Cerebellar degeneration-related proteins in anti-Yo associated paraneoplastic cerebellar degeneration

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Thesis for the degree of Philosophiae Doctor (PhD)
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SCIENTIFIC ENVIRONMENT

The work presented in this thesis was carried out at the Neurology Research Laboratory led by Prof. Christian A. Vedeler, Department of Neurology, Haukeland University Hospital, Bergen, and Neuro-SysMed, Centre of Excellence for Experimental Therapy in Neurology, Department of Neurology, Haukeland University Hospital, and the Department of Clinical Medicine, University of Bergen. Neuro-SysMed is supported as an FKB center (Centre for Clinical Treatment Research) by grants from The Research Council of Norway.

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Co-supervisors: Dr. Sonia Gavasso and ScD. Manja Schubert

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Already since attending Akademiet videregående skole I knew that I wanted to pursue a career in science. This is largely due to the influence from inspiring teachers, and I have to mention one in particular, Kugarajh Kanaganayagam. He even lectured me and my good friend, Amalie, in physics and maths during Saturday mornings so that we would be able to pursue our passions. Thank you for motivating and believing in us.

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LIST OF PUBLICATIONS

The thesis is based on the following papers:

I. Krakenes, T., Herdlevaer, I., Raspotnig, M., Haugen, M., Schubert, M., & Vedeler, C. A. (2019). CDR2L Is the Major Yo Antibody Target in Paraneoplastic Cerebellar Degeneration. *Ann Neurol*. doi:10.1002/ana.25511

II. Herdlevær, I., Kråkenes, T., Schubert, M., & Vedeler, C. A. (2020). "Localization of CDR2L and CDR2 in paraneoplastic cerebellar degeneration." Annals of clinical and translational neurology **7**(11): 2231-2242.

III. Herdlevær, I., Haugen, M., Mazengia K., Totland, C., Vedeler, C. A. (2021). "Paraneoplastic Cerebellar Degeneration: The Importance of Including CDR2L as a Diagnostic Marker." Neurology(R) neuroimmunology & neuroinflammation 8(2).

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LIST OF ABBREVIATIONS

BBB	Blood-brain barrier
CBA	Cell-based assay
CDR2	Cerebellar degeneration-related protein 2
CDR2L	Cerebellar degeneration-related protein 2-like
CNS	Central nervous system
CSF	Cerebrospinal fluid
eIF4A3	Eukaryotic initiation factor 4A-III
ER	Endoplasmic reticulum
GABA	Gamma-aminobutyric acid
HEK	Human embryonic kidney
Ig	Immunoglobulin
IHC	Immunohistochemistry
IIF	Indirect immunofluorescence
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
PBS	Phosphate-buffered saline
PAMP	Pathogen-associated molecular pattern
PCD	Paraneoplastic cerebellar degeneration
PNS	Paraneoplastic neurological syndromes
PRR	Pattern recognition receptor
rpS6	Ribosomal protein S6
SDS	Sodium dodecyl sulfate
SRSF2	Serine/arginine-rich splicing factor 2
STED	Super-resolution microscopy
TBS	Tris-buffered saline
TNF	Tumor necrosis factor
WB	Western blot

ABSTRACT

Background: Paraneoplastic neurological syndromes (PNS) are rare immune-mediated diseases triggered by cancer, and characterized by circulating onconeural antibodies directed against antigens expressed by neurons and tumor cells. One of the most common forms of PNS is paraneoplastic cerebellar degeneration (PCD). In patients with PCD and ovarian or breast cancer, the dominant onconeural antibody is anti-Yo, detected in serum and cerebrospinal fluid (CSF). Anti-Yo targets two intracellular antigens, cerebellar degeneration-related protein 2 (CDR2) and CDR2-like (CDR2L), expressed in the nucleus and cytoplasm of Purkinje neurons in the cerebellum, respectively. The interaction between anti-Yo and CDR proteins is thought to mediate Purkinje neuron dysfunction and death, leaving the patients in a severely disabled state.

The pathomechanisms underlying PCD, including the cellular functions and molecular mechanisms driven by the CDR proteins, are limited. About ten years ago, CDR2L was identified as a new target associated with PCD, and its specific location and neuronal functions remains elusive. Herein, we aim to draw a clearer picture of the anti-Yo associated neurodegeneration by addressing which onconeural antigen is the major target of Yo antibodies, identifying the subcellular localization of CDR2 and CDR2L and interaction partners. Finally, we evaluate the possibility of including CDR2L as a diagnostic marker when diagnosing patients with PCD.

Aims: The overarching aim of this work was to gain in depth knowledge about CDR2 and CDR2L in anti-Yo associated PCD.

Materials and methods: In paper I, anti-Yo reactivity towards CDR2 and CDR2L was addressed by staining cerebellar sections and cancer cells, and performing immunoprecipitation and fluorescent immunoblotting analysis. HepG2 cells were transfected to investigate whether Yo antibodies detect recombinant forms of the proteins. In paper II, mass spectrometry based proteomics was used to determine antibody specificity, and to identify CDR2 and CDR2L protein binding partners.

Findings were further investigated by co-immunoprecipitation, proximity ligation assay and co-localization studies using super-resolution microscopy. Immunochemistry and super-resolution microscopy enabled determination of the subcellular localizations of CDR2 and CDR2L. In paper III, the potential of including CDR2L as a diagnostic marker in PCD was assessed by developing two in-house techniques, cell-based assay for CDR2L and western blot analysis of recombinant CDR2 and CDR2L.

Results: We show that CDR2L is the major Yo antigen and that including CDR2L as a marker in the diagnosis of PCD greatly improves test specificity. Furthermore, the subcellular location of CDR2L is found to be in the cell cytoplasm, more specifically in direct contact with the ribosomal subunit protein, rpS6. CDR2 localizes to the nucleus in contact with nuclear speckle proteins, SON and eIF4A3.

Conclusions: Identifying CDR2L as the major Yo antigen is an important finding in the work of increasing test specificity for PCD diagnosis, and is essential for further investigation of PCD pathogenesis. However, a central role of CDR2 in PCD can not be excluded. By determining the subcellular locations of the proteins and binding partners, we are one step closer in understanding the functions of CDR2 and CDR2L in anti-Yo associated PCD.

1. INTRODUCTION

1.1 The nervous system

The nervous system is a complex network of neurons that transmit signals between different parts of the body and coordinate the body's functions. The nervous system has two structural components, the peripheral nervous system and the central nervous system (CNS).

