is vaccination that elicits strong specific immune responses to cancer antigens like MAGE-3 and NY-ESO-1 (Schreiber, Old et al. 2011). Immunization with HuD DNA has been shown to retard tumour growth in mice (Carpentier, Rosenfeld et al. 1998; Ohwada, Nagaoka et al. 1999). Another approach involves adoptive transfer of in vitro expanded, naturally arising or genetically engineered tumour-specific lymphocytes. Also therapeutic administration of monoclonal antibodies like Rituximab (against CD20 in leukaemia and lymphoma cells) and Herceptin (against HER2 on breast cancer cells), has shown promising results (Schreiber, Old et al. 2011).

2. ATM and CCDC104

Dysregulation of genes that control cell-cycle progression and DNA repair is a hallmark of tumour genesis. These defects also have an impact on neurons under certain conditions. Cell-cycle reactivation in neurons has been associated with degeneration of Purkinje cells and neuronal apoptosis (Staropoli 2008). The DNA repair kinase ATM plays a role both in neurons and in cancer. ATM is activated as a response to double-stranded DNA breaks. Following DNA break, ATM is autophosphorylated, and this activates the protein. ATM activation further triggers an ATM dependent phosphorylation cascade of substrates downstream of ATM (Lavin and Kozlov 2007). ATM can also phosphorylate the p53 tumour suppressor, and the ATM-p53 pathway is involved in maintaining glucose homeostasis (Armata, Golebiowski et al. 2010). Mutations in ATM lead to a rare human disease called ataxia telangiectasia. This disease has its onset in early childhood and is characterized by extreme cellular sensitivity to radiation, predisposition to cancer and neurodegeneration, particularly of the Purkinje cells, with subsequent ataxia. About 30% of all patients with ataxia telangiectasia develop cancer, usually lymphoma, and people that are heterozygous for ATM mutations have increased risk of developing breast cancer. Such patients also express a mild form of immunodeficiency with decreased levels of IgA, IgE and IgG2 (Ball and Xiao 2005).

Little information exists on the coiled-coil domain-containing 104 (CCDC104) protein. It is located on chromosome 2 p16.1, and two different isoforms have been verified. Isoform 1 has 342 amino acids and a molecular weight of 39 kDa, whereas isoform 2 has 367 amino acids and a molecular weight of 42 kDa (Strausberg, Feingold et al. 2002; Clark, Gurney et al. 2003; Hillier, Graves et al. 2005). In addition, there are several unverified isoforms (Ensembl geneID: ENSG00000163001) that code for proteins with estimated molecular weights of 25, 26 and 36 kDa. CCDC104 can be phosphorylated on Ser201 by ATM, which is activated as a response to double-stranded DNA breaks, or ATR (ATM-Rad3-related), which is associated with single-stranded DNA breaks (Matsuoka, Ballif et al. 2007). CCDC104 also has a potential role in the mitogen activated protein kinase (MAPK) pathway. A yeast two-hybrid screen found that CCDC104 interacted with the Rho GTPase RAC1 (Bandyopadhyay, Chiang et al. 2010). RAC1 works upstream of p38MAPK, and p38MAPK can be regulated by ATM and ATR as a response to DNA damage (Reinhardt and Yaffe 2009). Mass spectrophotometric studies have also revealed that CCDC104 can be phosphorylated on Ser85 and Ser147 in testis (Gauci, Helbig et al. 2009; Huttlin, Jedrychowski et al. 2010). These studies further showed that testis-specific phosphorylated proteins in general are involved in meiosis and cell cycle regulation and DNA damage and repair, while the nonphosphorylated testis-specific proteins are enriched in spermatogenesis and microtubule-based movements. This implicates a role for CCDC104 in cell cycle regulation.

3. Paraneoplastic neurological syndromes

PNS are rare side effects of cancer in which the tumour expresses proteins normally only expressed in the nervous system. The tumour proteins are identical to the neuronal proteins, but for unknown reasons these proteins are identified as foreign and an immune attack is mounted. The body produces antibodies and activates T cells towards these proteins in an attempt to control tumour growth. Somehow, these antibodies and T cells cross the blood-brain barrier and cross-react with proteins in the nervous system. This cross-reaction leads to loss of neuronal cells and development of neurological symptoms (Darnell and Posner 2003). Figure 3 gives a schematic illustration of proposed pathogenic mechanisms in PNS.

The most common paraneoplastic antibodies are anti-Hu, anti-Yo, anti-CRMP5, anti-amphiphysin, anti-Ma2 and anti-Ri. If a patient harbours any of these antibodies and shows neurological symptoms, the patients are diagnosed with PNS regardless of the detection of a tumour or not (Graus, Delattre et al. 2004). Tumours commonly involved in PNS express neuroendocrine proteins (e.g. SCLC and neuroblastoma), affect organs with immunomodulatory properties (thymoma) or contain mature or immature neuronal tissues (teratomas). About 3-5 % of all patients with SCLC and 15-20 % of patients with thymoma develop PNS. Less than 1 % of the patients with other types of tumour develop paraneoplastic symptoms (Dalmau and Rosenfeld 2008).

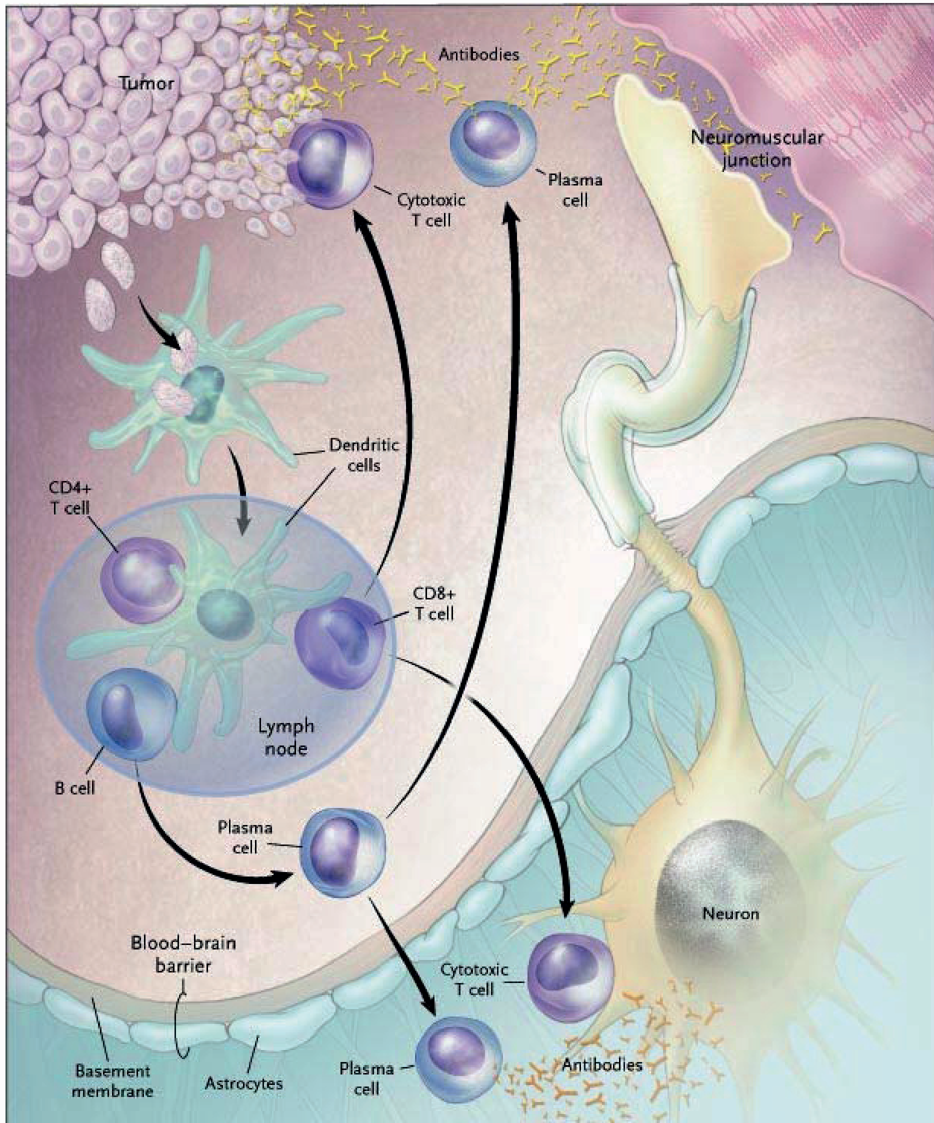


Figure 3: ***Proposed mechanism for paraneoplastic neurological syndromes.*** A tumour outside the nervous system expresses a neuronal protein that is recognized as nonself by the immune system. Dendritic cells (DC) phagocytose apoptotic tumour cells, migrate to the lymph nodes and activate antigen-specific CD4+, CD8+ and B cells. The B cells mature into antibody-producing plasma cells. The antibodies and the cytotoxic T cells slow the tumour growth, but they also react with the nervous system. Some antibodies react with peripheral neurones like the neuromuscular junction. Others cross the blood-brain-barrier and attack antigen expressing neurons. Image from (Darnell and Posner 2003).

Although paraneoplastic antibodies are detected in less than 1 % of all tumour patients, the detection of these antibodies is an important diagnostic tool. The clinical manifestations of PNS often appear early in the cancer development, while the cancer is still small (Graus, Keime-Guibert et al. 2001; Shams'ili, Grefkens et al. 2003), and the type of antibody can give indications as to where the cancer originated (Darnell and Posner 2003; Pittock, Kryzer et al. 2004). The tumour can then be identified at an earlier stage, specific cancer treatment can be started, and the chance for better recovery is increased. Anti-Hu and anti-Yo are the most common antibodies found (Giometto, Grisold et al. 2010). Anti-Hu is often associated with SCLC, while anti-Yo is normally associated with ovarian and breast cancer (Manley, Smitt et al. 1995; Monstad, Knudsen et al. 2009). As the detection of paraneoplastic antibodies precedes the cancer in about 2/3 of the cases, routine follow-up of patients with paraneoplastic antibodies where no cancer is detected should be performed for at least 4 years (Graus, Keime-Guibert et al. 2001; Vedeler, Antoine et al. 2006; Dalmau and Rosenfeld 2010; Giometto, Grisold et al. 2010). Table 1 lists an overview of the most common paraneoplastic antibodies and their associated cancers.

Table 1: ***Overview of paraneoplastic antibodies and associated cancer***

Antibody	Syndrome	Cancer
Hu	PEM, PCD, myelitis, PSN, autonomic dysfunction	SCLC, other
Yo	PCD	Ovarian, Breast
CRMP5	PEM, PCD, chorea, optic and peripheral neuropathy	SCLC, thymoma, other
Ma2	Different forms of encephalitis	Germ-cell tumours of the testis, other solid tumours
Ri	PCD, brainstem encephalitis, POM	Breast, ovarian, SCLC
Amphiphysin	Stiff person syndrome, PEM	Breast

PEM – paraneoplastic encephalomyelitis, PCD – Paraneoplastic cerebellar degeneration, PSN – paraneoplastic sensory neuronopathy, POM – paraneoplastic opsoclonus- myoclonus, SCLC- small cell lung cancer

PNS is a heterogeneous group of syndromes, and therefore Graus et al (2004) set up a list of criteria to define PNS. This study divided PNS into definite and possible PNS based on the detection of paraneoplastic antibodies, neurological symptoms and presence or absence of cancer. Syndromes that are often associated with cancer and paraneoplastic antibodies are defined as classical PNS. Among these are paraneoplastic encephalomyelitis, paraneoplastic limbic encephalitis, paraneoplastic cerebellar degeneration, paraneoplastic sensory neuronopathy and paraneoplastic opsoclonus-myoclonus. Lambert-Eaton myasthenic syndrome and dermatomyositis are also characterized as classical PNS, but they are less often associated with cancer (Vedeler, Antoine et al. 2006). Non-classical syndromes are diseases in which the patients show diverse neurological symptoms as a response to cancer, but paraneoplastic antibodies are not always detected (Graus, Delattre et al. 2004). Giometto et al. (2010) reported that 18 % of all patients with definite PNS had no paraneoplastic antibodies.

PNS can affect all parts of the central or peripheral nervous system. Anti-Yo mediated paraneoplastic cerebellar degeneration especially affects the Purkinje cells in the cerebellum leading to ataxia due to loss of Purkinje cells (Storstein, Krossnes et al. 2009). In limbic encephalitis, the medial temporal lobes are affected. The symptoms can be psychological (anxiety, depression), but also short-time memory loss and dementia. Paraneoplastic limbic encephalitis is associated with anti-Hu, anti-Ma2, anti-CRMP5, anti-amphiphysin and anti-Ri (Vedeler, Antoine et al. 2006; Grisold, Giometto et al. 2011). Paraneoplastic encephalomyelitis affects most of the central nervous system, especially the limbic system, cerebellum, basal ganglia, brainstem and spinal cord, and is associated with anti-Hu, anti-CRMP5, anti-Ri, anti-Ma2 and anti-amphiphysin (Graus, Delattre et al. 2004; Rosenfeld and Dalmau 2010).

Sensory and autonomic nerves can also be affected. Paraneoplastic sensory neuronopathy can affect limb, trunk and cranial nerves, and the patients complain of pain, numbness and sensory deficits. This is most often associated with anti-Hu or anti-

CRMP5. Paraneoplastic opsoclonus-myoclonus affects eye movement, often followed by myoclonus and truncal ataxia and is most often associated with anti-Ri, anti-Hu, anti-amphiphysin or anti-Ma2 (Vedeler, Antoine et al. 2006; Rosenfeld and Dalmau 2010).

Common for most patients with PNS of the central nervous system is the rapid development of symptoms and signs of inflammation in the CSF, such as pleocytosis, increased protein concentration, high IgG index and CSF-specific oligoclonal bands. Some of these bands represent Hu or Yo antibodies (Storstein, Monstad et al. 2004). Infiltrates of mononuclear cells, neurophagic nodules, neuronal degeneration, microglia proliferation and gliosis are present, such as in paraneoplastic cerebellar degeneration (Storstein, Krossnes et al. 2009). Patients with antibodies against intracellular antigens often have CD4+ and CD8+ T cell infiltrates in the brain (Rosenfeld and Dalmau 2010). Several studies indicate that PNS is T cell mediated. Activated CD4+ T cells have been found in the cerebrospinal fluid of patients with paraneoplastic cerebellar degeneration (Albert, Austin et al. 2000), while cytotoxic T cells that recognize CDR2 have been found in the blood of anti-Yo positive patients with paraneoplastic cerebellar degeneration (Albert, Darnell et al. 1998; Tanaka, Tanaka et al. 2001; Santomasso, Roberts et al. 2007). However, the functions of the cytotoxic T cells in PNS remain uncertain. Ma1-activated CD4+ cells have been found to induce encephalomyelitis in mice (Pellkofer, Schubart et al. 2004). Tani et al. (2008) found that SCLC patients with LEMS and Hu or Yo antibodies had lower levels of T_{reg}^{Foxp3+} cells than SCLC patients without PNS. They concluded that low levels of T_{reg} cells may be caused by an immune regulatory dysfunction in PNS (Tani, Tanaka et al. 2008). It has also been demonstrated that epithelial ovarian cancer patients with a high CD8+/ T_{reg} ratio have improved prognosis (Sato, Olson et al. 2005).