1.1.1 The peripheral nervous system

The peripheral nervous system consists of nerves that branches from the brain and spinal cord (the central nervous system), and has two functional subdivisions, a sensory component and a motor component. The sensory component includes sensory (afferent) neurons that link receptors on the body's surface or within tissues to the CNS. The motor component of the peripheral nervous system can be divided into the somatic and autonomic motor divisions. The somatic motor system involves voluntary movements driven by motor (efferent) neurons that connect the CNS to skeletal muscles. The autonomic motor system includes cells that innervate involuntary structures, such as smooth muscles, cardiac muscles and glands within the body.[1]

1.1.2 The central nervous system

The CNS comprises the spinal cord and the brain. The brain is divided into three major structures: the forebrain, the midbrain and the hindbrain.[1] The forebrain includes thalamus, hypothalamus, and cerebrum, which encompasses the cerebral cortex and subcortical structures, such as the hippocampus, amygdala, and basal ganglia. The thalamus and hypothalamus are in charge of homeostasis (e.g. respiration, blood pressure, body temperature) and relay information to the cerebral cortex from other brain regions. The cerebral cortex is important for conscious thought, perception, and learning. It comprises a six-layered structure, referred to as neocortex, each layer with its own composition of neuronal cells. The hippocampus is important for memory formation, the amygdala processes emotional information, and the basal ganglia are a group of subcortical nuclei responsible for motor control.[2] The midbrain connects the

forebrain to the hindbrain. The midbrain and the hindbrain compose the brainstem. The hindbrain involves the medulla, pons and cerebellum, each with its own unique structure and function. Together they maintain balance, posture, coordinate movement, relay sensory information and assist in autonomic functions, such as breathing, heart rate and blood pressure.[3] The cerebellum is essential for coordination and planning of movements, and will be addressed in the following.

Cerebellum

The human cerebellum has mainly been recognized as the regulator of highly skilled movements, especially the planning and execution of complex spatial and temporal sequences of movement. In the last ten years there has been an increasing focus on studying the role of the cerebellum in cognition, thus challenging the view that the cerebellum soley contributes to planning and execution of movements.[4-6]

The cerebellum contains three cortical layers: the molecular layer, the Purkinje neuron layer and the granular layer (Fig 1.1.2).[7] The innermost cortical layer is the granular layer, containing mossy fibers, the cell bodies of granule cells, and Golgi cells. The middle cerebellar layer is the Purkinje layer, containing the cell bodies of Purkinje neurons and Bergmann glial cells. The outermost cortical layer is the molecular layer, containing the dendrites of Purkinje neurons and the inhibitory stellate and basket cells.[7]

Mossy fibers are axons that originate from multiple brainstem nuclei and synapse on neurons in the deep cerebellar nuclei and on granule cells. The granule cells constitute the majority of neurons in the brain and give rise to axons called parallel fibers that ascend to the molecular layer of the cerebellar cortex and synapse onto Purkinje neuron dendrites[8]. Parallel fibers activate stellate and basket cells, which provide an inhibitory input to Purkinje neuron dendrites. Purkinje neurons are also innervated by climbing fibers, which arise from the inferior olivary nucleus in the medulla oblongata. The convergence of parallel fibers and climbing fibers to Purkinje neurons has been proposed to be important for cerebellar learning.[9] Purkinje neuron axons project into the deep cerebellar nuclei, where they exert inhibitory effects via release of the

inhibitory neurotransmitter, gamma-aminobutyric acid (GABA). Purkinje neurons constitute the sole output of the cerebellar cortex, which is wholly inhibitory.[8] Neurons in the deep cerebellar nuclei receive excitatory input from mossy and climbing fibers. An interplay between these inhibitory and excitatory projections serve to shape the discharge patterns to the thalamus.[10]

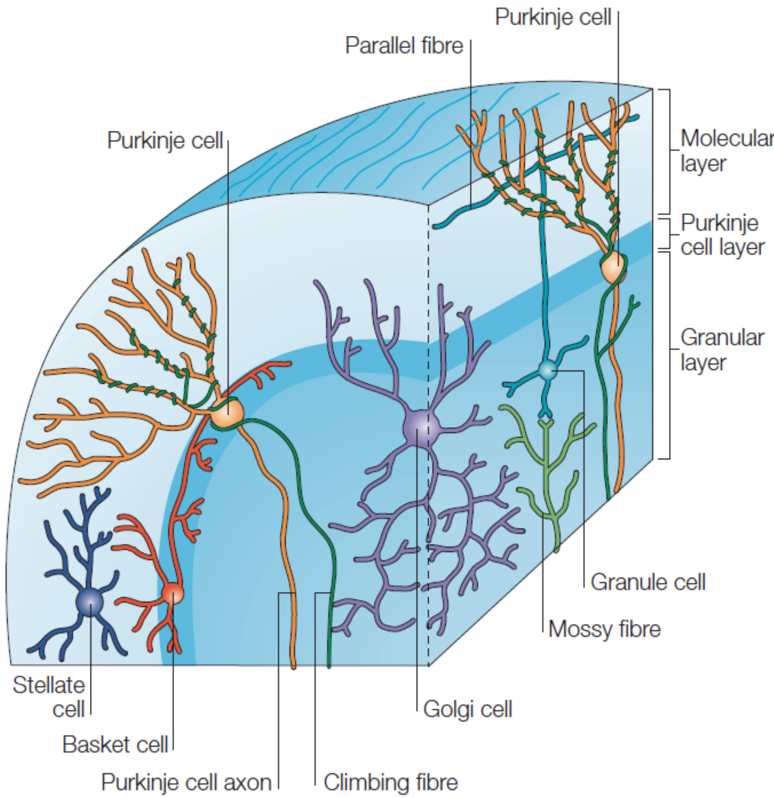


Figure 1.1.2: Neural circuit of the cerebellum. Reprinted with permission from Springer Nature, License #5031381453503: Nature reviews neuroscience, Anatomical and physiological foundations of cerebellar information processing, Apps et al. 2005.[11]

1.2 Neurons and glial cells

Cells of the nervous system can be divided into two main categories: neurons and glial cells. The glia to neuron ratio varies across the different human brain structures, with an almost equal amount in the cerebral cortex, six times as many glial cells in the spinal cord and around 20 times as many neurons in the cerebellum.[12]

1.2.1 Neurons

Neurons have diverse molecular, morphological and functional properties, but they all contain four distinct regions: the dendrites, the cell body, the axon and axon terminals.[13] Interneuronal communication is required for normal brain function and relies on two types of signaling mechanisms – action potentials and synaptic transmissions. Action potentials are electrical signals conducted from the cell body towards the axon terminals. Action potentials can trigger two main modalities of synaptic transmission, chemical and electrical.[14] At chemical synapses, information is conveyed via the release of neurotransmitters from presynaptic neurons to postsynaptic neurons via functional junctions (synapses). Chemical synapses can be of excitatory (e.g. glutamate) or inhibitory (e.g. GABA) nature depending on the released neurotransmitter, and can undergo response-dependent modifications to alter their activity pattern, referred to as synaptic plasticity. At electrical synapses there is a direct connection between the cytoplasm of a presynaptic and postsynaptic neuron via intracellular channels called gap junctions.[14]

Purkinje neurons

Purkinje neurons are characterized by their unique fan-shaped structure (Fig 1.2.1). The soma of Purkinje neurons are located in a single-layer manner, making up the Purkinje neuron layer of the cerebellum. One or two primary dendrites extend from the soma to form highly branched, non-overlapping dendrites which aborize into the molecular layer. Parallel fibers pass perpendicularly through Purkinje neuron dendrites in a grid-like structure, making up approximately 100,000 synaptic connections. The Purkinje neuron axon extends through the granular layer to the deep cerebellar nuclei.[15]

Purkinje neurons represent the sole output of the cerebellar cortex.[16] It follows that disturbances of Purkinje neuron signalling will cause severe symptoms. These include reduced coordination and fine movement (cerebellar ataxia), involuntary eye movement (nystagmus), and dysfunction of articulation (dysarthria).[17] Purkinje neurons are prone to damage caused by toxins (ethanol), autoimmune diseases (gluten

ataxia), genetic mutations (spinocerebellar ataxia) and neurodegenerative diseases (multiple system atrophy).[16]

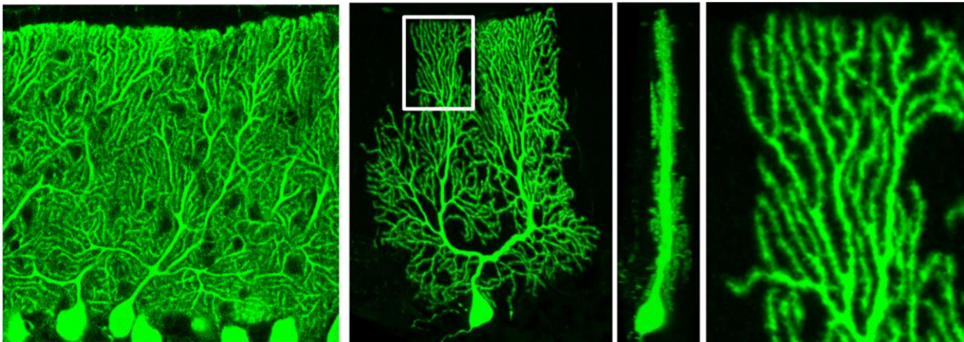


Figure 1.2.1: Purkinje neuron with its unique fan-shaped structure and extensive dendritic branches. Reprinted with permission from Springer Nature, License #5031380743925: Springer Nature, Dendritic Self-Avoidance and Morphological Development of Cerebellar Purkinje Cells, Fujishima et al. 2018.[15]

1.2.2 Glial cells

Glial cells are non-neuronal, supportive cells of the nervous system. They are involved in homeostasis (synaptic action, neurotransmitter uptake, maintaining the ionic and water milieu), aiding in defence, and repair of injured nerves.[18] There are two classes of glial cells in the mature CNS: macroglia (astrocytes and oligodendrocytes) and microglia. Astrocytes function in appropriate maintenance of chemical environments for neuronal signaling and blood-brain barrier (BBB) maintenance. Oligodendrocytes are myelin-producing cells that insulate axons, thereby increasing the transmission of action potentials.[19] Microglia are the resident immune cells of the CNS, with three essential functions: 1) sensing environmental changes, 2) physiologic housekeeping, and 3) defence against modified or harmful non-self antigens.[20]

1.3 The immune system of the central nervous system

The blood-brain barrier

The BBB is a semipermeable border that regulates the entry of immune cells into the CNS, and strictly controls the exchange of substances between the CNS and the blood (Fig 1.3).[21] This tight barrier consists of endothelial cells that express tight junction

proteins and restricts the passage of solutes. Neurons and astrocytes also reside in close proximity to the BBB. This allows for neurons to respond to changes in the local milieu, whereas astrocytes help regulate ion and water flux.[22] Thus, the BBB provides anatomical and physical protection for the CNS, which is of particular importance since BBB dysfunction plays a key role in neurodegenerative disorders by affecting movement of immune cells and immune mediators into the brain.[23]

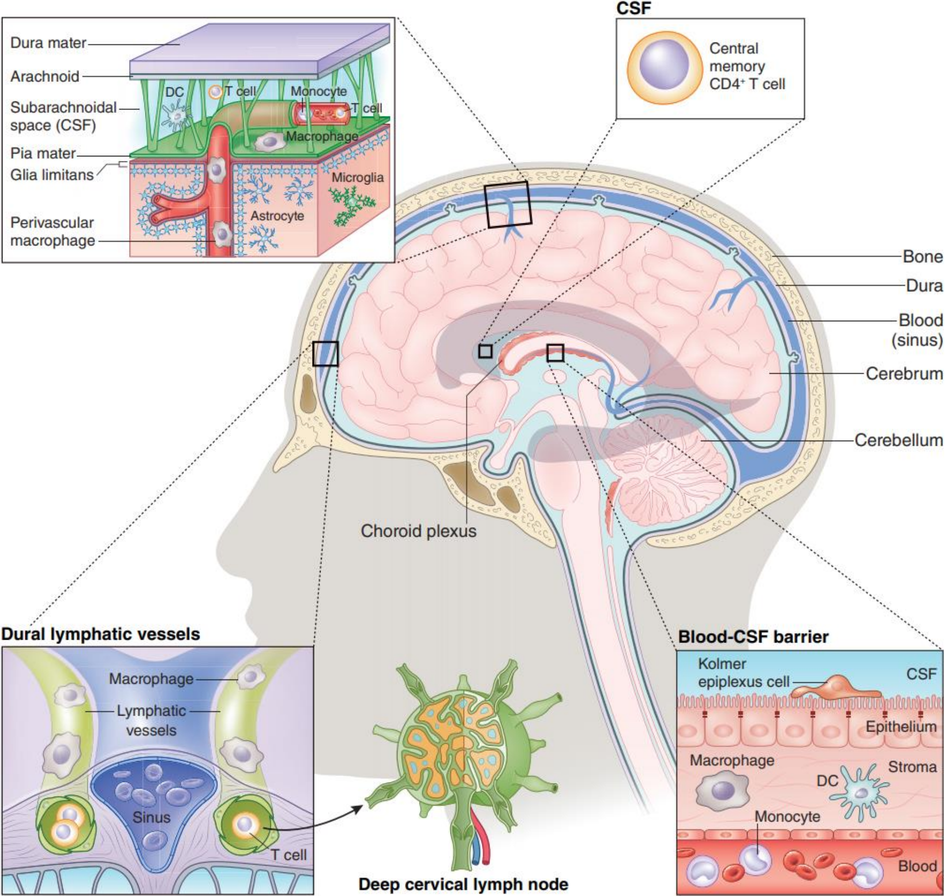


Figure 1.3: Immune system in the central nervous system during homeostasis in a non diseased brain. Reprinted with permission from Springer Nature, License #5031890971092: Nature neuroscience, The role of peripheral immune cells in the CNS in steady state and disease, Prinz and Priller 2017.[24]

The meninges

The meninges are composed of three protective tissue layers: the dura mater, the arachnoid mater and the pia mater, which surround and support the brain and spinal

cord. The subarachnoid space exist between the arachnoid mater and pia mater, and consist of CSF, major blood vessels and cisterns (enlarged pockets of CSF).[25]

The ventricular system

The ventricular system is responsible for the production, transport and reabsorption of CSF. It is composed of a communicating network of cavities located in the core of the forebrain and brainstem, the ventricles. The choroid plexus is a vascular structure present in all four ventricles that produce CSF.[25]

The blood-cerebrospinal fluid barrier

Choroid plexus epithelial cells and tight junctions establish the blood-CSF barrier and regulate the passage of solutes from blood into CSF.[26] The blood-CSF barrier has also been shown to regulate the entry of leukocytes to the CNS.[27]

The glymphatic system

Interstitial fluid, fluid between blood vessels and cells, and CSF drain to lymph nodes by separate ways, but an exchange system between the two has been identified, called the glymphatic system. This system provides efficient elimination of soluble proteins and metabolites from the CNS, essential for maintaining brain homeostasis.[28]