Many tumours associated with PNS express one or more of the onconeural antigens, and some patients even harbour paraneoplastic antibodies without developing neurological symptoms (Storstein, Monstad et al. 2011). Why some patients develop

PNS, while others do not, remains uncertain, but the HLA haplotype has been suggested to be important. The frequency of the HLA-DQ2^{+} haplotype is higher in PNS patients with anti-Hu (de Graaf, de Beukelaar et al. 2010), while the frequency of HLA-A2.1, HLA-A24 or HLA-B27 haplotypes is higher in patients with anti-Yo mediated PCD (Albert, Darnell et al. 1998; Sutton, Steele et al. 2004; Santomasso, Roberts et al. 2007; Carpenter, Vance et al. 2008).

Some studies suggest that tumour expression of onconeural antigens invoke the body's tumour immunity response. Patient with paraneoplastic antibodies often have smaller tumours, and in some cases the tumour disappears (Darnell and DeAngelis 1993; Mason, Graus et al. 1997). However, the loss of neurones is permanent, and in many cases the neuronal damage has been so devastating that the patients have severely reduced life quality or sometimes die as a consequence of the paraneoplastic disease itself.

It is difficult to treat the neurological manifestations in PNS since the neuronal damage usually is irreversible. In a study of anti-Hu positive encephalomyelitis patients only 7 % showed neurological improvement while 47 % remained stable after tumour remission (Sillevis Smitt, Grefkens et al. 2002). The best way of combating PNS is tumour removal. However, even if the tumour is successfully removed the antibodies may persist, but usually in low titres. Immunesuppressive treatment is usually beneficial in patients with PNS that affect the peripheral nerves (e.g. Lambert Eaton myasthenic syndrome, myasthenia gravis and stiff person syndrome) and in patients with antibodies directed towards ion channels and surface antigens. PNS that affect the central nervous system is more difficult to treat. Corticosteroids, intravenous IgG and plasma exchange are often used as immunotheraphy in PNS (Vedeler, Antoine et al. 2006; Rosenfeld and Dalmau 2010; Grisold, Giometto et al. 2011).

3.1 Paraneoplastic cerebellar degeneration

Paraneoplastic cerebellar degeneration usually affects the entire cerebellum. The symptoms appear subacutely within weeks with dizziness, nausea and vomiting followed by gait unsteadiness. The symptoms rapidly develop into ataxia, diplopia, dysarthia and dysphagia. Further on in the disease course, cerebellar atrophy can be detected by magnetic resonance imaging. The symptoms usually occur before detection of the tumour. Yo, Tr, VGCC and Zic antibodies are usually associated with paraneoplastic cerebellar degeneration, but patients with Hu, CRMP5, amphiphysin, Purkinje cell cytoplasmic antibody type 2 and ANNA-3 antibodies have also been described (Mason, Graus et al. 1997; Dalmau and Rosenfeld 2008; Rosenfeld and Dalmau 2010). Some patients with Hodgkin's disease and cerebellar ataxia harbour antibodies towards mGluR1 (Sillevis Smitt, Kinoshita et al. 2000). In many cases the paraneoplastic diagnosis precedes the tumour diagnosis (Mason, Graus et al. 1997; Shams'ili, Grefkens et al. 2003).

One of the most prominent markers of paraneoplastic cerebellar degeneration is loss of Purkinje cells. Often the granule cells are lost as well. CD8+ cells are found in the cerebellum. This may be associated with inflammatory infiltrates in the cerebellar cortex, deep cerebellar nuclei and inferior olivary nuclei, and diffuse microglial activation has been observed. In some cases there are changes in the corticospinal and spinocerebellar tracts and dorsal columns as well (Storstein and Vedeler 2007; Dalmau and Rosenfeld 2008; Storstein, Krossnes et al. 2009).

The median survival for patients with paraneoplastic cerebellar degeneration is 13-22 months. Longer survival time has been observed in patients with breast cancer than in patients with tumours in the female genital organs (100 months vs. 22 months) (Rojas, Graus et al. 2000; Shams'ili, Grefkens et al. 2003; Storstein and Vedeler 2007). While patients with anti-Hu associated paraneoplastic cerebellar degeneration have as little as 7 months median survival, patients with Ri and Tr antibodies usually have longer survival (Shams'ili, Grefkens et al. 2003). This probably reflects the

underlying cancer, as anti-Hu is usually associated with SCLC. Patients with paraneoplastic cerebellar degeneration and ovarian cancer often have smaller tumours than patients without PCD, but the cancers are still at an advanced stage at the time of diagnosis. A few patients demonstrate improvement of symptoms if treated with intravenous IgG after tumour removal if the treatment is started early (Shams'ili, Grefkens et al. 2003; Schessl, Schuberth et al. 2010).

3.2 Paraneoplastic antibodies and antigens

Several neuronal antibodies are associated or may be associated with cancer (Raspotnig, Vedeler et al. 2011). The well-characterized paraneoplastic antibodies that are associated with cancer in most cases are anti-Hu, anti-Yo, anti-CRMP5, anti-Ri, anti-amphiphysin and anti-Ma1/2 as well as anti-Tr and anti-recoverin (Musunuru and Darnell 2001; Sutton 2002; Graus, Delattre et al. 2004).

There are also several other neuronal antibodies that are associated with neurological diseases, but less often with tumour. An example is AChR antibodies that are associated with myasthenia gravis. Thymoma and AChR antibodies are found in approximately 15% of the cases. Titin antibodies are also associated with thymoma in these patients (Vincent, Willcox et al. 1998). VGCC antibodies are associated with Lambert Eaton myasthenic syndrome, and SCLC is found in about 60% of the cases (Takamori 2008). Antibodies against the VGKC complex, such as anti-LGI1 and anti-caspr2 are associated with limbic encephalitis and Morvan syndrome respectively, but cancer is rarely the underlying cause in these patients (Irani, Bien et al. 2011). Furthermore, NMDAR antibodies are associated with brainstem encephalitis, and in women older than 18 years teratomas may be found in 50% of the cases, whereas tumours are rarely found in children with NMDAR encephalitis. AMPA receptor, GABA receptor and Glycine receptor antibodies may also be found in patients with encephalitis and in some of these an underlying cancer can be detected (Rosenfeld and Dalmau 2010).

In the following a brief overview of the well-characterized paraneoplastic antibodies and their respective onconeural antigens will be outlined.

The proteins involved in PNS are almost exclusively expressed in immuneprivileged tissues like the brain and the testis. Onconeural antigens are found in the tumour of all patients with antibody-mediated PNS, and many cancer patients without antibodies also express the same proteins. The genes that code for the onconeural proteins are not mutated in the tumour, so PNS can not be explained by infrequent expression or by mutations in the genes encoding for these antigens (Darnell and Posner 2003; Totland, Aarskog et al. 2011). Most paraneoplastic antibodies are directed towards intracellular proteins and common for many of these antibodies is that they are directed towards the functional domains of the onconeural proteins (Sakai, Ogasawara et al. 1993; Sodeyama, Ishida et al. 1999; Geis, Weishaupt et al. 2010).

Hu and Ri proteins are neuron-specific RNA binding proteins. RNA binding proteins are important regulators of gene expression and act at all levels: transcription, processing, transport, localization, stability and translation of RNA. In humans, there are four members of the Hu family, HuR, which is non-neuronal, and HuB, HuC and HuD, which are expressed in the brain (Musunuru and Darnell 2001). Hu antibodies recognize all members of the Hu family and specifically bind to the first two RNA-binding motifs of the Hu proteins (Manley, Smitt et al. 1995; Sodeyama, Ishida et al. 1999). This could suggest that Hu antibodies may affect the RNA-binding properties of Hu. Hu proteins have been localized to the nuclei, with weaker staining of the cytoplasm in central and peripheral nervous tissue (Dalmau, Furneaux et al. 1992). Others have reported that in the dorsal root ganglia, Hu proteins are mainly cytoplasmic and associated with the Golgi apparatus and mitochondria. Nuclear localization has been found in some, but not all, adult sensory neurons, especially in the nuclear pores, suggesting a role in nucleocytoplasmic shuttling (Fornaro, Raimondo et al. 2007).

HuD is the antigen that is mainly involved in paraneoplastic diseases, and it is expressed by all SCLC (Dalmau, Furneaux et al. 1992). Hu antibodies have been found in up to 25 % of all SCLC patients, and about 15 % of the patients develop Hu antibodies without showing neurological symptoms (Graus, Dalmau et al. 1997; Mason, Graus et al. 1997; Musunuru and Darnell 2001; Monstad, Drivsholm et al. 2004). These patients have limited-stage disease and some have improved clinical diagnosis. Hu antibodies are associated with various types of PNS, such as encephalomyelitis, and the patients may have symptoms where the peripheral nerves, cerebellum, brainstem and the limbic system are affected (Graus, Keime-Guibert et al. 2001; Musunuru and Darnell 2001).

The development of a SCLC mouse model has given new understanding of HuD expression in SCLC (Meuwissen, Linn et al. 2003). All mouse SCLC tumours also express HuD. Interestingly, 14 % of the mice developed antibodies towards HuD, and Hu antibodies could arise up to 100 days before the cancer was clinically detectable (Kazarian, Calbo et al. 2009).

Ri antibodies are associated with paraneoplastic opsoclonus-myoclonus and most often seen in patients with SCLC or gynaecological cancer. Anti-Ri recognizes two proteins termed Nova-1 (50-55 kDa) and Nova-2 (70-80 kDa). Nova proteins are mainly localized to the nucleus, but can also be found in somato-dendritic compartments. Nova-1 is expressed in hindbrain and spinal cord, while Nova-2 can be found where Nova-1 is not expressed. The Nova proteins contain 3 RNA-binding motifs known as the KH-domains (Musunuru and Darnell 2001). Ri antibodies recognize the KH3 domain of Nova-1 and inhibit the RNA-binding properties *in vitro* (Buckanovich, Yang et al. 1996).

CRMP5 is a 62 kDa protein that is mainly localized to the dendrites of oligodendro-cytes and neurones in the cerebral cortex, hippocampus and cerebellum (Fukada, Watakabe et al. 2000; Bretin, Reibel et al. 2005). CRMP5 has been found to regulate neurite outgrowth in developing neurones and is also important for proper development of the Purkinje cells during dendritic branching of Purkinje cells (Brot,

Rogemond et al. 2010; Yamashita, Mosinger et al. 2011). CRMP5 antibodies are associated with cerebellar ataxia, chorea, myasthenia gravis, Lambert Eaton myasthenic syndrome, and peripheral neuropathy. SCLC and thymoma are the two types of cancer that are most often associated with CRMP5 antibodies (Monstad, Drivsholm et al. 2008; Honnorat, Cartalat-Carel et al. 2009).

Ma antibodies recognize two homologous proteins, Ma1 and Ma2. Ma1 is a 37 kDa protein that is expressed in brain and testis, and the protein is mainly localized to the nuclei and nucleoli and to a lesser degree to the cytoplasm (Dalmau, Gultekin et al. 1999). Anti-Ma1 mainly attacks the brainstem and cerebellum and is associated with several different forms of cancer (Dalmau, Gultekin et al. 1999). Ma2 is a 40 kDa protein that shows nuclear and cytoplasmic distribution in neurons in the brain, spinal cord, dorsal root ganglia, intestinal autonomic neurons and adrenal medullary ganglion. Some neurons in the cerebrum also show cytoplasmic distribution (Voltz, Gultekin et al. 1999; Sahashi, Sakai et al. 2003). Ma2 antibodies are mainly associated with testicular cancer and brain stem encephalitis or limbic encephalitis (Voltz, Gultekin et al. 1999). Anti-Ma2 has also been reported in breast cancer (Sahashi, Sakai et al. 2003). Ma2 is expresses by most small intestine neuroendocrine tumours, and about 50 % of these harbour Ma2 antibodies (Cui, Hurtig et al. 2011). The functions of Ma proteins are largely unknown, but it has recently been shown that Ma1 promotes neuronal cell death through it's BH3-like sequence (Chen and D'Mello 2010). Adoptive transfer of a Ma1 reactive Th1 effector CD4+ T cells induced encephalomyelitis in rats, but neuronal degeneration was not induced (Pellkofer, Schubart et al. 2004).

Amphiphysin is localized to the cytoplasmic side of the synaptic terminals, and shows a widespread distribution throughout the central nervous system (Lichte, Veh et al. 1992). Amphiphysin is also expressed in normal testis and in breast tumours, but low levels of amphiphysin have also been observed in normal tissue such as breast tissue (Floyd, Butler et al. 1998). Amphiphysin antibodies are associated with stiff person syndrome, and most commonly found in association with breast cancer

(Floyd, Butler et al. 1998). The antibodies are directed towards the SH3 domain of amphiphysin leading to synaptic inhibition (Geis, Weishaupt et al. 2010). There have been many unsuccessful attempts to make an animal model for PNS, but anti-amphiphysin is an exception. In a recent report, purified plasma IgG from patients with amphiphysin antibodies and stiff person syndrome were injected into the subarachnoid space of rats. The rats subsequently developed symptoms similar to those seen for stiff person syndrome (Geis, Weishaupt et al. 2010).

Paraneoplastic antibodies are mainly of the IgG1 subclass, a subclass that can fix complement. Lower levels of IgG2, IgG3 and IgG4 have also been reported in some patients with PNS (Amyes, Curnow et al. 2001; Greenlee, Boyden et al. 2001). Many patients with PNS are not positive for the well-characterized paraneoplastic antibodies (Giometto, Grisold et al. 2010). This may be because they harbour so far unidentified antibodies. It is therefore important to search for new antibodies that may be clinically significant for PNS.

It has been speculated whether the presence of paraneoplastic antibodies is associated with improved survival. Some studies indicate that patients with SCLC and anti-Hu have smaller tumours and that the tumours are restricted to the chest (Mason, Graus et al. 1997). Other studies indicate that these patients show better response to therapy and improved survival (Winter, Sekido et al. 1993; Graus, Dalmau et al. 1997). Patients with CRMP5 antibodies have longer survival than patients with Hu antibodies (Honnorat, Cartalat-Carel et al. 2009). A study of 200 SCLC patients showed no correlation between the presence of Hu or VGCC antibodies and improved survival (Monstad, Drivsholm et al. 2004). Furthermore, there has not been any correlation between the presence of Ri, CRMP5 or any paraneoplastic antibodies and survival for SCLC patients (Knudsen, Monstad et al. 2006; Monstad, Drivsholm et al. 2008; Monstad, Knudsen et al. 2009). In a study of patients with small intestine neuroendocrine tumours, those that harboured Ma2 antibodies had a lower survival rate and were more prone to tumour recurrence than those without Ma2 antibodies (Cui, Hurtig et al. 2011).