1.4 The immune system

Immunity is derived from the Latin word *immunitas*, and refers to protection from infectious disease. The cells and molecules responsible for immunity constitute the immune system.[29] The skin, respiratory or intestinal tract, and other areas are protected against microbes, viruses, cancer cells and toxins, by the immune systems two fundamental lines of defense: innate immunity and adaptive immunity.[30]

1.4.1 Innate immunity

Innate immunity provides the initial defence against infections and consists of four types of barriers: anatomic, physiologic, phagocytic and inflammatory. The anatomic barrier comprises intact epithelial surfaces that forms physical barriers between microbes in the external environment and host tissue. The physiological barrier refers

to changes in body temperature to inhibit growth of some pathogens, acidic pH in the stomach to kill undigested microbes or chemical mediators, such as lysozyme digestion. Cells with specialized phagocytic functions, primarily neutrophils and macrophages, ingest and sometimes digest whole organisms or foreign particles, which are important functions in innate immunity. Finally, blood and extracellular fluids contain molecules that recognize microbes and promote host defense, including recruitment of immune cells. This inflammatory barrier is sometimes called the humoral branch of innate immunity, and can be compared to the humoral branch of adaptive immunity.[30] Innate immune responses do not require prior exposure to microbes and there is no prominent change in the magnitude upon repeated exposure, which contrasts adaptive immunity.

This first line of host defense highly depends on pattern recognition receptors (PRRs) recognizing unique microbial molecules, called pathogen-associated molecular patterns (PAMPs). Different microbes produce varying PAMPs, such as cell-wall components, lipopolysaccharides, on gram-negative bacteria.[30] Upon PAMP-PRR interactions, intracellular signaling cascades are triggered, resulting in expression of various inflammatory molecules, such as cytokines and chemokines.[31] Cytokines orchestra proinflammatory or anti-inflammatory host responses depending on the nature of the target cell and nature of the activating signal.[32] Tumor necrosis factor (TNF), interleukin 1 (IL-1) and interleukin 6 (IL-6) are among the key inflammatory cytokines. These cytokines are involved in local inflammation and recruitment of phagocytic cells and antigen-presenting cells. Dendritic cells are conventional antigen-presenting cells that express a group of proteins called major histocompatibility complex (MHC). After digesting an antigen into peptides, dendritic cells, present these MHC bound peptides to T cells. Thus, antigen-presenting cells play an important role in the crosstalk between innate and adaptive immunity.

1.4.2 Adaptive immunity

Adaptive immunity is critical when innate immunity fails to eliminate the infectious agents. The main characteristics of adaptive immunity is the ability to distinguish between different self and non-self substances (antigens), its specificity, and its ability

to respond more vigorously upon subsequent infection, its memory.[33] There are two broad classes of adaptive immunity: cell-mediated immunity and humoral immunity.

Cell-mediated immunity

Cell-mediated immunity is mediated by T cells that derive from hematopoietic stem cells in the bone marrow and mature in the thymus.[30] T cells recognize intracellular microbes that are inaccessible to circulating antibodies, and promote destruction of the microbes or infected cells.[29] Fragments of the microbes are displayed on MHC molecules by antigen-presenting cells. This MHC/antigen complex is recognized by unique antigen-binding receptors, T cell receptors (TCR), together with co-stimulatory receptors, cluster of differentiation (CD). This interaction leads to T cell activation, proliferation, differentiation and cytokine release.[34]

T cells can differentiate into functionally different populations, such as T helper (Th) cells ($CD4^+$ cells), cytotoxic T cells ($CD8^+$ cells) and regulatory T cells, depending on the interaction between TCRs with peptides presented by class I or class II MHC molecules.[30] $CD8^+$ cytotoxic T cells are activated by the interaction with peptides bound to MHC class I molecules, whereas $CD4^+$ Th cells are activated by the interaction to peptides on MHC class II molecules. Upon activation, $CD8^+$ cytotoxic T cells eliminate reservoirs of infection by destructing cells infected by viruses or bacteria. Unlike $CD8^+$ cytotoxic T cells, $CD4^+$ Th cells have no cytotoxic or phagocytic activity and cannot directly kill infected cells. Instead, $CD4^+$ Th cells secrete cytokines that recruit other cells to aid in these tasks, such as helping phagocytes to kill infectious pathogens. Regulatory T cells, a subset of $CD4^+$ T cells, function mainly to suppress immune responses and prevent responses to self-antigens. $CD4^+$ T cells activate both cellular and humoral immune responses.[30, 33]

Humoral immunity

B cells are derived from hematopoietic stem cells in the bone marrow. This is also where they mature and assemble their antigen receptors, surface immunoglobulin (Ig).[33] Ig molecules are composed of two identical 50 kDa heavy chains and two identical 25 kDa light chains connected by disulfide bonds. Both the heavy and light

chains consist of amino terminal portions that vary in amino acid sequence between antibody molecules, and that participate in antigen recognition.[33] Ig, also known as antibodies, are synthesized by B cells in two forms: membrane bound antibodies on the surface of B cells, or secreted antibodies that neutralize toxins and target them for phagocytosis.[29] Upon activation, B cells proliferate and differentiate into antibody secreting plasma cells. These cells produce large amounts of antibodies, are short lived and often undergo apoptosis after the immune response is eliminated. B cells can also differentiate into memory B cells that respond faster and more effectively upon re-exposure.[29, 30]

The complement system is an important effector mechanism of both innate and adaptive immunity. It is composed of over 25 plasma and cell surface proteins that interact with one another and with other molecules of the immune system to eliminate microbes. Three major pathways ensure complement activation: alternative, classical and lectin pathways. The alternative pathway is activated by complement protein C3 binding to microbial surfaces, the classical pathway by C1 binding to antigen-antibody complexes and the lectin pathway by binding of microbial polysaccharides to mannose-binding lectin, ficolins or collectins. Only the classical pathway relies on antibody binding for activation. Despite which pathways are generated, the final steps and the complement activated are the same. The proteolysis of C3 results in activation of C5 convertase, which cleaves C5 to C5a and C5b. Generation of the C5a fragment is important in the formation of membrane pore complexes in cell membranes of microbial targets, which kills the targets by osmotic lysis.[33]

1.4.3 Antibodies and antigens

As noted, antibodies consist of two heavy and two light chains bound by disulfide bonds (Fig 1.4.3). The antigen-antibody binding site consists of two variable (V) domains, the light chain, V_L, and the heavy chain, V_H. These domains contain three sub-regions that are highly variable among different antibodies, known as hypervariable regions. Furthermore, the variable regions form the fragment antigen-binding (Fab) region through its non-covalent interaction with the constant C_L and C_H1 domains. The Fab region mediates specific recognition of antigens.[35]

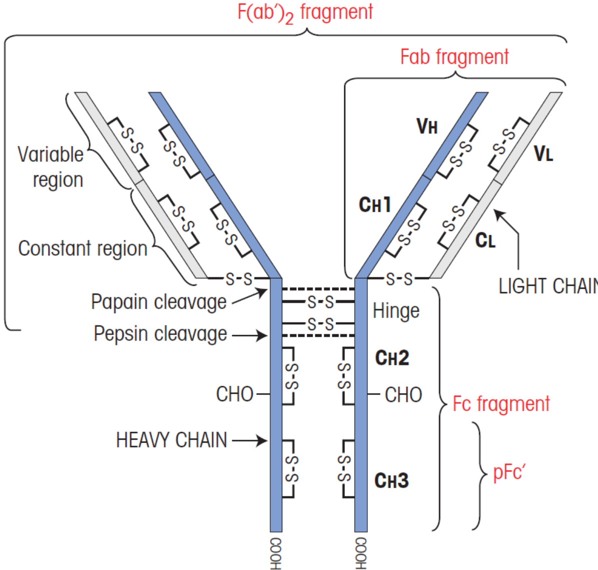


Figure 1.4.3: Structure of an antibody. The Y shaped antibody consists of two heavy and two light chains connected via disulfide bonds. V, variable; C, constant; Fab, antigen-binding fragment; Fc, crystallization fragment. Reprinted with permission from John Wiley and Sons, License #5044400418103. John Wiley and Sons, Roitt's Essential Immunology, 12th Edition, Burton et al. 2011.[36]

The heavy chain constant regions can self-associate and crystallize to form the crystallization fragment (Fc) region. This region is distant from the antigen-antibody site and interacts directly with other elements of the immune system, including binding to Fc receptors (FcRs) and activating the complement system.[30, 37]

Antibody molecules are divided into different classes (isotypes), IgA, IgD, IgE, IgG, and IgM, depending on the structure of their heavy chain constant region. These classes have different biological functions, mainly mediated by binding of the constant regions of the heavy chain to FcRs.[37]

FcRs are membrane molecules expressed by several cells, among others, B cells, macrophages and neutrophils, that aid in the recognition of the Fc region of several Ig classes and subclasses. These receptors constitute critical elements for activating or down-modulating immune responses and combine humoral and cell-mediated immunity. Thus, impared regulation by FcRs can lead to hyperreactivity to foreign or self-antigens.[38]

Antibodies have many practical applications in medical diagnosis, therapy and research. A key to understanding the various functions of antibodies in numerous applications is to determine the epitopes (binding sites) they recognize. Two classes of epitopes exist: linear epitopes, in which a stretch of continuous amino acids are sufficient for binding, and conformational epitopes, where protein folding enables amino acid residues to form an antigenic surface that can be bound by the antibody. Binding of an epitope to its antibody depends on the spatial arrangement of the epitope and non-covalent, reversible binding between the antigen and antibody. The strength of the interaction between one epitope and one antibody reflects the affinity of the antibody, whereas the strength of the antibody binding to all available epitopes on an antigen refers to its avidity.[39, 40] Antibodies can cross-react with similar epitopes on other antigens, but most often with lower affinity. The ability the antibody has to recognize a specific epitope in the presence of other epitopes, reflects the antibody`s specificity.[39]

Monoclonal and polyclonal antibodies can be purchased from commercial companies for research purposes. Monoclonal antibodies have often been preferred due to their homogeneity and consistency, making them suitable when studying changes in protein-interactions or conformational changes. The drawback concerning the use of monoclonal antibodies is that small structural changes in the epitope or conformational changes, due to altered pH, salt concentration or post-translational modification, can markedly affect the function of the antibody. Polyclonal antibodies, on the other hand, are less prone to such variations in the cellular surroundings, due to its heterogeneity and ability to recognize numerous antigenic epitopes.[39] Thus, the choice of antibody will greatly depend on the application.

1.4.4 Autoimmunity

One of the remarkable features of the immune system is its ability to react against foreign antigens while not reacting harmfully towards self-antigens. Such immune tolerance is regulated at the stage of immature B cell development by clonal deletion. Clonal deletion involves apoptosis of B cells that express receptors against self-antigens before they mature into immunocompetent cells, thereby maintaining self-

tolerance.[41] In the thymus, T cells with potential reactivity against self-antigens are deleted through negative selection.[42] Abnormalities in the regulation of these mechanisms may result in autoimmunity, the failure to maintain self-tolerance.[29]

Effector mechanisms, such as circulating autoantibodies, autoreactive T cells and formation of immune complexes, contribute to various autoimmune diseases.[29] Autoantibodies are antibodies that react against self-antigens and that can be pathogenic.[43] Serum or cerebrospinal fluid (CSF) autoantibodies have become a potent diagnostic marker for autoimmune diseases.[44] Paraneoplastic cerebellar degeneration, described in chapter 1.5.2, are rare immune-mediated syndromes associated with the production of autoantibodies and antigen-specific T cells.

1.4.5 Neuroinflammation

Neuroinflammation is defined as inflammatory responses within the brain and spinal cord, triggered by aging, neurodegenerative diseases, injury or infection, and mediated by cytokines and chemokines.[45, 46] Cytokines and chemokines regulate inflammatory responses throughout the body and are crucial for the brain's immune function by acting as neuromodulators in neurodevelopment, neuroinflammation and synaptic transmission.[46] Upon pathological states within the nervous system, microglia are activated. This leads to the production and release of proinflammatory cytokines and chemokines that trigger astrocytes and infiltration of peripheral immune cells to respond to the pathologic state. In general, this activation aids in protecting and repairing the CNS.[45] Nonetheless, prolonged inflammation, chronic microglia activation and continual recruitment of effector cells can ultimately result in nerve injury and a well-balanced interplay between proinflammatory factors and repair is essentially defining the outcome of the neuroinflammatory process.[45, 46]

1.5 Paraneoplastic neurological syndromes

Paraneoplastic neurological syndromes (PNS) are rare immune-mediated disorders that affect the nervous system in patients with cancer.[47] These disorders are not caused by the direct tumor invasion, metastasis or consequence of treatment, but as a result of the immune response against neuronal proteins expressed by the tumor.[48, 49] Essentially any region of the nervous system can be affected by PNS, ranging from involving single cell types (e.g. Purkinje neurons in the cerebellum) to multiple levels of the nervous system (e.g. encephalomyelitis).[47, 48] Although all parts of the nervous system can be affected, eight syndromes have been classified as "classical" PNS: encephalomyelitis, limbic encephalitis, paraneoplastic cerebellar degeneration, opsoclonus–myoclonus, sensory neuronopathy, chronic gastrointestinal pseudo obstruction, Lambert–Eaton myasthenic syndrome, and dermatomyositis.[50]

Pathophysiology

PNS are mainly autoimmune but the roles of humoral and cellular mediated immunity remain unresolved, largely due to the complicated features of the different syndromes.[47] A model has been proposed (Fig 1.5), in which immature dendritic cells phagocytose apoptotic tumor cells, mature, and migrate to lymph nodes, whereby they activate $CD4^+$ and $CD8^+$ T cells, and B cells.[47, 51] Activated tumor-specific T cells can trigger a feedback loop by inducing apoptosis and thereby amplifying the antitumor immune response, or cross the BBB and become an effector in specific brain regions depending on the nature of the PNS.[47, 52] A humoral mediated response has been proposed due to the detection of a greater portion of IgG in CSF compared to the serum, thus indicating that the antibody is being selectively transported across the BBB or is produced by the CNS and projected into the CSF.[53] Most probably, clones of B cells cross the BBB to produce elevated titers of the specific antibody in the CNS.[53] In other instances of PNS, antibodies in association with a $CD8^+$ T cell response is likely the main effector in pathogenesis.[54]