3.2.1 Yo antibodies

Sera from patients with paraneoplastic cerebellar degeneration have been shown to react with proteins of 34 (CDR1) and 62 kDa (CDR2) in Purkinje cell extract (Cunningham, Graus et al. 1986; Furneaux, Dropcho et al. 1989), and antibodies towards these antigens are called Yo antibodies. They are mainly associated with ovarian or breast cancer, but they have also been found in patients with adenocarcinomas and lymphomas. Yo antibody frequency has been associated with the FIGO stage of the tumour. With higher FIGO stage, both the frequency of Yo positive patients and the amount of antibody increased (Monstad, Storstein et al. 2006).

Most patients with Yo antibodies are women, but some cases of men with Yo antibodies have also been reported (Debes, Lagarde et al. 2007; Matschke, Kromminga et al. 2007). Many of the patients have intrathecal production of Yo antibodies (Stich, Graus et al. 2003; Storstein, Monstad et al. 2004). Monstad et al. (2006) found by using a sensitive immunoprecipitation technique that 2.3 % of the patients with ovarian cancer and 1.6 % of the patients with breast cancer in their cohort harboured Yo antibodies. Yo antibodies have also been found several years after removal of the initial tumour without recurrence of a new cancer (Shams'ili, Grefkens et al. 2003) .

Yo antibodies most commonly appear alone (Pittock, Kryzer et al. 2004; Storstein, Monstad et al. 2011). Antibodies against nuclear antigens have been found in 37 % and cytoplasmic antibodies in 42 % of Yo positive sera, but no specific correlation was detected (Aguirre-Cruz, Charuel et al. 2005). However, more patients with PNS harboured nuclear antigen antibodies compared to the general population, which suggests that PNS patients have a higher risk of developing autoimmune diseases (Aguirre-Cruz, Charuel et al. 2005). Amyes et al. (2001) reported that Yo antibodies were restricted to the IgG1 subclass, while others have observed lower levels of IgG2 and IgG3 in co-existence with IgG1 (Greenlee, Boyden et al. 2001). Interestingly,

one study has reported that 2 of 6 Yo-positive patients co-expressed IgG and IgM Yo isotypes, while one patient had IgG and IgA Yo isotypes (Smith, Finley et al. 1988). This suggests that the immune reaction in patients with Yo antibodies follows a normal antibody maturation pattern, but that the clinical symptoms associated with the antibodies are first detected later in the disease progress.

In immunohistochemical staining if brain sections Yo antibodies mainly bind to the cytoplasm of Purkinje cells but staining of cytoplasmic elements in hippocampal neurons, spinal cord neurons, the dorsal root ganglion, the nerve root, and Schwann cells in peripheral nerves have been reported (McKeon, Tracy et al. 2011). Ultrastructural studies have shown that Yo antibodies bind to the ribosomes of rough endoplasmatic reticulum and to free ribosomes in Purkinje cells (Hida, Tsukamoto et al. 1994). Anti-Yo recognizes the leucine-zipper motif of CDR2, and it has been suggested that Yo antibodies binding to this motif may affect gene transcription by inhibiting binding to a suitable partner protein (Sakai, Ogasawara et al. 1993). Recently, Greenlee et al. (2010) showed that Purkinje cells incorporate IgG, and that Yo antibodies accumulate in the cells and trigger Purkinje cell death in a nonapoptotic manner.

The exact function of Yo antibodies is not understood. Several studies indicate that Yo antibodies alone are not sufficient to cause disease. Establishing animal models for anti-Yo mediated paraneoplastic cerebellar degeneration have been unsuccessful. Trials where Yo antibodies were injected into the brain (in occipital cerebellar parenchyma or frontal horn of the lateral ventricle) showed that anti-Yo was taken up by the Purkinje cells, but Yo antibodies were unable to induce neurological symptoms (Graus, Illa et al. 1991; Tanaka, Tanaka et al. 1994). Immunization with recombinant CDR2 protein resulted in the production of high titer of Yo antibodies without inducing neurological symptoms in different MHC-strains of mice (Tanaka, Tanaka et al. 1994; Tanaka, Tanaka et al. 1995). Further, neither injection of CDR2 reactive lymphocytes in the brain, nor intravenous injection caused neurological damage (Tanaka, Tanaka et al. 1995).

Activated T cells probably play a part in the pathogenesis. Activated CD4+ T cells have been found in the cerebrospinal fluid of patients with paraneoplastic cerebellar degeneration, and treatment with immunosuppressants reduced the number of T cells and stabilized the neurological symptoms (Albert, Austin et al. 2000). Storstein et al. (2009) reported that patients with Yo and paraneoplastic cerebellar degeneration had loss of Purkinje cells and microglia activation. CD8+ T cells were found in cerebellar parenchyma, brainstem and medulla oblongata, but B cells or IgG were not found in the cerebellum. Some patients with paraneoplastic cerebellar degeneration and HLA-A2.1 haplotype harbour cytotoxic T cells that can lyse CDR2 expressing HeLa cells. In this study, purified T cells from these patients were incubated with dendritic cells pulsed with CDR2 epitopes. The most promising epitope was located at the amino acids 289-297 (Albert, Darnell et al. 1998). Other studies have not been able to replicate these results.

Sutton et al. (2004) found that patients with paraneoplastic cerebellar degeneration and anti-Yo express the HLA-A2.1 haplotype, but they found no cytotoxic T cells reactive with CDR2 epitopes. Furthermore, they found that only 2 of 9 patients had tumour infiltrating T cells. In another study, no CDR2-specific CD8+ T cells were found in paraneoplastic cerebellar degeneration (Carpenter, Vance et al. 2008). However, results from one study indicate that the most promising HLA-A2.1 T cell epitope is located at aa 290-298, not 289-297. The cytotoxic T cells that recognized aa 290-298 could also bind aa 289-297, but with lower binding affinity (Santomasso, Roberts et al. 2007). This may account for the different results. T cell clones that recognise the 290-298 epitope were also able to lyse CDR2-expressing tumour cell lines (Santomasso, Roberts et al. 2007). One study found that 2 of 3 patients with paraneoplastic cerebellar degeneration and the HLA-A24 or HLA-B27 haplotype had cytotoxic T cell activity towards a peptide (AYRARALEL) located at aa 242 (Tanaka, Tanaka et al. 2001).

4. The CDR proteins

Expression cloning studies with sera from patients with Yo antibodies have lead to the identification of three cerebellar degeneration-related (CDR) antigens, CDR1, CDR2 and CDR2L.

4.1 CDR1

CDR1, also known as CDR34, was first identified by Dropcho et al. (1987). The gene is located at Xq27.1-q27.2 near the fragile X syndrome locus, FRAXA, and the entire protein is encoded for by a single exon (Chen, Rettig et al. 1990; Siniscalco, Oberle et al. 1991). It is highly conserved among mouse and humans. CDR1 is 262 amino acids long and the molecular weight is approximately 34 kDa. It is a unique protein and contains 34 inexact hexamer amino acid repeats. These repeats compose more than 90 % of the protein (Dropcho, Chen et al. 1987). Tandem repeats are common in many parasite proteins and are quite immunogenic (Rubio, Bolchi et al. 2009; Dangoudoubiyam, Vemulapalli et al. 2010), maybe due to increased antibody avidity caused by bivalent interactions with the repetitive segments of the proteins (Valiente-Gabioud, Veaute et al. 2010).

CDR1 mRNA is strongly expressed in human cerebellar cortex and cerebral hemisphere cortex, while in mouse the expression is stronger in the cerebellum than in the cerebrum. Small amounts of CDR1 mRNA have been found in human lung, kidney and heart muscles, but not in mice. High levels of CDR1 mRNA has also been found in several neuroblastoma cell lines, in renal cell carcinoma lines and in astrocytoma, melanoma and lung carcinoma lines (Dropcho, Chen et al. 1987). The CDR1 protein is strongly expressed in Purkinje cells, and Western blot analysis has shown that CDR1 is expressed in tumour tissue from patients with paraneoplastic cerebellar degeneration, but not in tumour tissue from patients without paraneoplastic cerebellar degeneration, or in normal tissue (Furneaux, Dropcho et al. 1989). CDR1

mRNA upregulation has also been associated with other neurodegenerative disorders like prion diseases (Satoh and Yamamura 2004).

4.2 CDR2

CDR2, also known as CDR62, was first cloned by Sakai et al. (1990). It is a 454 aa long protein with an estimated molecular weight of 52 kDa, but various studies have reported it to be 52-62 kDa (Furneaux, Rosenblum et al. 1990; Sakai, Mitchell et al. 1990; Corradi, Yang et al. 1997; Darnell, Albert et al. 2000). The structure and function of the protein is largely unknown, but the protein contains a leucine-zipper motif at aa 172-192, suggesting that it might be involved in transcriptional regulation (Fathallah-Shaykh, Wolf et al. 1991). Proteins that contain a leucine-zipper motif often interact with other leucine-zipper proteins and form homodimers or heterodimers through the leucine-zipper motifs. These proteins often participate in transcriptional regulation. CDR2 lacks the typical leucine-zipper DNA binding motifs, but contains two putative DNA binding domains and has been shown to bind DNA (Fathallah-Shaykh, Wolf et al. 1991).

CDR2 mRNA is detected in many tissues, especially testis and spleen, but until now protein expression has only been reported in testis, brain and tumour tissue from patients with paraneoplastic cerebellar degeneration and ovarian or breast cancers, as well as in renal cancer (Furneaux, Rosenblum et al. 1990; Corradi, Yang et al. 1997; Balamurugan, Luu et al. 2009). Further studies have revealed mRNA and protein expression in the Purkinje cells, deep cerebellar nuclei, the brain stem and some cortical neurons, but not in hippocampus, basal ganglia and most of the neocortex (Corradi, Yang et al. 1997). In the testis, the CDR2 protein was located to the outermost cell layer in the seminiferous tubules, probably in spermatogonia (the least differentiated type in the germ-cell layer) (Corradi, Yang et al. 1997). The differences between CDR2 mRNA expression and protein expression suggest posttranslational regulation of the CDR2 protein expression. Further, Corradi et al. (1997) found that even though Yo antibodies recognize several CDR proteins, only the *CDR2* gene is transcribed in tumours from patients with paraneoplastic cerebellar degeneration.

This implicates CDR2 as the target in anti-Yo mediated paraneoplastic cerebellar degeneration. However, they only examined three patients. Furneaux et al. (1989) identified one patient with ovarian tumour which expressed CDR1 in addition to CDR2 (Furneaux, Dropcho et al. 1989), so one can not rule out that other tumour samples can express other CDR proteins.

Balamurugan et al. (2009) found that 54 % of the papillary renal cell carcinomas expressed CDR2. This is similar to the findings for breast and ovarian cancers where 25 % of the breast cancers and more than 60 % of the ovarian cancers expressed CDR2 protein, while normal ovarian tissue did not (Darnell, Albert et al. 2000). Even more interesting is it that they observed low levels of CDR2 in normal kidney (Balamurugan, Luu et al. 2009). CDR2 protein expression was similar in several breast carcinoma cell lines and in HeLa, colorectal, hepatoma and osteosarcoma cell lines, as it was in ovarian adenocarcinoma tumour cell lines. This indicates that CDR2 is more widely expressed than previously reported.

The function of CDR2 is largely unknown, but there is some evidence of that CDR2 can homodimerize through its leucine-zipper motif (Takanaga, Mukai et al. 1998; Okano, Park et al. 1999), and that it has transcriptional activity (Takanaga, Mukai et al. 1998; O'Donovan, Diedler et al. 2010). CDR2 has been associated with regulation of genes involved in chromosomal, chromatin and nucleosome regulation as well as in cell cycle and mitotic biology (O'Donovan, Diedler et al. 2010). It is speculated whether anti-Yo can bind to the leucine-zipper motif of CDR2, inhibit homodimerization and repress gene transcription. CDR2 has been found to interact with the serine/threonine kinase PKN that phosphorylates CDR2 (Takanaga, Mukai et al. 1998). In addition CDR2 has been found to interact with several proteins involved in transcriptional regulation, like two members of the MORF4 family (MRG X and MRG15) (Sakai, Shirakawa et al. 2002; Sakai, Kitagawa et al. 2004), NF-κ B (Sakai, Kitagawa et al. 2001) and PHD1 (Balamurugan, Luu et al. 2009). The transcriptional activities of these proteins are down-regulated in the presence of CDR2 (Sakai, Kitagawa et al. 2001; Sakai, Shirakawa et al. 2002; Sakai, Kitagawa et

al. 2004; Balamurugan, Luu et al. 2009), while presence of Yo antibodies abolishes this down-regulation (Sakai, Kitagawa et al. 2004).

CDR2 has been found to interact with c-Myc in Purkinje cells (Okano, Park et al. 1999). C-Myc is a potent transcription factor regulating many genes, and c-Myc is involved in several different processes like cell transformation, cell proliferation and apoptosis (Zornig and Evan 1996). CDR2 has recently been found to be upregulated during mitosis (O'Donovan, Diedler et al. 2010). In the interphase CDR2 is localized to the cytoplasm, while c-Myc resides in the nucleus. Confocal studies have shown that during mitosis, the nuclear envelope dissolves and c-Myc and CDR2 colocalize proximal to the spindle poles (O'Donovan, Diedler et al. 2010). CDR2 is important for proper spindle formation as CDR2 knockdown resulted in multipolar spindle formation. After mitosis CDR2 is rapidly degraded by APC/C-mediated polyubiquitination and proteosomal degradation. (O'Donovan, Diedler et al. 2010). CDR2 has three cell cycle regulated phosphorylation sites, Ser309-311, close to one of its destruction boxes. These sites are phosphorylated in HeLa cells in the G1 phase, but not in the mitotic phase (Dephoure, Zhou et al. 2008). It might be that phosphorylation of these serines is a signal for degradation, while the unphosphorylated forms can interact with c-Myc.

CDR2's interaction with c-Myc affects the transcriptional properties of c-Myc. In some cases CDR2 enhances c-Myc transcriptional activity, while in other cases c-Myc dependent transcription is down-regulated (Okano, Park et al. 1999; O'Donovan, Diedler et al. 2010). The CDR2 c-Myc interaction is regulated through the leucine-zipper domains of both CDR2 and c-Myc, and c-Myc interacts with the lower band of the CDR2 double band. Anti-Yo sera binds specifically to the leucine-zipper domain, and this binding inhibits the CDR2 c-Myc interaction (Okano, Park et al. 1999).

An animal model of SV40 T antigen (Tag) transgenic mice showed ataxia and loss of Purkinje cells (Feddersen, Ehlenfeldt et al. 1992). Tag, like c-Myc, is a protooncogen with transcriptional function. In Purkinje cells Tag can reactivate the cell cycle and induce DNA synthesis which results in DNA fragmentation and subsequent cell death

in postmitotic Purkinje cells (Feddersen, Clark et al. 1995). There are many examples of that reactivation of the cell cycle in neurons is thought to be associated with cell death (Staropoli 2008). It may therefore be that CDR2 restrains c-Myc's transcriptional activity and that anti-Yo mediated disruption of this interaction activates c-Myc, with subsequent reactivation of the cell cycle, and thereby leads to Purkinje cell death.