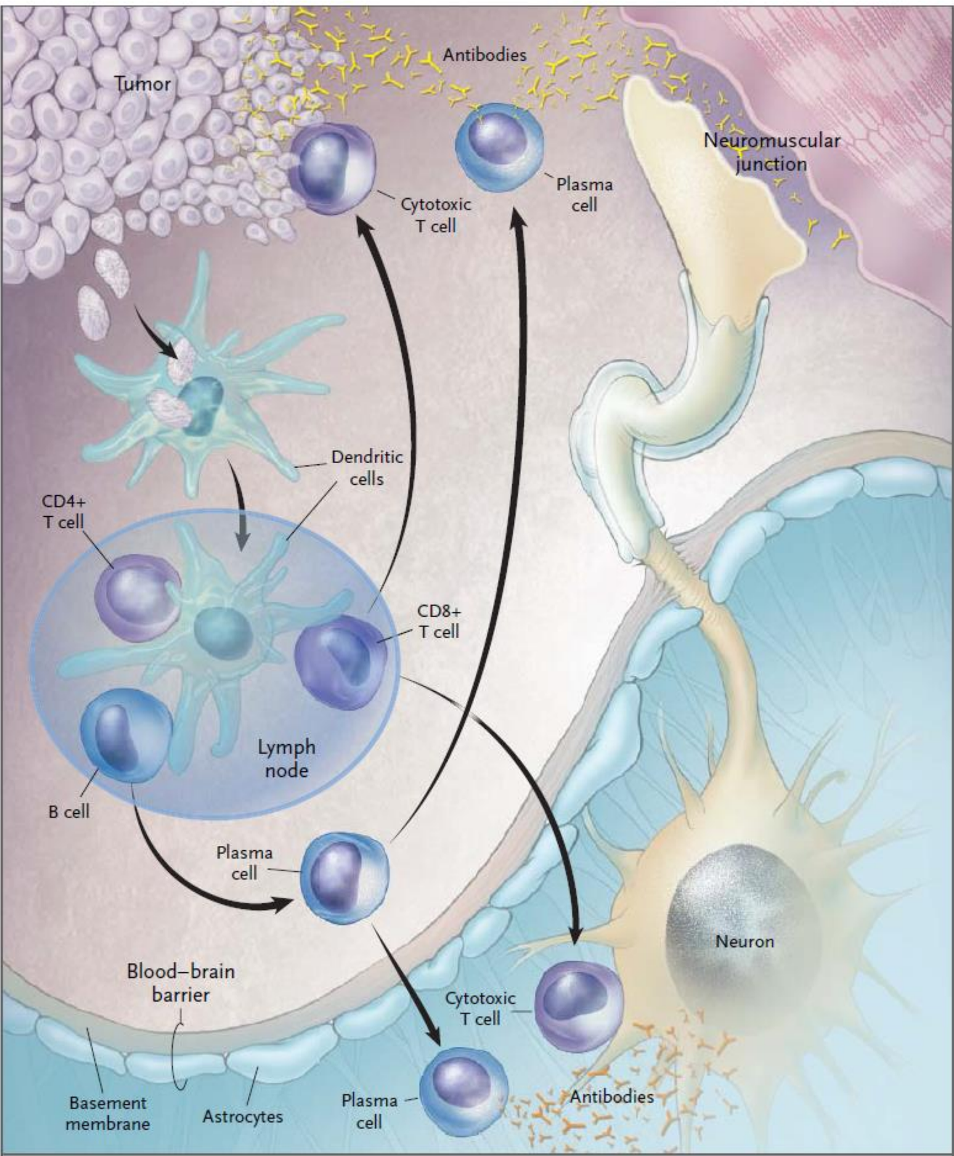


Figure 1.5: Proposed model for pathogenesis of paraneoplastic neurological syndromes. Dendritic cells phagocytose tumor cells, migrate to lymph nodes and present tumor antigens to $CD4^+$ and $CD8^+$ T cells and B cells. $CD8^+$ T cells mature into cytotoxic T cells that can attack tumor cells, or neurons expressing antigens they share with the tumor. B cells mature into antibody-secreting plasma cells that can react against the tumor, portions outside the nervous system, such as the neuromuscular junction or in some instances attack neurons directly. Reproduced with permission from Darnell and Posner 2003,[47] Copyright Massachusetts Medical Society.

Antibodies as biomarkers of PNS

Paraneoplastic autoimmunity can be directed against intracellular antigens or cell-surface antigens.[55] Paraneoplastic antibodies, also called onconeural antibodies, that target intracellular antigens may not have a direct pathogenic effect, as antigen-specific $CD8^+$ T cells probably exert a cytotoxic effect causing neuronal death.[55] These onconeural antibodies almost always indicate the presence of an underlying cancer. Thus, even though these antibodies may not be pathogenic in nature, they serve as excellent biomarkers for PNS.[56] Some of the most well characterized onconeural antibodies include, Hu, Yo, Ri, collapsin response mediator protein 5 (CRMP5) and Ma1/Ma2 (Table 1.5).[56] Hu antibodies, which target all neurons, are associated with widespread PNS such as encephalomyelitis and sensory neuropathy. Yo antibodies, on the other hand, mainly target Purkinje neurons and are associated with a limited PNS called paraneoplastic cerebellar degeneration.[57] PNS associated with antibodies directed against intracellular antigens are characterized by poor response to treatment and relentless progression due to the rapid neuronal loss.[55]

Autoantibodies targeting cell-surface antigens can occur in patients with or without cancer. The frequency and type of cancer can vary according to the autoantibody, with over 50% of patients with anti-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and anti-$GABA_B$ receptor having underlying malignant tumors, and less than 5% of patients with anti-leucine-rich, glioma inactivated 1 (LGI1) having a underlying, often non-malignant, tumor.[55, 56] Antibodies that target cell-surface antigens are considered to have a direct pathogenic effect, but this is reversible in many instances. Thus, PNS associated with these autoantibodies are more likely to respond to immunotherapy.[47, 54, 55, 58, 59]

Antibodies associated with PNS detected in patient sera or CSF are extremely useful diagnostic biomarkers.[60] Identification of onconeural antibodies with intracellular antigens reliably predicts that the PNS is paraneoplastic, and guides the search for an underlying tumor.[56] Several screening tests have been developed, including indirect immunofluorescence (IIF) of rat brain sections, western blot analysis, cell-based assays (CBAs) and commercial line immunoassays.[44] Especially commercially available line

immunoassays provide rapid and easy detection of several antibodies simultaneously, but the diagnostic value has been questioned due limited information regarding test reliability, sensitivity and specificity.[61]

Table 1.5: PNS autoantibodies, their antigens and associated tumors.

Antibody	Antigen	PNS	Associated tumor
Antibodies targeting intracellular antigens			
Yo	CDR2L/CDR2	PCD	Ovarian, breast and fallopian tube
Hu	HuD	Encephalomyelitis, sensory neuronopathy	SCLC, NSCLC and extrathoracic cancers
Ri	NOVA1/NOVA2	Brainstem encephalitis, opsoclonus	Breast, lung and other cancers
SOX1	SOX1	LEMS, PCD	SCLC, NSCLC and extrathoracic cancers
Amphiphysin	Amphiphysin	SPS, encephalomyelitis, sensorimotor neuropathy	SCLC, breast and other cancers
MAP1B	MAP1B	Encephalomyelitis and/or sensorimotor neuropathy	SCLC, NSCLC, and extrathoracic cancers
CRMP5	CRMP5	Encephalomyelitis, sensorimotor neuropathy	SCLC, NSCLC, thymoma and extra-thoracic cancers
Ma1/Ma2	Ma1/Ma2	Limbic encephalitis and brainstem encephalitis	Testicular, lung and other cancers
Tr	DNER	PCD	Hodkin lymphoma
Antibodies targeting cell-surface antigens			
AMPAR	GluA1/GluA2	Limbic encephalitis and non-focal encephalitis	Lung, thymus, breast and other cancers
$GABA_BR$	B1 ($GABA_BR$ subunit)	Limbic encephalitis, cerebellar ataxia, opsoclonus myoclonus syndrome	SCLC
NMDAR	GluN1	Encephalitis	Teratoma
mGlur5	mGlur5	Non-focal encephalitis	Hodgkin lymphoma
VGCC P/Q	P/Q-type VGCC	LEMS, PCD	SCLC
LGl1		Limbic encephalitis	Thymoma

AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; CDR2, cerebellar degeneration-related protein 2; CDR2L, CDR2-like; CRMP5, collapsin response mediator protein 5; DNER, delta and notch-like epidermal growth factor-related receptor; $GABA_BR$, gamma-aminobutyric acid type B receptor; GluA1, glutamate receptor 1; GluA2, glutamate receptor 2; GluN1, NMDAR subunit NR1; HuD, Hu-antigen D; LEMS, Lambert-Eaton myasthenic syndrome; LGl1, leucine-rich, glioma inactivated 1; MAP1B, microtubule-associated protein 1B; mGlur5, metabotropic glutamate receptor 5; NMDAR, N-methyl-D-aspartate receptor; NOVA, neuro-oncological ventral antigen; NSCLC, non-small-cell lung carcinoma; PCD, paraneoplastic cerebellar degeneration; SCLC, small-cell lung carcinoma; SOX1, SRY-related high mobility group box 1; SPS, stiff-person syndrome; VGCC P/Q, P/Q-type voltage-gated calcium channel. Reprinted by permission from Springer Nature, License #5031901477248: Nature reviews clinical oncology, Paraneoplastic neurological syndromes in the era of immune-checkpoint inhibitors, Graus et al. 2019.[56]

Clinical features and diagnosis

The diagnosis of PNS is based on the type of syndrome (classic or non-classic), presence of onconeural antibodies, clinical symptoms and potential underlying cancer.[56] A definite diagnosis of PNS can be made if: 1) a neurological syndrome appears with the detection of well characterized onconeural antibodies (such as anti-Hu or anti-Yo), 2) a classical syndrome and a tumor is identified, regardless of the presence or absence of a onconeural antibody, 3) a non-classical syndrome that resolves or greatly improves after cancer treatment without simultaneous immunotherapy, or 4) a none-classical syndrome with onconeural antibodies and cancer develops within five years of diagnosing the neurological disorder.[17] The clinical symptoms of PNS are diverse, but some features are common.[47] In two-thirds of the cases, the diagnosis of PNS precedes the diagnosis of the cancer.[62, 63] Detection of one or more paraneoplastic antibodies in serum or CSF prompts targeted tumor screening, since such antibodies often are associated with certain cancer types.[55]

Treatment

The two main treatment strategies involve immunotherapy and tumor treatment to remove the source of the antigenic stimulation. A combination approach might be favorable to improve the neurological outcome.[47, 55] The most commonly used immunotherapies include corticosteroids, plasma exchange, intravenous immunoglobulins and rituximab.[54] The response to treatment varies largely; PNS associated with antibodies to cell-surface antigens showing a greater response compared to PNS associated with antibodies to intracellular antigens.[54]

Immune checkpoint inhibitors (ICIs) are monoclonal antibodies that target cytotoxic T-lymphocyte antigen (CTLA4), programmed cell death 1 (PD-1) protein or PD-1 ligand and promotes T cell activation and initiate tumor cell elimination.[64] The introduction of ICIs has revolutionized the treatment across a wide range of malignancies, resulted in unprecedented survival rates and durable responses.[65] However, due to its generalized immune activation and lack of specificity, ICIs are

associated with a high risk of immune-related adverse effects, some of which can manifest as PNS.[56, 66]

1.5.2 Paraneoplastic cerebellar degeneration

Paraneoplastic cerebellar degeneration (PCD) is one of the most frequently detected PNS, characterized by rapidly progressing ataxia, nystagmus and dysarthria. PCD is associated with numerous different onconceural antibodies, the most well characterized being anti-Yo, anti-Hu, anti-Ri and anti-Tr. The tumors most commonly associated with these antibodies are SCLC (anti-Hu), gynecological and breast cancer (anti-Yo and anti-Ri), and Hodgkin's lymphoma (anti-Tr).[67] Patients with PCD experience progressive symptoms from weeks to months, and due to the irreversible damage to the cerebellum, most patients are significantly disabled within the time the symptoms stabilize (within 6 months).[67] There are no preventative measures and prognosis is poor.[67]

Anti-Yo associated paraneoplastic cerebellar degeneration

Anti-Yo was identified as an immunological marker for PCD by Greenlee and Brashear in 1983.[57] Sera from two patients with ovarian cancer and PCD was used to stain human cerebellum sections. The sera contained an antibody termed anti-Yo, which produced cytoplasmic staining of Purkinje neurons and neurons within deep cerebellar nuclei.[57] Yo antibodies could also be detected in CSF, and often in higher titers compared to sera.[53, 68] Immunostaining of post-mortem cerebellar sections from the patients identified a complete loss of Purkinje neurons, and a thinning of the granular cell layer (loss of granule cells), a characteristic feature of patients diagnosed with anti-Yo associated PCD.[57, 62, 69]

Cerebellar degeneration related-proteins

Extensive work in the late 1980`s and beginning of the 1990`s led to the identification of two anti-Yo target proteins: cerebellar degeneration-related protein 1 (CDR1) and cerebellar degeneration-related protein 2 (CDR2).[70-74] In 1996, a third protein was

identified, first termed cerebellar degeneration-related protein 3, now referred to as CDR2-like (CDR2L), due to its 45% sequence identity with CDR2.[75, 76]

CDR1 is a 34 kDa protein, consisting of 34 inexact repetitive hexapeptides that account for approximately 50% of the amino acid sequence.[72, 77] Although CDR1 is expressed in the cytosol and dendrites of Purkinje neurons, and in ovarian and breast cancer tissue, no clear association with PCD has been identified.[77] Thus, CDR1 will not be addressed in further detail, as it is beyond the scope of this work.