Common for many of the association partners found for CDR2 is that they are involved in gene transcription, and CDR2 can be involved in down-regulating their transcriptional function. There are also several examples of that induction of Yo antibodies in the cells can interfere with CDR2's ability to down-regulate transcription factors. This is probably due to binding of anti-Yo to the leucine-zipper motif of CDR2. The transcription factors will then remain active and the result is unwanted and unregulated gene transcription.

4.3 CDR2L

The *CDR2L* gene is located at q25.1 at chromosome 17, and the nucleotide sequence has been described (Fathallah-Shaykh, Finizio et al. 1996; Strausberg, Feingold et al. 2002; Gerhard, Wagner et al. 2004; Ota, Suzuki et al. 2004). The nucleotide sequence codes for a 465 aa long protein with an estimated molecular weight of 53 kDa. The protein can be phosphorylated on several different serines (Cantin, Yi et al. 2008). CDR2 and CDR2L have approximately 50 % sequence similarity, and it is therefore reasonable to assume that Yo antibodies also recognize CDR2L. Both CDR2 and CDR2L mRNA expression has been detected in the cerebellum, but only CDR2 mRNA expression has been found in ovarian tumours from patients with paraneoplastic cerebellar degeneration. This makes CDR2 more likely to be the main target for Yo antibodies (Corradi, Yang et al. 1997). Results from the Human Protein Atlas imply that CDR2L is widely distributed in medium to low levels in many organs, but CDR2L is strongly expressed in the Purkinje cells and the digestive tract system and medium expressed in the ovaries, breast tissue and testis. Subcellular localization

studies suggest that the protein is mainly located to the cytoplasm (www.proteinatlas.org).

5. Immunological methods

5.1.1 In vitro transcription/translation and immunoprecipitation assay

Early detection of cancer can usually reduce cancer-related mortality. The detection of cancer-associated biomarkers is an important tool in this search. An effective screening method must have high specificity and sensitivity. In order to be universally applied it should also be cost effective and have no side effects for the patients. Autoantibodies against tumour-associated antigens are more stable and specific than other serum-derived proteins. They are therefore often used as biomarkers for cancer (Anderson and LaBaer 2005).

The most applied methods for detecting onconeural antibodies are western blot, line-blot and indirect immunofluorescence of sections of the brain. These methods have a sensitivity of approximately 30 %, and more than 95 % specificity (Tampoia, Zucano et al. 2010). With a sensitivity of only 30 % there is a huge potential for developing new, more sensitive techniques.

Routine screening for autoantibodies often rely on solid-phase assays like immuno-blotting or ELISA. These methods work best for antibodies with high affinity and high titres that are not sensitive to conformational epitopes. For instance, the solid-phase based assays are not reliable for detection of insulin specific autoantibodies, and for this a radioimmunopreciptitation assay (RIA) has been developed (Falorni, Ortqvist et al. 1995). We have modified this method so that it can be used to detect paraneoplastic antibodies. This is an in vitro transcription/translation and immunoprecipitation (ITT) assay (Storstein, Monstad et al. 2004; Knudsen, Monstad et al. 2006; Monstad, Storstein et al. 2006; Monstad and Vedeler 2006). In brief, recombinant 35S-labeled onconeuroproteins were produced by adding a plasmid

containing the cDNA of interest to a rabbit reticulocyte lysate transcription/translation system (Promega). The recombinant proteins were produced in mammalian cell lysates which make the proteins more like proteins produced in mammalian cells, and the proteins probably have their native conformations. This allows the antibodies to recognize both linear and conformational epitopes. The protein is also in solution which makes every part of the protein accessible to the antibodies.

Radiolabelled proteins and patient sera diluted 1:10 were incubated over night before the bound antigen-antibody complexes were immunoprecipitated by adding an equal volume of a protein-A Sepharose mixture in 96-wells filtration plates. Each sample was tested in triplicate. The mixture was incubated at 4°C for 45 min, washed and dried. Scintillation fluid was added and the emitted radioactivity, which is a measure of the bound antibody-antigen complexes, was measured in a β-counter as counts per minute (cpm). Pooled sera from 100 blood donors were used as negative control, while rabbit sera containing antibodies raised against the proteins of interest were used as positive controls. An index was calculated from the following formula:

$$Antibody\ \ index = \frac{cpm\ sample - cpm\ negative\ control}{cpm\ positive\ control - cpm\ negative\ control} \times 1000$$

A selection of blood donors were tested individually and a cut-off was set to mean value of the blood donors antibody index + 5 SD. The method combines high specificity and sensitivity, and since it can be performed in 96-well plates it has huge capacity for analysing multiple samples in a short time.

The ITT assay has been successfully used to detect Hu (Monstad, Drivsholm et al. 2004; Storstein, Monstad et al. 2004), Yo (Monstad, Storstein et al. 2006), Ri (Knudsen, Monstad et al. 2006), CRMP5 (Monstad, Drivsholm et al. 2008), amphiphysin and Ma2 (Monstad, Knudsen et al. 2009) antibodies and has proven to be more sensitive than the established techniques, immunofluorescence and immunoblot. By using this assay it was found that 25 % of the SCLC patients harboured Hu antibodies compared to 19 % detected by the conventional methods

(Monstad, Drivsholm et al. 2004). In ovarian cancer it was detected that 2.3 % of the patients harboured Yo antibodies compared to 0.9 % detected by established methods (Monstad, Storstein et al. 2006). The ITT assay correlated best with the other methods when Ri antibodies were detected. The ITT method detected Ri antibodies in 4.5 % of the patients with SCLC, 0.8% of the patients with breast cancer and 0.2 % of the patients with ovarian cancer. All but one of the positive sera was confirmed by at least one of the other immune assays (Knudsen, Monstad et al. 2006; Storstein, Monstad et al. 2011). The ITT assay detected CRMP5 antibodies in 5 % of the SCLC patients and 12 % of the patients with thymoma, and was proven to be far more sensitive than line blot and immunofluorescence (Monstad, Drivsholm et al. 2008). In a large retrospective study of paraneoplastic antibodies in cancer patients it was found that the ITT technique detected paraneoplastic antibodies in 11.9 % of the sera compared to 7 % of the sera by immunofluorescence and 6.3 % by immunoblot. The ITT assay detected paraneoplastic antibodies in 45 % more sera than the established techniques (Storstein, Monstad et al. 2011).

The diagnostic value of the ITT technique can be debated. Even though the technique detects more positive sera than the other immunological methods only a few of the patients showed neurological signs. Most of these antibodies were also found by the established techniques. However, CRMP5 is an exception. In a case study of a patient with limbic encephalitis and high levels of CRMP5 antibodies, the antibodies could only be detected by ITT (Monstad, Nostbakken et al. 2009). Further, in a retrospective study of paraneoplastic antibodies in cancer patients the ITT technique detected 18 sera with CRMP5 antibodies. Immunofluorescence and immunoblot did not pick up 13 of these. Eight of the patients had PNS, six of them classical (Storstein, Monstad et al. 2011). This suggests that the current techniques used to identify CRMP5 antibodies are not sensitive enough. It may be that CRMP5 antibodies are directed towards conformational epitopes that will not be picked up by solid phase assays. In addition conventional methods did not detect 3 of 5 patients with anti-amphiphysin and PNS, 4 of 21 patients with anti-Yo and PNS, 2 of 20 patients with anti-Hu and PNS and 1 of 5 patients with anti-Ma2 and PNS (Storstein,

Monstad et al. 2011). These results suggest that improvement of the current techniques is important for better diagnostics in PNS.

6. Aims (papers 1 - 3)

Aim 1: The functional relevance of paraneoplastic antibodies is unclear. Since high avidity antibodies may be of pathogenic importance, the aim of paper 1 was to study the avidity of Yo and Hu antibodies.

Aim 2: The reason why only some tumour patients produce paraneoplastic antibodies is not known. The aim of paper 2 was therefore to study if the production of Yo antibodies was related to differences in the CDR2 cDNA sequence, mRNA or protein levels in ovarian cancer.

Aim 3: As the functional role of paraneoplastic antibodies is largely unknown, insight gained by identifying associated antibodies may help to clarify the pathogenesis of PNS. The aim of paper 3 was therefore to identify antibodies that could be associated with anti-Yo.

Results and discussion

6.1 Paper I: Hu and Yo antibodies have heterogeneous avidity

The aim of paper I was to study the avidity of Yo and Hu antibodies. Avidity measurements of paraneoplastic antibodies had never been performed before this study. Other studies have shown that pathogenic antibodies often have high avidity and are associated with disease onset (Comin, Yuki et al. 2006) or that increasing avidity can predict the duration of the disease (Hedman, Lappalainen et al. 1989). We therefore studied if the avidity of paraneoplastic antibodies could give more information about the antibodies role in PNS. Since anti-Hu and anti-Yo are the most common paraneoplastic antibodies (Giometto, Grisold et al. 2010), we chose to compare the avidity of these antibodies.

We compared the differences in avidity among patients with Hu or Yo antibodies by adding a washing step with various concentrations of urea in two techniques that is used to test for paraneoplastic antibodies, namely immunofluorescence of rat brain and ITT (Storstein, Monstad et al. 2004; Monstad, Storstein et al. 2006). Urea dissociation studies for determining antibody avidity has been performed in both ELISA and immunofluorescence studies previously (Gray 1995; Rossi 1998). We found great variability in the avidity within each patient group which suggests that patients have heterogeneous anti-Hu or anti-Yo avidity. This may reflect differences in the patient's immune response, the severity of the disease or different time points of sampling.

In the urea immunofluorescence approach fixed rat cerebellum sections were incubated over night with anti-Hu or anti-Yo containing patient sera diluted 1/500. The slides were washed the following day and treated with various concentrations of urea. From our studies we found that 8M urea gave highest discrepancy between high

and low avidity antibodies. 8M urea has also been the chosen urea concentration in other studies for determining avidity by immunofluorescence (Gray 1995). At 8M urea the immunofluorescence signal was lost for 11 of the anti-Hu positive sera, while the other 10 were unaffected. Binding was abolished for 2 of the Yo-positive sera while 12 anti-Yo sera showed no change in binding at 8M urea. The differences in avidity was significantly greater for Hu than for Yo antibodies ($p=0.034$).

The ITT immunoprecipitation method was used to obtain a more quantitative value of the antibody avidity. The ITT-method was performed as previously described, but we added an extra washing step with various concentrations of urea. Again, 1M and 4M urea had little effect on dissociation of the antigen-antibody complex, while 8M urea gave a significant effect and was chosen for determining avidity. Yo antibodies in general had higher avidity than Hu antibodies. Low avidity antibodies have previously been defined as antibodies with an avidity index of ≤ 30 % while high avidity antibodies have an avidity index of ≥ 50 %. (Hedman, Lappalainen et al. 1989). There was a significant association between the results from the avidity immunofluorescence assay and the avidity ITT assay. The lowest avidity indexes coincided with the negative immunofluorescence findings ($p=0.008$ for anti-Hu and $p= 0.02$ for anti-Yo). The avidity index was ≤30 % for anti-Hu and ≤ 24 % for anti-Yo antibody binding that was abolished at 8M urea in the avidity immunofluorescence test. In both groups we found patients that harboured high avidity antibodies, but patients with Yo antibodies in general had higher avidity than patients with Hu antibodies. By avidity ITT we found that 11 of 21 Hu positive patients harboured low avidity antibodies while 4 of 21 had high avidity. In comparison, 2 of 14 Yo positive patients had low avidity antibodies, while 7 of 14 had high avidity antibodies. The other patients had intermediate avidity antibodies. We therefore concluded that the avidity of Hu and Yo antibodies are heterogeneous.

Heterogeneous avidity variances in patients with antibodies towards the same antigen are not uncommon. For instance, sera from patients with aquaporin-4 antibodies showed different binding affinities (Crane, Lam et al. 2011). The heterogeneous

avidity may reflect that the disease has progressed for a longer period in patients with Yo antibodies and in patients with high avidity anti-Hu as the avidity of antibodies often increase with time from onset. Low-avidity antibodies are usually found within the first months after onset, while high avidity antibodies develop further out in the course of the disease (Hedman and Seppala 1988; Hedman, Lappalainen et al. 1989; Blackburn, Besselaar et al. 1991; Gray 1995; Rossi 1998).

There are many examples of patients developing paraneoplastic antibodies before the underlying cancer is detected (Graus, Keime-Guibert et al. 2001; Shams'ili, Grefkens et al. 2003). In a mouse model of SCLC, Hu antibodies were detected up to 100 days before a cancer could be detected (Kazarian, Calbo et al. 2009). It is reasonable to assume that the same can be true for anti-Yo associated cancers. In this context it is interesting to observe that one study found that of 6 patients with paraneoplastic cerebellar degeneration with IgG Yo antibodies, 2 of these also had IgM antibodies and one had IgA antibodies (Smith, Finley et al. 1988). This implies that the antibody avidity maturation in patients with PNS follow the general antibody maturation pattern. Other studies have only been able to detect paraneoplastic antibodies of IgG isotypes, mainly IgG1 (Amyes, Curnow et al. 2001; Greenlee, Boyden et al. 2001). The symptoms of PNS probably occur after the switch from IgM to IgG, and may be the reason why other isotypes are not observed.

The higher avidity of Yo antibodies can also indicate that Yo antibodies are more pathogenic than Hu antibodies. Pathogenic antibodies often have high avidity, and in a mouse model of Guillain-Barré syndrome it was found that the presence of high-avidity antibodies coincided with onset of disease (Comin, Yuki et al. 2006). High avidity anti-β2-GPI antibodies have been associated with thrombosis and kidney disease in anti-phospholipid syndrome, while patients with pure systemic lupus erythematosus harboured low avidity antibodies (Cucnik, Kveder et al. 2004). High affinity insulin antibodies increased the risk of developing diabetes (Achenbach, Koczwara et al. 2004). Greenlee et al. (2010) showed that anti-Yo uptake in Purkinje cells on cerebellar slices killed the Purkinje cells in less than 72 hours. This

implicates that Yo antibodies are pathogenic. In this context it is also of interest to measure the antibody avidity of patients with paraneoplastic antibodies, but without neurological symptoms.

We also measured the time-dependent avidity changes in some patients with Hu and Yo antibodies. These studies showed that while the avidity indexes increased with time for most patients with Hu antibodies, the avidity indexes for patients with Yo antibodies were fairly constant. This further indicates that one may discover Hu antibodies at an earlier time point in the disease progress, while the Yo antibodies have persisted for a while before the neurological symptoms develop.

8M urea was used to break antigen-antibody complexes. Urea is a chaotrop that can disrupt the hydrogen bonds in the antigen-antibody complexes. It can also partially unfold the tertiary structure of proteins. We used 8M urea which can be quite harsh for proteins. However, it is not likely that these conditions had a significant impact on the results. In both the Hu and Yo groups we found patients with antibodies that were unaffected at 8M urea which makes it unlikely that the differences in avidity was due to harsh conditions. Both Hu and Yo antibodies can bind to linear epitopes (Sakai, Ogasawara et al. 1993; Manley, Smitt et al. 1995; Sodeyama, Ishida et al. 1999). It is therefore unlikely that the antibody-antigen binding would be affected by a potential unfolding. 8M urea has also been used successfully to disrupt antigen-antibody binding without causing irreversible changes to the antigen in other avidity assays (Hedman and Seppala 1988; Blackburn, Besselaar et al. 1991; Gray 1995; Ono, Lafer et al. 2004; Smolander, Koskinen et al. 2010). Furthermore, we have recently done a Biacore study were we measured the antigen-antibody binding capacity of purified IgG from anti-Yo positive patients to purified recombinant CDR2 (manuscript in preparation). This study showed a significant correlation between the Biacore and ITT avidity measurements. It is therefore likely that the heterogeneity seen in patients with Yo and Hu antibodies was due to differences among the patients and not the strong urea concentration. An ITT-urea test is therefore reliable for measuring onconeural antibody avidity.

One problem with avidity measurements is the variation in different techniques used to measure avidity. Some use a fixed serum dilution and determine the avidity index as the difference between the measured signals in the wells with urea compared to the signal in the wells without urea (Rossi 1998; Cucnik, Kveder et al. 2004). Others suggest that the avidity index is dependent on serum dilution and recommend using end-point titrations for avidity testing (Hedman, Lappalainen et al. 1989; Romero-Steiner, Holder et al. 2005; Smolander, Koskinen et al. 2010). This observation probably had minor impact on our results since we used high dilutions of the serum samples, i.e. 1/500 in the immunofluorescence and 1/1000 in the ITT-avidity assay.

6.2 Paper II: CDR2 antigen and Yo antibodies

CDR2 mRNA is widely distributed in several tissues, but protein expression has only been described in immuneprivileged tissues like the brain and testis, in ovarian, breast and renal cell tumour tissues and some cancer cell lines (Corradi, Yang et al. 1997; Darnell, Albert et al. 2000; Balamurugan, Luu et al. 2009). It is speculated whether this discrepancy between the mRNA distribution and the observed protein expression is due to posttranslational modifications in the tissues. While more than 60 % of the patients with ovarian cancer express CDR2, only 2.3 % of these patients develop Yo antibodies (Darnell, Albert et al. 2000; Monstad, Storstein et al. 2006). In paper II we investigated whether the production of Yo antibodies in some ovarian cancer patients was related to variants in the cDNA sequence or to differences in the CDR2 mRNA or protein levels among the patients.

In SCLC various levels of HuD expression reflecting the degree of neuroendocrine differentiation of the tumours have been reported (King 1997), and several mutations in the HuD gene in neuroendocrine tumours have been observed (D'Alessandro, Muscarella et al. 2009). We wanted to investigate whether there were similar differences in the CDR2 gene and if such differences could explain why some patients develop Yo antibodies and some do not. In our study we enrolled 16 patients with ovarian cancers, 5 had Yo antibodies and 2 of these had paraneoplastic cerebellar degeneration. We found no differences in the CDR2 cDNA among the 16

patients that could indicate an alternative CDR2 protein variant for any of the examined patients. However, we did not examine the CDR2 promoter regions so we can not rule out that these areas contain mutations important for changes in CDR2 expression.

The tissues from the two patients with paraneoplastic cerebellar degeneration were formalin fixed and we did not purify RNA from these samples. Total RNA was extracted from the 14 other ovarian cancers, but no differences in CDR2 mRNA levels could be observed among the patients. Also various normal human tissues expressed similar levels CDR2 mRNA. This is in accordance with a previous study where CDR2 mRNA was found to be evenly distributed in mouse tissue (Corradi, Yang et al. 1997). We found that all 16 ovarian cancers examined expressed CDR2 protein. This result differs slightly from previous findings where only 62 % of all ovarian cancers expressed CDR2 (Darnell, Albert et al. 2000). This may be due to the fact that Darnell et al. (2000) used Yo sera from patients with paraneoplastic cerebellar degeneration to identify the CDR2 protein in western blot. To avoid the interference of the 55 kDa human IgG band they had to deplete the human IgG. We used a monoclonal mouse antibody against CDR2 and could therefore avoid the IgG depletion step. It may therefore be that some of the CDR2 expression in Dalmau's experiments was lost during the IgG depletion. Another reason why we found that CDR2 was present in various tissues could be that the monoclonal antibody was more sensitive than Yo sera. It is, however, not surprising that CDR2 was found in all tumour samples, since CDR2 is expressed in almost all forms of renal carcinomas (Balamurugan, Luu et al. 2009) and HuD in all SCLC tested (Dalmau, Graus et al. 1995).

By western blot we did not find any differences in the protein expression levels among the patients. CDR2 expression was similarly strong in patients with Yo antibodies as in those without Yo antibodies. This suggests that other mechanisms are involved in the development of paraneoplastic cerebellar degeneration. Dysregulation of B and T cells has been suggested as mediators of paraneoplastic pathogenesis. In

anti-Hu associated syndromes both dysregulation of regulatory T cells and association of specific HLA-class alleles have been implicated (Tani, Tanaka et al. 2008; de Graaf, de Beukelaar et al. 2010). In patients with paraneoplastic cerebellar degeneration activated CD4+ and CD8+ cells as well as microglia activation in the neural tissue have been found (Albert, Austin et al. 2000; Storstein, Krossnes et al. 2009). A higher frequency of HLA-A2.1, HLA-A24 or HLA-B27 haplotypes have been observed among patients with anti-Yo mediated paraneoplastic cerebellar degeneration (Albert, Darnell et al. 1998; Sutton, Steele et al. 2004; Santomasso, Roberts et al. 2007; Carpenter, Vance et al. 2008). These patients have been shown to harbour cytotoxic T cells that have the potential to lyse CDR2 expressing cells (Albert, Darnell et al. 1998; Sutton, Steele et al. 2004; Santomasso, Roberts et al. 2007).

We found that western blot with a monoclonal CDR2 antibody on 3 different cancer cell extracts (ovarian, lung and neuroblastoma) recognized CDR2 expression as a double band. The highest one, with a molecular weight of 62 kDa, was the strongest for all three cell extracts. Such double bands have been observed in lysates of other cancer cell lines (Balamurugan, Luu et al. 2009), but the exact identity of these bands have not been described. The double bands could suggest modifications of CDR2 in the cells. It may also be that these antibodies recognize both CDR2 and CDR2L. The leucine-zipper domain of CDR2 and CDR2L has high sequence similarity, and antibodies directed towards this domain would probably identify both proteins. A weaker band in the same height as the highest band in the cell extracts was observed in cerebellum, while no CDR2 expression was observed in the cerebrum. These findings are consistent with previous findings as CDR2 expression has been found in many cancer cell lines, as well as in Purkinje cells in the cerebellum (Corradi, Yang et al. 1997; Balamurugan, Luu et al. 2009).

We found that intracellular localization studies of CDR2 with a polyclonal antibody confirmed that CDR2 was expressed in both the ovarian and lung cancer cell line as well as in HeLa cells. In all cell lines, the CDR2 antibody showed a granular staining,

mainly in the cytoplasm. These findings are in accordance with Hida et al. (1994) who found that Yo antibodies localised to the ribosomes of rough endoplasmatic reticulum and to free ribosomes in Purkinje cells. Staining of HeLa cells with a monoclonal antibody showed similar results.

In ovarian and colon cancer biopsies from patients without paraneoplastic cerebellar degeneration we found nuclear and cytoplasmic CDR2 staining of cancer cells with the polyclonal CDR2 antibody. Ovarian tissue from two patients with paraneoplastic cerebellar degeneration and Yo antibodies showed nuclear CDR2 staining of the cancer cells. The monoclonal CDR2 antibody 4F5 (Abnova) that O´Donovan et al. (2010) used to stain HeLa cells is directed against the same peptide sequence as sc-100320 (Abcam) that we used to stain HeLa cells. Both antibodies showed cytoplasmic CDR2 localization. Interestingly, patient sera with antibodies towards both CDR2 and CDR2L stained nucleus in some cells, but the cytoplasm in other cells, while the monoclonal antibody and a T7CDR2 construct both localized CDR2 to the cytoplasm (O'Donovan, Diedler et al. 2010). This indicates that CDR2 is transported to the nucleus during cell division.

The ovarian biopsies used in our study also contained normal stromal and epithelial cells. We found weak CDR2 staining of the normal stromal cells and strong staining of the normal epithelial cells. These findings were confirmed by the fact that western blot of normal ovarian tissue lysate also showed CDR2 expression. However, these results are not surprising. Amphiphysin has been found in both normal and breast cancer tissue (Floyd, Butler et al. 1998). Weak CDR2 staining has been observed in normal kidney lysate (Balamurugan, Luu et al. 2009), and according to the Human Protein Atlas, CDR2 is widely expressed in normal tissue, including normal ovary follicle and stromal cells. This suggests that we have to re-evaluate the dogma that CDR2 is only expressed in cancer tissue and immuneprivileged sites, and that the model for PNS is even more complicated than previously thought.

The pathogenesis of paraneoplastic cerebellar degeneration is probably not only related to the CDR2 expression and Yo antibody synthesis, but also to immune

dysregulation, such as antigen presentation and cooperation between B and T cells. Several reports have suggested that patients with specific HLA alleles are more prone to PNS and activated T cells directed towards paraneoplastic antigens have been found in many patients with PNS (Albert, Darnell et al. 1998; Albert, Austin et al. 2000; Tanaka, Tanaka et al. 2001; Pellkofer, Schubart et al. 2004; Santomasso, Roberts et al. 2007; Tani, Tanaka et al. 2008; de Graaf, de Beukelaar et al. 2010).

6.3 Paper III: Antibody to CCDC104 is associated with a paraneoplastic antibody to CDR2 (anti-Yo)

Identification of new antibodies is very important in the search for new antigens that can be used as cancer biomarkers, as they may give improved insight into the etiology of PNS. In paper III we described a patient with Yo antibodies and ovarian cancer who also harboured antibodies towards an unidentified protein. Anti-Yo positivity was observed by immunohistochemistry on rat cerebellum, and was confirmed by a line blot with recombinant onconeural proteins. A western blot screening of the patient's serum on rat cerebellar extract revealed a distinct band of approximately 39 kDa in addition to the expected Yo band of 52 kDa. To identify this unknown protein the patient serum was used to screen a cDNA expression library from rat cerebellum. Such expression libraries have been used to identify many new antigens (Dropcho, Chen et al. 1987; Sakai, Mitchell et al. 1990; Fathallah-Shaykh, Wolf et al. 1991; Lichte, Veh et al. 1992).

The screening resulted in the identification of a protein called coiled-coil domain containing 104 (CCDC104). Very little is known about this protein. By sequence alignment it was found that the protein is highly conserved among mammalians with 85 % sequence identity and 93 % amino acid sequence conservation. When proteins show such a degree of conservation it often implies that they have important biological functions.

No expression studies of CCDC104 had ever been done before, and we wanted to investigate which tissues the protein was expressed in. We expressed recombinant

full-length CCDC104 protein, and this protein was used to produce antibodies in rabbits. Due to the similarities between the different CCDC104 isoforms this antibody can probably recognize all CCDC104 isoforms. We found that the CCDC104 protein was widely expressed in several different tissues. Expression of the 39 kDa isoform was especially strong in the testis and spleen, but could also be observed in various parts of the brain. This is in accordance with the findings of the Human Protein Atlas that finds strong staining of cells in the seminiferous ducts. The seminiferous ducts are located in the testes, and the meiosis take place here. In this context it is interesting to notice that CCDC104 is heavily phosphorylated in the testis and that testis-specific phosphorylated proteins often are involved in meiosis, cell cycle regulation and DNA damage repair (Gauci, Helbig et al. 2009; Huttlin, Jedrychowski et al. 2010).

We also observed several isoforms of CCDC104. A 42 kDa band was observed in the lung and pancreas and a 36 kDa band was seen in the heart. The observation of the 36 kDa variant in the heart is further supported by findings of the Human Protein Atlas where an antibody that only identifies the 39 and 42 kDa isoforms does not stain heart muscle cells, while an antibody that also recognizes the three other potential isoforms shows strong staining of myocytes. In the brain we found that the 39 kDa isoform was widely expressed in almost all sections of newborn rat brain with the exception of the spinal cord and the frontal cortex. The main expression pattern of CCDC104, i.e. testis and brain, was similar to the expression pattern of many other paraneoplastic proteins such as CDR2, amphiphysin and Ma1 (Corradi, Yang et al. 1997; Floyd, Butler et al. 1998; Dalmau, Gultekin et al. 1999).

Determination of the intracellular localization of CCDC104 was done in a neuroblastoma cell line. A rabbit peptide antibody raised towards CCDC104 was used for specific detection. The study showed that CCDC104 is mainly localized to the nucleus, but parts of the cytoplasm were also weakly stained. Later studies of the CCDC104 expression in other cell lines, like ovarian cancer, HeLa and a lung cancer cell line showed a similar staining pattern (unpublished results). Subcellular

localization studies, published by the Human Protein Atlas also showed similar results in other cell lines. This implies that CCDC104 is a protein that is widely distributed in many different cell lines and organs.

To investigate whether CCDC104 antibodies could be a new paraneoplastic marker, we collected serum samples from patients with the most common forms of cancers that are associated with PNS, namely breast, lung and ovarian cancer. About 25 % of these sera contained other paraneoplastic antibodies. Sera from 300 blood donors were used as negative controls. The reason why we picked 5 SD as cut-off value instead of 3 SD as has been done in other assays (Knudsen, Monstad et al. 2006; Monstad, Storstein et al. 2006; Monstad, Drivsholm et al. 2008; Monstad, Knudsen et al. 2009) was that the mean value of the blood donors was very low. When we used only 3 SD many "positives" turned out not to react with recombinant CCDC104 in western blot. When we used a cut-off with 5 SD, most of the positive patients reacted with recombinant CCDC104, while none of the patients below the cut-off did.

All sera were screened for CCDC104 antibodies by the ITT assay. Nine of the ten patients that were positive by the ITT technique bound specifically to recombinant CCDC104 in western blot. CCDC104 antibodies were found in 10.5 % (4 of 38 sera) of the patients with Yo antibodies. CCDC104 antibodies were not found in the 158 patients with other paraneoplastic antibodies. We also found CCDC104 antibodies in 1.1 % of the patients with different forms of cancer. To be able to confirm that an antibody has a diagnostic relevance one needs to identify the right group of patients, and examine a large number of patients. The detection of CCDC104 antibodies was not correlated to any specific form of cancer, no neurological symptoms were reported for the patients who only harboured CCDC104 antibodies and CCDC104 antibodies were also detected in 2 of 300 (0.7 %) of the blood donor controls. However, the presence of paraneoplastic antibodies has also been observed in other controls. CRMP5 antibodies have been detected in 2 of 300 blood donors while anti-Ma2 and anti-amphiphysin were detected in 1 of 300 blood donors (Monstad, Knudsen et al. 2009). CCDC104 antibodies are probably not directly related to PNS,

but we only examined a limited number of patients within each cancer group and we only examined groups of patients with cancers that are commonly associated with PNS.

CCDC104 has been shown to be phosphorylated by ATM and ATR, two proteins that are both associated with various forms of DNA break repair. Furthermore, it has been shown that patients with ataxia telangiectasia have loss of Purkinje cells and ataxia, symptoms that are common in patients with Yo antibodies. We do not know the function of CCDC104, but the fact that it is regulated by ATM, and that ATM dysregulation gives increased risk of cancer and loss of Purkinje cells, makes it likely that CCDC104 also has a role in cancer development and maintenance of Purkinje cells. In this context it is interesting that we found two antibodies towards proteins that may have regulating functions in the same cells. Both CDR2 and CCDC104 are highly expressed in the seminiferous ducts (Corradi, Yang et al. 1997), and CDR2 is expressed during mitosis (O'Donovan, Diedler et al. 2010). It is not unlikely that they both have a role in mitosis and meiosis. We have recently detected an accumulation of CCDC104 around the spindle poles in dividing cells (manuscript in preparation) which supports this assumption. It is also interesting to note that CDR2 is important for proper spindle formation. CDR2 knockdown results in an increased number of spindle poles in single cells (O'Donovan, Diedler et al. 2010). This further strengthens the relationship between CDR2 and CCDC104, and supports our findings that CCDC104 and CDR2 antibodies co-exist. It would therefore be very interesting to further see if knockdown of CCDC104 would give similar findings as shown for CDR2.

7. Summary and future perspectives

Paraneoplastic antibodies and their association with specific forms of cancer have been known for decades, but still very little is know about the mechanisms behind the diseases, why only some people develop PNS, the role of the antibodies and the main functions of the onconeural proteins. In this study we have tried to further elucidate the properties of Yo antibodies, potential antibodies that coexist with anti-Yo and whether mutations in the CDR2 gene or differences in CDR2 transcription and expression could explain why some patients develop PNS.

Paper I: In this study the avidity of 21 patients with Hu antibodies and 14 patients with Yo antibodies was compared. We found that Yo antibodies generally had higher avidity than Hu antibodies, but there was great avidity variance within each group. We found no association between antibody avidity and type of cancer. However, when we measured avidity changes in patients over time, we saw that Hu antibodies followed normal avidity maturation pattern with low avidity index in the earliest samples and a time-dependent increase in the avidity index. The Yo antibody avidity, however, remained high. This suggests that Hu antibodies are detected at an earlier time-point than Yo antibodies, and this finding may have a prognostic importance.

Paper 1 reported a small study of the avidity of Hu and Yo antibodies. A larger study may be performed to see if this trend is significant. Surface-plasmon resonance studies would give a more functional approach for studying the avidities of these and other paraneoplastic antibodies (manuscript in preparation). Furthermore, we now have more serum samples from patients for a longitudinal study where it is possible to look for the avidity during the course of PNS. However, this is only known from the onset of the neurological disease because the onset of cancer is unknown.

Paper II: Ovarian tumours from 16 patients were screened for differences in CDR2 cDNA sequence, mRNA expression level and protein expression. Five of the 16 patients had Yo antibodies, and two of the anti-Yo positive patients had paraneoplastic cerebellar degeneration. We found no difference in the CDR2 cDNA

sequence in the tumours of any of the patients that could explain a possible alternative transcription of the CDR2 gene. Neither did we see any difference in the mRNA expression levels in the ovarian tumours compared to various normal tissues. CDR2 protein expression was approximately similar in all tumours. Interestingly, we found CDR2 protein expression in several cancer cell lines, in prostate cancer and in normal ovarian tissue. This suggests that CDR2 expression is more abundant than previously reported, and it is even expressed in normal tissue. Differences in the immune regulation may therefore be of importance in the development of paraneoplastic antibodies and PNS.

Even though we have some knowledge about CDR2, much remains to be resolved. There is still uncertainty about which tissues that express CDR2, its exact intracellular location, its functional role and potential interaction partners and pathways. Even less is known about the role of Yo antibodies in paraneoplastic cerebellar degeneration. With new commercial antibodies available it is reasonable to re-evaluate previous results about CDR2 expression, localization and function.

So far there is almost no knowledge about the other CDR proteins. A few explorational studies about CDR1 have been performed, but since the early nineties no new information has been revealed. It is reasonable to assume that CDR1 also has a role in paraneoplastic cerebellar degeneration, since some patients with this disease harbour antibodies against CDR1. CDR1 mRNA has also been found to be upregulated in several neurodegenerative diseases. There are no commercial antibodies available for CDR1. Developing functional CDR1 antibodies is therefore necessary to characterize CDR1. Furthermore, production of CDR1 protein is necessary to screen for CDR1 antibodies in sera from patients with various tumours. Identifying potential interaction partners would also give a better understanding of the functions of CDR1. A yeast two-hybrid system may be used to identify such interaction partners.

Little is also known about CDR2L. We know that the protein shares high amino acid sequence similarity with CDR2. A search in web-based databases implies that

CDR2L has a higher expression in the Purkinje cells than CDR2, and that CDR2L localization pattern resembles that seen for Yo antibodies. We have recently found that patients with paraneoplastic cerebellar degeneration also harbour CDR2L antibodies (manuscript in preparation). A better characterization of CDR2L is therefore also important for the understanding of paraneoplastic cerebellar degeneration. However, the functions of CDR2L are not known. This can be further studied by using a yeast two-hybrid system to look for possible interaction partners with CDR2L.

Paper III: A patient with Yo-associated paraneoplastic cerebellar degeneration also harboured antibodies to an unknown protein. This protein was identified as CCDC104. CCDC104 is a well conserved protein that is mainly localized in the nucleus, and it is expressed in many tissues, especially in brain and testis. We found CCDC104 antibodies in the sera of 8 of 756 cancer patients and in 2 of 300 blood donors. There was no association between cancer or PNS and CCDC104 antibodies. However, there was a significant association between anti-Yo and anti-CCDC104. 10.5 % of the anti-Yo positive patients also harboured CCDC104 antibodies. The co-existence of these antibodies indicates similar biological functions and recent studies suggest that they are both involved in the cell cycle.

CDR2 is probably associated with correct spindle formation during mitosis. Recent findings in our laboratory imply that CCDC104 is localized to the centeromers during mitosis (manuscript in preparation). The fact that a significant proportion of patients with Yo antibodies also harbour CCDC104 antibodies, the observation that CDR2 and CCDC104 have similar distribution pattern and that they are both involved in mitosis makes CCDC104 an interesting interaction partner for CDR2. Unfortunately, there is presently little information available also for this protein. One approach would be to investigate CCDC104's function in mitosis, for example by knocking down CCDC104 in cell cultures.

References

Achenbach, P., K. Koczwara, et al. (2004). "Mature high-affinity immune responses to (pro)insulin anticipate the autoimmune cascade that leads to type 1 diabetes." J Clin Invest **114**(4): 589-97.

Aguirre-Cruz, L., J. L. Charuel, et al. (2005). "Clinical relevance of non-neuronal auto-antibodies in patients with anti-Hu or anti-Yo paraneoplastic diseases." J Neurooncol **71**(1): 39-41.

Alarcon-Segovia, D. (2001). "Antinuclear antibodies: to penetrate or not to penetrate, that was the question." Lupus **10**(5): 315-8.

Albert, M. L., L. M. Austin, et al. (2000). "Detection and treatment of activated T cells in the cerebrospinal fluid of patients with paraneoplastic cerebellar degeneration." Ann Neurol **47**(1): 9-17.

Albert, M. L., J. C. Darnell, et al. (1998). "Tumor-specific killer cells in paraneoplastic cerebellar degeneration." Nat Med **4**(11): 1321-4.

Amyes, E., J. Curnow, et al. (2001). "Restricted IgG1 subclass of anti-Yo antibodies in paraneoplastic cerebellar degeneration." J Neuroimmunol **114**(1-2): 259-64.

Anderson, K. S. and J. LaBaer (2005). "The sentinel within: exploiting the immune system for cancer biomarkers." J Proteome Res **4**(4): 1123-33.

Ang, C. W., B. C. Jacobs, et al. (2004). "The Guillain-Barre syndrome: a true case of molecular mimicry." Trends Immunol **25**(2): 61-6.

Armata, H. L., D. Golebiowski, et al. (2010). "Requirement of the ATM/p53 tumor suppressor pathway for glucose homeostasis." Mol Cell Biol **30**(24): 5787-94.

Balamurugan, K., V. D. Luu, et al. (2009). "Onconeuronal cerebellar degeneration-related antigen, Cdr2, is strongly expressed in papillary renal cell carcinoma and leads to attenuated hypoxic response." Oncogene **28**(37): 3274-85.

Ball, L. G. and W. Xiao (2005). "Molecular basis of ataxia telangiectasia and related diseases." Acta Pharmacol Sin **26**(8): 897-907.

Bandyopadhyay, S., C. Y. Chiang, et al. (2010). "A human MAP kinase interactome." Nat Methods **7**(10): 801-5.

Bhat, R. and L. Steinman (2009). "Innate and adaptive autoimmunity directed to the central nervous system." Neuron **64**(1): 123-32.

Blackburn, N. K., T. G. Besselaar, et al. (1991). "Differentiation of primary cytomegalovirus infection from reactivation using the urea denaturation test for measuring antibody avidity." J Med Virol **33**(1): 6-9.

Bretin, S., S. Reibel, et al. (2005). "Differential expression of CRMP1, CRMP2A, CRMP2B, and CRMP5 in axons or dendrites of distinct neurons in the mouse brain." J Comp Neurol **486**(1): 1-17.

Brot, S., V. Rogemond, et al. (2010). "CRMP5 interacts with tubulin to inhibit neurite outgrowth, thereby modulating the function of CRMP2." J Neurosci **30**(32): 10639-54.

Buckanovich, R. J., Y. Y. Yang, et al. (1996). "The onconeural antigen Nova-1 is a neuron-specific RNA-binding protein, the activity of which is inhibited by paraneoplastic antibodies." J Neurosci **16**(3): 1114-22.

Cantin, G. T., W. Yi, et al. (2008). "Combining protein-based IMAC, peptide-based IMAC, and MudPIT for efficient phosphoproteomic analysis." J Proteome Res **7**(3): 1346-51.

Caponi, L., C. Anzilotti, et al. (2007). "Antibodies directed against ribosomal P proteins cross-react with phospholipids." Clin Exp Immunol **150**(1): 140-3.

Carpenter, E. L., B. A. Vance, et al. (2008). "Functional analysis of CD8+ T cell responses to the onconeural self protein cdr2 in patients with paraneoplastic cerebellar degeneration." J Neuroimmunol **193**(1-2): 173-82.

Carpentier, A. F., M. R. Rosenfeld, et al. (1998). "DNA vaccination with HuD inhibits growth of a neuroblastoma in mice." Clin Cancer Res **4**(11): 2819-24.

Chen, H. L. and S. R. D'Mello (2010). "Induction of neuronal cell death by paraneoplastic Ma1 antigen." J Neurosci Res **88**(16): 3508-19.

Chen, Y. T., W. J. Rettig, et al. (1990). "Cerebellar degeneration-related antigen: a highly conserved neuroectodermal marker mapped to chromosomes X in human and mouse." Proc Natl Acad Sci U S A **87**(8): 3077-81.

Chistiakov, D. A. (2003). "Thyroid-stimulating hormone receptor and its role in Graves' disease." Mol Genet Metab **80**(4): 377-88.

Christen, U., E. Hintermann, et al. (2009). "Viral triggers for autoimmunity: is the 'glass of molecular mimicry' half full or half empty?" J Autoimmun **34**(1): 38-44.

Clark, H. F., A. L. Gurney, et al. (2003). "The secreted protein discovery initiative (SPDI), a large-scale effort to identify novel human secreted and transmembrane proteins: a bioinformatics assessment." Genome Res **13**(10): 2265-70.

Comin, R., N. Yuki, et al. (2006). "High affinity of anti-GM1 antibodies is associated with disease onset in experimental neuropathy." J Neurosci Res **84**(5): 1085-90.

Corradi, J. P., C. Yang, et al. (1997). "A post-transcriptional regulatory mechanism restricts expression of the paraneoplastic cerebellar degeneration antigen cdr2 to immune privileged tissues." J Neurosci **17**(4): 1406-15.

Crane, J. M., C. Lam, et al. (2011). "Binding affinity and specificity of neuromyelitis optica autoantibodies to aquaporin-4 m1/m23 isoforms and orthogonal arrays." J Biol Chem **286**(18): 16516-24.

Cucnik, S., T. Kveder, et al. (2004). "High avidity anti-beta 2-glycoprotein I antibodies in patients with antiphospholipid syndrome." Ann Rheum Dis **63**(11): 1478-82.

Cui, T., M. Hurtig, et al. (2011). "Paraneoplastic antigen Ma2 autoantibodies as specific blood biomarkers for detection of early recurrence of small intestine neuroendocrine tumors." PLoS One **5**(12): e16010.

Cunningham, J., F. Graus, et al. (1986). "Partial characterization of the Purkinje cell antigens in paraneoplastic cerebellar degeneration." Neurology **36**(9): 1163-8.

D'Alessandro, V., L. A. Muscarella, et al. (2009). "Molecular analysis of the HuD gene in neuroendocrine lung cancers." Lung Cancer.

Dalmau, J., H. M. Furneaux, et al. (1992). "The expression of the Hu (paraneoplastic encephalomyelitis/sensory neuronopathy) antigen in human normal and tumor tissues." Am J Pathol **141**(4): 881-6.

Dalmau, J., F. Graus, et al. (1995). "Major histocompatibility proteins, anti-Hu antibodies, and paraneoplastic encephalomyelitis in neuroblastoma and small cell lung cancer." Cancer **75**(1): 99-109.

Dalmau, J., S. H. Gultekin, et al. (1999). "Ma1, a novel neuron- and testis-specific protein, is recognized by the serum of patients with paraneoplastic neurological disorders." Brain **122 (Pt 1)**: 27-39.

Dalmau, J. and M. R. Rosenfeld (2008). "Paraneoplastic syndromes of the CNS." Lancet Neurol **7**(4): 327-40.

Dalmau, J. and M. R. Rosenfeld (2010). "Update on paraneoplastic neurologic disorders." Community Oncol **7**(5): 219-224.

Dangoudoubiyam, S., R. Vemulapalli, et al. (2010). "Molecular cloning of an immunogenic protein of Baylisascaris procyonis and expression in Escherichia coli for use in developing improved serodiagnostic assays." Clin Vaccine Immunol **17**(12): 1933-9.

Darnell, J. C., M. L. Albert, et al. (2000). "Cdr2, a target antigen of naturally occuring human tumor immunity, is widely expressed in gynecological tumors." Cancer Res **60**(8): 2136-9.

Darnell, R. B. (1998). "Immunologic complexity in neurons." Neuron **21**(5): 947-50.

Darnell, R. B. and L. M. DeAngelis (1993). "Regression of small-cell lung carcinoma in patients with paraneoplastic neuronal antibodies." Lancet **341**(8836): 21-2.

Darnell, R. B. and J. B. Posner (2003). "Paraneoplastic syndromes involving the nervous system." N Engl J Med **349**(16): 1543-54.

De Baets, M. and M. H. Stassen (2002). "The role of antibodies in myasthenia gravis." J Neurol Sci **202**(1-2): 5-11.

de Graaf, M. T., J. W. de Beukelaar, et al. (2010). "HLA-DQ2+ individuals are susceptible to Hu-Ab associated paraneoplastic neurological syndromes." J Neuroimmunol **226**(1-2): 147-9.

Debes, J. D., S. M. Lagarde, et al. (2007). "Anti-Yo-associated paraneoplastic cerebellar degeneration in a man with adenocarcinoma of the gastroesophageal junction." Dig Surg **24**(5): 395-7.

Dephoure, N., C. Zhou, et al. (2008). "A quantitative atlas of mitotic phosphorylation." Proc Natl Acad Sci U S A **105**(31): 10762-7.

Devey, M. E., K. M. Bleasdale-Barr, et al. (1990). "Antibodies of different human IgG subclasses show distinct patterns of affinity maturation after immunization with keyhole limpet haemocyanin." Immunology **70**(2): 168-74.

Di Simone, N., M. P. Luigi, et al. (2007). "Pregnancies complicated with antiphospholipid syndrome: the pathogenic mechanism of antiphospholipid antibodies: a review of the literature." Ann N Y Acad Sci **1108**: 505-14.

Doyle, H. A. and M. J. Mamula (2005). "Posttranslational modifications of self-antigens." Ann N Y Acad Sci **1050**: 1-9.

Drachman, D. B. (1994). "Myasthenia gravis." N Engl J Med **330**(25): 1797-810.

Dropcho, E. J., Y. T. Chen, et al. (1987). "Cloning of a brain protein identified by autoantibodies from a patient with paraneoplastic cerebellar degeneration." Proc Natl Acad Sci U S A **84**(13): 4552-6.

Falorni, A., E. Ortqvist, et al. (1995). "Radioimmunoassays for glutamic acid decarboxylase (GAD65) and GAD65 autoantibodies using 35S or 3H recombinant human ligands." J Immunol Methods **186**(1): 89-99.

Fathallah-Shaykh, H., S. Wolf, et al. (1991). "Cloning of a leucine-zipper protein recognized by the sera of patients with antibody-associated paraneoplastic cerebellar degeneration." Proc Natl Acad Sci U S A **88**(8): 3451-4.

Fathallah-Shaykh, H. M., J. Finizio, et al. (1996). Cloning and characterization of a second leucine zipper protein recognized by the sera of the patients with antibody associated paraneoplastic cerebellar degeneration., EMBL/GenBank/DDBJ databases.

Feddersen, R. M., H. B. Clark, et al. (1995). "In vivo viability of postmitotic Purkinje neurons requires pRb family member function." Mol Cell Neurosci **6**(2): 153-67.

Feddersen, R. M., R. Ehlenfeldt, et al. (1992). "Disrupted cerebellar cortical development and progressive degeneration of Purkinje cells in SV40 T antigen transgenic mice." Neuron **9**(5): 955-66.

Floyd, S., M. H. Butler, et al. (1998). "Expression of amphiphysin I, an autoantigen of paraneoplastic neurological syndromes, in breast cancer." Mol Med **4**(1): 29-39.

Fornaro, M., S. Raimondo, et al. (2007). "Neuron-specific Hu proteins sub-cellular localization in primary sensory neurons." Ann Anat **189**(3): 223-8.
Frisoni, L., L. McPhie, et al. (2005). "Nuclear autoantigen translocation and autoantibody opsonization lead to increased dendritic cell phagocytosis and presentation of nuclear antigens: a novel pathogenic pathway for autoimmunity?" J Immunol **175**(4): 2692-701.
Fukada, M., I. Watakabe, et al. (2000). "Molecular characterization of CRMP5, a novel member of the collapsin response mediator protein family." J Biol Chem **275**(48): 37957-65.
Furneaux, H. M., E. J. Dropcho, et al. (1989). "Characterization of a cDNA encoding a 34-kDa Purkinje neuron protein recognized by sera from patients with paraneoplastic cerebellar degeneration." Proc Natl Acad Sci U S A **86**(8): 2873-7.
Furneaux, H. M., M. K. Rosenblum, et al. (1990). "Selective expression of Purkinje-cell antigens in tumor tissue from patients with paraneoplastic cerebellar degeneration." N Engl J Med **322**(26): 1844-51.
Galon, J., A. Costes, et al. (2006). "Type, density, and location of immune cells within human colorectal tumors predict clinical outcome." Science **313**(5795): 1960-4.
Gauci, S., A. O. Helbig, et al. (2009). "Lys-N and trypsin cover complementary parts of the phosphoproteome in a refined SCX-based approach." Anal Chem **81**(11): 4493-501.
Geis, C., A. Weishaupt, et al. (2010). "Stiff person syndrome-associated autoantibodies to amphiphysin mediate reduced GABAergic inhibition." Brain **133**(11): 3166-80.
Gerhard, D. S., L. Wagner, et al. (2004). "The status, quality, and expansion of the NIH full-length cDNA project: the Mammalian Gene Collection (MGC)." Genome Res **14**(10B): 2121-7.
Giometto, B., W. Grisold, et al. (2010). "Paraneoplastic neurologic syndrome in the PNS Euronetwork database: a European study from 20 centers." Arch Neurol **67**(3): 330-5.
Graus, F., J. Dalmau, et al. (1997). "Anti-Hu antibodies in patients with small-cell lung cancer: association with complete response to therapy and improved survival." J Clin Oncol **15**(8): 2866-72.
Graus, F., J. Y. Delattre, et al. (2004). "Recommended diagnostic criteria for paraneoplastic neurological syndromes." J Neurol Neurosurg Psychiatry **75**(8): 1135-40.
Graus, F., I. Illa, et al. (1991). "Effect of intraventricular injection of an anti-Purkinje cell antibody (anti-Yo) in a guinea pig model." J Neurol Sci **106**(1): 82-7.
Graus, F., F. Keime-Guibert, et al. (2001). "Anti-Hu-associated paraneoplastic encephalomyelitis: analysis of 200 patients." Brain **124**(Pt 6): 1138-48.
Gray, J. J. (1995). "Avidity of EBV VCA-specific IgG antibodies: distinction between recent primary infection, past infection and reactivation." J Virol Methods **52**(1-2): 95-104.
Greenlee, J. E., J. W. Boyden, et al. (2001). "Antibody types and IgG subclasses in paraneoplastic neurological syndromes." J Neurol Sci **184**(2): 131-7.
Greenlee, J. E., S. A. Clawson, et al. (2010). "Purkinje cell death after uptake of anti-Yo antibodies in cerebellar slice cultures." J Neuropathol Exp Neurol **69**(10): 997-1007.
Grisold, W., B. Giometto, et al. (2011). "Current approaches to the treatment of paraneoplastic encephalitis." Ther Adv Neurol Disord **4**(4): 237-48.
Hedman, K., M. Lappalainen, et al. (1989). "Recent primary toxoplasma infection indicated by a low avidity of specific IgG." J Infect Dis **159**(4): 736-40.
Hedman, K. and I. Seppala (1988). "Recent rubella virus infection indicated by a low avidity of specific IgG." J Clin Immunol **8**(3): 214-21.

Hida, C., T. Tsukamoto, et al. (1994). "Ultrastructural localization of anti-Purkinje cell antibody-binding sites in paraneoplastic cerebellar degeneration." Arch Neurol **51**(6): 555-8.

Hill, K. E., S. A. Clawson, et al. (2009). "Cerebellar Purkinje cells incorporate immunoglobulins and immunotoxins in vitro: implications for human neurological disease and immunotherapeutics." J Neuroinflammation **6**: 31.

Hillier, L. W., T. A. Graves, et al. (2005). "Generation and annotation of the DNA sequences of human chromosomes 2 and 4." Nature **434**(7034): 724-31.

Honnorat, J., S. Cartalat-Carel, et al. (2009). "Onco-neural antibodies and tumour type determine survival and neurological symptoms in paraneoplastic neurological syndromes with Hu or CV2/CRMP5 antibodies." J Neurol Neurosurg Psychiatry **80**(4): 412-6.

Hughes, E. G., X. Peng, et al. (2010). "Cellular and synaptic mechanisms of anti-NMDA receptor encephalitis." J Neurosci **30**(17): 5866-75.

Huttlin, E. L., M. P. Jedrychowski, et al. (2010). "A tissue-specific atlas of mouse protein phosphorylation and expression." Cell **143**(7): 1174-89.

Irani, S. R., C. G. Bien, et al. (2011). "Autoimmune epilepsies." Curr Opin Neurol **24**(2): 146-53.

Kazarian, M., J. Calbo, et al. (2009). "Immune response in lung cancer mouse model mimics human anti-Hu reactivity." J Neuroimmunol **217**(1-2): 38-45.

Kazarian, M. and I. A. Laird-Offringa (2011). "Small-cell lung cancer-associated autoantibodies: potential applications to cancer diagnosis, early detection, and therapy." Mol Cancer **10**: 33.

Kim, Y. T. and G. W. Siskind (1978). "Studies on the control of antibody synthesis. XII. Genetic influences on antibody affinity." Immunology **34**(4): 669-78.

King, P. H. (1997). "Differential expression of the neuroendocrine genes Hel-N1 and HuD in small-cell lung carcinoma: evidence for down-regulation of HuD in the variant phenotype." Int J Cancer **74**(4): 378-82.

Kleine, T. O. and L. Benes (2006). "Immune surveillance of the human central nervous system (CNS): different migration pathways of immune cells through the blood-brain barrier and blood-cerebrospinal fluid barrier in healthy persons." Cytometry A **69**(3): 147-51.

Knudsen, A., S. E. Monstad, et al. (2006). "Ri antibodies in patients with breast, ovarian or small cell lung cancer determined by a sensitive immunoprecipitation technique." Cancer Immunol Immunother **55**(10): 1280-4.

Laffer, S., C. Lupinek, et al. (2008). "A high-affinity monoclonal anti-IgE antibody for depletion of IgE and IgE-bearing cells." Allergy **63**(6): 695-702.

Lavin, M. F. and S. Kozlov (2007). "ATM activation and DNA damage response." Cell Cycle **6**(8): 931-42.

Lazarus, J. H., A. B. Parkes, et al. (2002). "Postpartum thyroiditis." Autoimmunity **35**(3): 169-73.

Li, B., J. Chen, et al. (2008). "Measuring binding kinetics of surface-bound molecules using the surface plasmon resonance technique." Anal Biochem **377**(2): 195-201.

Libert, C., L. Dejager, et al. (2010). "The X chromosome in immune functions: when a chromosome makes the difference." Nat Rev Immunol **10**(8): 594-604.

Lichte, B., R. W. Veh, et al. (1992). "Amphiphysin, a novel protein associated with synaptic vesicles." EMBO J **11**(7): 2521-30.

Lopez, P. H., A. M. Villa, et al. (2002). "High affinity as a disease determinant factor in anti-GM(1) antibodies: comparative characterization of experimentally induced vs. disease-associated antibodies." J Neuroimmunol **128**(1-2): 69-76.

Lopez, P. H., G. Zhang, et al. (2010). "Passive transfer of IgG anti-GM1 antibodies impairs peripheral nerve repair." J Neurosci **30**(28): 9533-41.

Lutz, H. U. (2007). "Homeostatic roles of naturally occurring antibodies: an overview." J Autoimmun **29**(4): 287-94.

Manley, G. T., P. S. Smitt, et al. (1995). "Hu antigens: reactivity with Hu antibodies, tumor expression, and major immunogenic sites." Ann Neurol **38**(1): 102-10.

Mason, W. P., F. Graus, et al. (1997). "Small-cell lung cancer, paraneoplastic cerebellar degeneration and the Lambert-Eaton myasthenic syndrome." Brain **120 (Pt 8)**: 1279-300.

Matschke, J., A. Kromminga, et al. (2007). "Paraneoplastic cerebellar degeneration and anti-Yo antibodies in a man with prostatic adenocarcinoma." J Neurol Neurosurg Psychiatry **78**(7): 775-7.

Matsuoka, S., B. A. Ballif, et al. (2007). "ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage." Science **316**(5828): 1160-6.

McKeon, A., J. A. Tracy, et al. (2011). "Purkinje Cell Cytoplasmic Autoantibody Type 1 Accompaniments: The Cerebellum and Beyond." Arch Neurol.

Meuwissen, R., S. C. Linn, et al. (2003). "Induction of small cell lung cancer by somatic inactivation of both Trp53 and Rb1 in a conditional mouse model." Cancer Cell **4**(3): 181-9.

Monstad, S. E., L. Drivsholm, et al. (2008). "CRMP5 antibodies in patients with small-cell lung cancer or thymoma." Cancer Immunol Immunother **57**(2): 227-32.

Monstad, S. E., L. Drivsholm, et al. (2004). "Hu and voltage-gated calcium channel (VGCC) antibodies related to the prognosis of small-cell lung cancer." J Clin Oncol **22**(5): 795-800.

Monstad, S. E., A. Knudsen, et al. (2009). "Onconeural antibodies in sera from patients with various types of tumours." Cancer Immunol Immunother.

Monstad, S. E., J. K. Nostbakken, et al. (2009). "CRMP5 antibodies found in a patient with limbic encephalitis and myasthenia gravis." J Neurol Neurosurg Psychiatry **80**(2): 241-2.

Monstad, S. E., A. Storstein, et al. (2006). "Yo antibodies in ovarian and breast cancer patients detected by a sensitive immunoprecipitation technique." Clin Exp Immunol **144**(1): 53-8.

Monstad, S. E. and C. A. Vedeler (2006). "An immunoprecipitation assay for the detection of onconeural antibodies." Acta Neurol Scand Suppl **183**: 71-2.

Musunuru, K. and R. B. Darnell (2001). "Paraneoplastic neurologic disease antigens: RNA-binding proteins and signaling proteins in neuronal degeneration." Annu Rev Neurosci **24**: 239-62.

Nagata, S. (2010). "Apoptosis and autoimmune diseases." Ann N Y Acad Sci **1209**: 10-6.

Nangaku, M. and W. G. Couser (2005). "Mechanisms of immune-deposit formation and the mediation of immune renal injury." Clin Exp Nephrol **9**(3): 183-91.

O'Donovan, K. J., J. Diedler, et al. (2010). "The onconeural antigen cdr2 is a novel APC/C target that acts in mitosis to regulate c-myc target genes in mammalian tumor cells." PLoS One **5**(4): e10045.

Ohwada, A., I. Nagaoka, et al. (1999). "DNA vaccination against HuD antigen elicits antitumor activity in a small-cell lung cancer murine model." Am J Respir Cell Mol Biol **21**(1): 37-43.

Okano, H. J., W. Y. Park, et al. (1999). "The cytoplasmic Purkinje onconeural antigen cdr2 down-regulates c-Myc function: implications for neuronal and tumor cell survival." Genes Dev **13**(16): 2087-97.
Ono, E., M. M. Lafer, et al. (2004). "A simple and cheaper in house varicella zoster virus antibody indirect ELISA." Rev Inst Med Trop Sao Paulo **46**(3): 165-8.
Ota, T., Y. Suzuki, et al. (2004). "Complete sequencing and characterization of 21,243 full-length human cDNAs." Nat Genet **36**(1): 40-5.
Pellkofer, H., A. S. Schubart, et al. (2004). "Modelling paraneoplastic CNS disease: T-cells specific for the onconeuronal antigen PNMA1 mediate autoimmune encephalomyelitis in the rat." Brain **127**(Pt 8): 1822-30.
Persson, M. A., S. E. Brown, et al. (1988). "IgG subclass-associated affinity differences of specific antibodies in humans." J Immunol **140**(11): 3875-9.
Pittock, S. J., T. J. Kryzer, et al. (2004). "Paraneoplastic antibodies coexist and predict cancer, not neurological syndrome." Ann Neurol **56**(5): 715-9.
Raspotnig, M., C. A. Vedeler, et al. (2011). "Onconeural antibodies in patients with neurological symptoms: detection and clinical significance." Acta Neurol Scand Suppl(191): 83-8.
Reinhardt, H. C. and M. B. Yaffe (2009). "Kinases that control the cell cycle in response to DNA damage: Chk1, Chk2, and MK2." Curr Opin Cell Biol **21**(2): 245-55.
Roberts, W. K., I. J. Deluca, et al. (2009). "Patients with lung cancer and paraneoplastic Hu syndrome harbor HuD-specific type 2 CD8+ T cells." J Clin Invest **119**(7): 2042-51.
Rojas, I., F. Graus, et al. (2000). "Long-term clinical outcome of paraneoplastic cerebellar degeneration and anti-Yo antibodies." Neurology **55**(5): 713-5.
Romero-Steiner, S., P. F. Holder, et al. (2005). "Avidity determinations for Haemophilus influenzae Type b anti-polyribosylribitol phosphate antibodies." Clin Diagn Lab Immunol **12**(9): 1029-35.
Rosenfeld, M. R. and J. Dalmau (2010). "Update on paraneoplastic and autoimmune disorders of the central nervous system." Semin Neurol **30**(3): 320-31.
Rosenfeld, M. R. and J. Dalmau (2010). "Update on paraneoplastic neurologic disorders." Oncologist **15**(6): 603-17.
Rossi, C. L. (1998). "A simple, rapid enzyme-linked immunosorbent assay for evaluating immunoglobin G antibody avidity in toxoplasmosis." Diagn Microbiol Infect Dis **30**(1): 25-30.
Rubio, I., A. Bolchi, et al. (2009). "Potent anti-HPV immune responses induced by tandem repeats of the HPV16 L2 (20 -- 38) peptide displayed on bacterial thioredoxin." Vaccine **27**(13): 1949-56.
Sahashi, K., K. Sakai, et al. (2003). "Anti-Ma2 antibody related paraneoplastic limbic/brain stem encephalitis associated with breast cancer expressing Ma1, Ma2, and Ma3 mRNAs." J Neurol Neurosurg Psychiatry **74**(9): 1332-5.
Sakai, K., Y. Kitagawa, et al. (2001). "Suppression of the transcriptional activity and DNA binding of nuclear factor-kappa B by a paraneoplastic cerebellar degeneration-associated antigen." J Neuroimmunol **119**(1): 10-5.
Sakai, K., Y. Kitagawa, et al. (2004). "Effect of a paraneoplastic cerebellar degeneration-associated neural protein on B-myb promoter activity." Neurobiol Dis **15**(3): 529-33.
Sakai, K., D. J. Mitchell, et al. (1990). "Isolation of a complementary DNA clone encoding an autoantigen recognized by an anti-neuronal cell antibody from a patient with paraneoplastic cerebellar degeneration." Ann Neurol **28**(5): 692-8.

Sakai, K., T. Ogasawara, et al. (1993). "Analysis of autoantibody binding to 52-kd paraneoplastic cerebellar degeneration-associated antigen expressed in recombinant proteins." Ann Neurol **33**(4): 373-80.

Sakai, K., T. Shirakawa, et al. (2002). "Interaction of a paraneoplastic cerebellar degeneration-associated neuronal protein with the nuclear helix-loop-helix leucine zipper protein MRG X." Mol Cell Neurosci **19**(4): 477-84.

Santomasso, B. D., W. K. Roberts, et al. (2007). "A T-cell receptor associated with naturally occurring human tumor immunity." Proc Natl Acad Sci U S A **104**(48): 19073-8.

Sato, E., S. H. Olson, et al. (2005). "Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer." Proc Natl Acad Sci U S A **102**(51): 18538-43.

Satoh, J. and T. Yamamura (2004). "Gene expression profile following stable expression of the cellular prion protein." Cell Mol Neurobiol **24**(6): 793-814.

Schessl, J., M. Schuberth, et al. (2010). "Long-term efficiency of intravenously administered immunoglobulin in anti-Yo syndrome with paraneoplastic cerebellar degeneration." J Neurol.

Schreiber, R. D., L. J. Old, et al. (2011). "Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion." Science **331**(6024): 1565-70.

Schulz, T. F. (2009). "Cancer and viral infections in immunocompromised individuals." Int J Cancer **125**(8): 1755-63.

Shams'ili, S., J. Grefkens, et al. (2003). "Paraneoplastic cerebellar degeneration associated with antineuronal antibodies: analysis of 50 patients." Brain **126**(Pt 6): 1409-18.

Shao, L., H. Fujii, et al. (2009). "Deficiency of the DNA repair enzyme ATM in rheumatoid arthritis." J Exp Med **206**(6): 1435-49.

Sillevis Smitt, P., J. Grefkens, et al. (2002). "Survival and outcome in 73 anti-Hu positive patients with paraneoplastic encephalomyelitis/sensory neuronopathy." J Neurol **249**(6): 745-53.

Sillevis Smitt, P., A. Kinoshita, et al. (2000). "Paraneoplastic cerebellar ataxia due to autoantibodies against a glutamate receptor." N Engl J Med **342**(1): 21-7.

Simard, E. P. and E. A. Engels (2010). "Cancer as a cause of death among people with AIDS in the United States." Clin Infect Dis **51**(8): 957-62.

Siniscalco, M., I. Oberle, et al. (1991). "Physical and genetic mapping of the CDR gene with particular reference to its position with respect to the FRAXA site." Am J Med Genet **38**(2-3): 357-62.

Smith, J. L., J. C. Finley, et al. (1988). "Autoantibodies in paraneoplastic cerebellar degeneration bind to cytoplasmic antigens of Purkinje cells in humans, rats and mice and are of multiple immunoglobulin classes." J Neuroimmunol **18**(1): 37-48.

Smolander, H., J. O. Koskinen, et al. (2010). "A novel antibody avidity methodology for rapid point-of-care serological diagnosis." J Virol Methods **166**(1-2): 86-91.

Sodeyama, N., K. Ishida, et al. (1999). "Pattern of epitopic reactivity of the anti-Hu antibody on HuD with and without paraneoplastic syndrome." J Neurol Neurosurg Psychiatry **66**(1): 97-9.

Staropoli, J. F. (2008). "Tumorigenesis and neurodegeneration: two sides of the same coin?" Bioessays **30**(8): 719-27.

Steward, M. W., M. C. Reinhardt, et al. (1979). "The genetic control of antibody affinity. Evidence from breeding studies with mice selectively bred for either high or low affinity antibody production." Immunology **37**(3): 697-703.

Stich, O., F. Graus, et al. (2003). "Qualitative evidence of anti-Yo-specific intrathecal antibody synthesis in patients with paraneoplastic cerebellar degeneration." J Neuroimmunol **141**(1-2): 165-9.

Storstein, A., B. K. Krossnes, et al. (2009). "Morphological and immunohistochemical characterization of paraneoplastic cerebellar degeneration associated with Yo antibodies." Acta Neurol Scand **120**(1): 64-7.

Storstein, A., S. E. Monstad, et al. (2011). "Onconeural antibodies: improved detection and clinical correlations." J Neuroimmunol **232**(1-2): 166-70.

Storstein, A., S. E. Monstad, et al. (2004). "Paraneoplastic antibodies detected by isoelectric focusing of cerebrospinal fluid and serum." J Neuroimmunol **155**(1-2): 150-4.

Storstein, A., S. E. Monstad, et al. (2004). "Paraneoplastic antibodies against HuD detected by a sensitive radiobinding assay." J Neurol **251**(2): 197-203.

Storstein, A. and C. A. Vedeler (2007). "Paraneoplastic neurological syndromes and onconeural antibodies: clinical and immunological aspects." Adv Clin Chem **44**: 143-85.

Strausberg, R. L. (2005). "Tumor microenvironments, the immune system and cancer survival." Genome Biol **6**(3): 211.

Strausberg, R. L., E. A. Feingold, et al. (2002). "Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences." Proc Natl Acad Sci U S A **99**(26): 16899-903.

Sutton, I. (2002). "Paraneoplastic neurological syndromes." Curr Opin Neurol **15**(6): 685-90.

Sutton, I. J., J. Steele, et al. (2004). "An interferon-gamma ELISPOT and immunohistochemical investigation of cytotoxic T lymphocyte-mediated tumour immunity in patients with paraneoplastic cerebellar degeneration and anti-Yo antibodies." J Neuroimmunol **150**(1-2): 98-106.

Saalman, R., U. I. Dahlgren, et al. (2003). "Avidity progression of dietary antibodies in healthy and coeliac children." Clin Exp Immunol **134**(2): 328-34.

Takamori, M. (2008). "Lambert-Eaton myasthenic syndrome: search for alternative autoimmune targets and possible compensatory mechanisms based on presynaptic calcium homeostasis." J Neuroimmunol **201-202**: 145-52.

Takanaga, H., H. Mukai, et al. (1998). "PKN interacts with a paraneoplastic cerebellar degeneration-associated antigen, which is a potential transcription factor." Exp Cell Res **241**(2): 363-72.

Tampoia, M., A. Zucano, et al. (2010). "Diagnostic accuracy of different immunological methods for the detection of antineuronal antibodies in paraneoplastic neurological syndromes." Immunol Invest **39**(2): 186-95.

Tanaka, K., M. Tanaka, et al. (1995). "Trial to establish an animal model of paraneoplastic cerebellar degeneration with anti-Yo antibody. 2. Passive transfer of murine mononuclear cells activated with recombinant Yo protein to paraneoplastic cerebellar degeneration lymphocytes in severe combined immunodeficiency mice." Clin Neurol Neurosurg **97**(1): 101-5.

Tanaka, K., M. Tanaka, et al. (1994). "Passive transfer and active immunization with the recombinant leucine-zipper (Yo) protein as an attempt to establish an animal model of paraneoplastic cerebellar degeneration." J Neurol Sci **127**(2): 153-8.

Tanaka, M., K. Tanaka, et al. (1995). "Trial to establish an animal model of paraneoplastic cerebellar degeneration with anti-Yo antibody. 1. Mouse strains bearing different MHC molecules produce antibodies on immunization with recombinant Yo protein, but do not cause Purkinje cell loss." Clin Neurol Neurosurg **97**(1): 95-100.

Tanaka, M., K. Tanaka, et al. (2001). "Cytotoxic T cell activity against the peptide, AYRARALEL, from Yo protein of patients with the HLA A24 or B27 supertype and paraneoplastic cerebellar degeneration." J Neurol Sci **188**(1-2): 61-5.

Tani, T., K. Tanaka, et al. (2008). "Regulatory T cells in paraneoplastic neurological syndromes." J Neuroimmunol **196**(1-2): 166-9.

Totland, C., N. K. Aarskog, et al. (2011). "CDR2 antigen and Yo antibodies." Cancer Immunol Immunother **60**(2): 283-9.

Valiente-Gabioud, A. A., C. Veaute, et al. (2010). "Effect of repetitiveness on the immunogenicity and antigenicity of Trypanosoma cruzi FRA protein." Exp Parasitol **127**(3): 672-9.

van de Schans, S. A., D. J. van Spronsen, et al. (2010). "Excess of autoimmune and chronic inflammatory disorders in patients with lymphoma compared with all cancer patients: A cancer registry-based analysis in the south of the Netherlands." Autoimmun Rev **10**(4): 228-34.

van Houdt, I. S., B. J. Sluijter, et al. (2008). "Favorable outcome in clinically stage II melanoma patients is associated with the presence of activated tumor infiltrating T-lymphocytes and preserved MHC class I antigen expression." Int J Cancer **123**(3): 609-15.

Vedeler, C. A., J. C. Antoine, et al. (2006). "Management of paraneoplastic neurological syndromes: report of an EFNS Task Force." Eur J Neurol **13**(7): 682-90.

Vesely, M. D., M. H. Kershaw, et al. (2011). "Natural innate and adaptive immunity to cancer." Annu Rev Immunol **29**: 235-71.

Vincent, A., N. Willcox, et al. (1998). "Determinant spreading and immune responses to acetylcholine receptors in myasthenia gravis." Immunol Rev **164**: 157-68.

Voltz, R., S. H. Gultekin, et al. (1999). "A serologic marker of paraneoplastic limbic and brain-stem encephalitis in patients with testicular cancer." N Engl J Med **340**(23): 1788-95.

Winter, S. F., Y. Sekido, et al. (1993). "Antibodies against autologous tumor cell proteins in patients with small-cell lung cancer: association with improved survival." J Natl Cancer Inst **85**(24): 2012-8.

Wucherpfennig, K. W. (1994). "Autoimmunity in the central nervous system: mechanisms of antigen presentation and recognition." Clin Immunol Immunopathol **72**(3): 293-306.

Yamashita, N., B. Mosinger, et al. (2011). "CRMP5 (collapsin response mediator protein 5) regulates dendritic development and synaptic plasticity in the cerebellar Purkinje cells." J Neurosci **31**(5): 1773-9.

Zornig, M. and G. I. Evan (1996). "Cell cycle: on target with Myc." Curr Biol **6**(12): 1553-6.

Zuckier, L. S., E. Z. Berkowitz, et al. (2000). "Influence of affinity and antigen density on antibody localization in a modifiable tumor targeting model." Cancer Res **60**(24): 7008-13.