The biological functions of CDR2 is partly characterized through the identification of a leucine zipper motif in its amino acid sequence,[71, 78, 79] determination of potential protein binding partners and its expression in immune-privileged tissues.[80] The presence of a leucine zipper motif suggests that CDR2 is involved in transcription or regulate gene expression, as proteins harbouring a leucine zipper motif dimerize to other proteins harbouring this motif and function primarily as transcriptional regulators.[78, 81] Accordingly, proposed binding partners of CDR2, the serine-threonine protein kinase PKN, mortality factor-like protein MRGX, cell cycle related protein MRG15 and c-myc, are all involved in signal transduction or gene transcription.[81-85] A model for CDR2 in PCD pathogenesis has been proposed based on its interaction with c-myc. CDR2 and c-myc forms a complex in Purkinje neuron cytoplasm that prevents c-myc from translocating to the nucleus, where it acts to promote transcription and transduction of neuronal signals.[83] Binding of anti-Yo to CDR2 disrupts the CDR2-c-myc interaction and thereby results in aberrant gene transcription, which leads to induced apoptotic cell death of Purkinje neurons.[83]

CDR2 expression has been comprehensively studied at the mRNA and protein levels, as well as in cancerous and normal tissues. In 1997, Corradi et al., illustrated that the CDR2 protein expression is restricted to Purkinje neurons, brainstem neurons and testes in mice.[80] Since the brain and testes previously were considered as immune-privileged sites, these findings correspond well with the autoimmune model of PCD pathogenesis.[80] In 2000, a study reported that approximately 60% of human ovarian cancers and 20% of human breast cancers expressed CDR2.[86] Later, it was found that

CDR2 is also expressed in a type of kidney cancer, papillary renal cell carcinoma,[87] and in cell lines of lung, neuroblastoma, cervical, ovarian and colon cancer.[88] These findings imply that the CDR2 protein is expressed in various cancers. Furthermore, CDR2 has also been found in normal ovary tissue, raising the question why anti-Yo associated PCD is restricted to the nervous system.[88, 89]

An age dependent expression has been suggested based on western blot analysis of rat brain cortex, illustrating a down-regulation of the CDR2 protein after birth.[90] A similar pattern has been shown for CDR2 at the mRNA level in human cerebellum.[89] Overall, CDR2 mRNA is detected in nearly all tissues and this wider mRNA distribution compared to the protein, suggests that CDR2 undergoes post-translational modification, potentially phosphorylation by PKN or ubiquitination by anaphase-promoting complex/cyclosome (APC/C).[80, 84, 85]

The subcellular location of the anti-Yo antigen has been demonstrated by immunostaining of human and rat cerebellum sections using patient sera and an antibody from a PCD patient that showed 94% homology with the antigen recognized by Yo antibodies.[91, 92] The results imply that CDR2 localizes to the cytoplasm, and associates with membrane-bound and free ribosomes, and potentially to the Golgi apparatus, in Purkinje neurons.

For 30 years, research mainly focused on CDR2 as the main Yo antigen, partially based on the identification of CDR2 mRNA expressed in PCD-associated tumors.[80] In 2013, CDR2L was introduced as a new player in PCD after showing that only patients with Yo positive sera that contained both CDR2 and CDR2L antibodies had PCD.[75] Localization studies have shown that CDR2L antibodies stain human Purkinje neurons in a granular cytoplasmic pattern, similar to anti-Yo.[47, 75] In addition, CDR2L is more highly expressed in human Purkinje neurons than CDR2.[75] Recent studies have shown that not only CDR2 but also CDR2L is widely expressed in normal tissues and PCD associated tumors.[75, 89, 93] The exact subcellular localization of endogenous CDR2L is largely unknown, with only a proposed localization of recombinant CDR2L to the plasma membrane of transfected HeLa cells.[75] According to the Atlas of the developing

human brain database (www.brainspan.org), CDR2L mRNA levels increase with age, the opposite trend of what has been shown for CDR2 mRNA.

The CDR2 and CDR2L transcripts encode two proteins of 454 and 465 amino acids, with an estimated molecular weight of 62 kDa and 55 kDa, respectively (Appendices 9.2 to 9.4). CDR2L contains potential coiled-coiled regions, similar to CDR2.[75] Results from epitope mapping imply that anti-Yo do not recognize any common linear epitopes between CDR2 and CDR2L.[94]

Pathophysiology

The cellular and molecular mechanisms causing PCD pathogenesis is partially unknown. As for most or all PNS, PCD is also thought to be immune-mediated but the roles of humoral and cellular immunity is largely unresolved.[47] Anti-Yo is mainly of the IgG1 subclass, present in higher titers in CSF compared to serum of PCD patients. This high CSF/serum ratio of Yo antibodies and the presence of CSF oligoclonal IgG bands suggest that there is an ongoing Yo antibody synthesis and activation of B cells.[53, 95] However, the number of B cells detected in the cerebellum of post-mortem sections from PCD patients is low. This can merely be due to the patients already being at the final "burn-out" stage and that a humoral immune response may take place earlier in the pathogenesis.[69, 96, 97]

The presence of antigen (CDR2)-specific $CD8^+$ T cells have been reported in several studies of PCD patients,[51, 52, 96] both in ovarian tumors and in autopsy studies of the human cerebellum.[69] These observations have led to a proposed pathomechanism in which apoptotic tumor cells that express the antigen are phagocytosed by antigen-presenting cells that migrate to the lymph nodes and activate antigen-specific cytotoxic T cells. Activated T cells can then migrate back to the tumor site and thus elicit the immune response, or potentially cross the BBB and attack Purkinje neurons.[47, 51] However, these findings were contradicted in later studies, in which $CD8^+$ T cells specific for CDR2 could not be found.[98, 99]

Greenlee et al., used organotypic slice cultures of rat cerebellum to show that administration of anti-Yo via serum, CSF or purified IgG resulted in Purkinje neuron

death, thus implying a direct damaging effect of Yo antibodies.[100, 101] An adsorption experiment was performed to show that the observed cytotoxic effect was caused by a specific binding of anti-Yo to its antigen, CDR2.[102] However, others found that co-expression of CDR2 and CDR2L antibodies is necessary for rat Purkinje neuron staining and that the observed staining by Greenlee et al. (2010) most likely was due to the presence of CDR2L antibodies in the serum and CSF.[75, 100]

Other studies have investigated the role of microglia in PCD. Despite extensive microglia activation in the human cerebellar white matter and the presence of microglia nodules in the Purkinje neuron layer of PCD patients,[103] immunofluorescence studies of organotypic slice cultures of rat cerebellum suggest that microglia infiltration occurs after Purkinje neuron death.[75, 102]

Another disease model suggests that dysregulation of calcium homeostasis upon anti-Yo-antigen binding causes Purkinje neuron death.[104] Incubating rat cerebellar organotypic slice cultures with human patient sera or rabbit CDR2 and CDR2L antibodies, leads to altered calcium buffering capacity due to calbindin malfunction and subsequent Purkinje neuron death.[101, 104]

Several attempts to develop animal models that mimic PCD have failed.[97] Guinea pigs, rats and mice injected with IgG from PCD patients show IgG immunoreactivity in Purkinje neuron cytoplasm but no clinical or pathologic evidence of Purkinje neuron loss.[105-108] A mouse model of paraneoplastic diseases was generated, in which Purkinje neurons and implanted breast tumors expressed the same antigen, namely hemagglutinin.[109] Mice were transferred with hemagglutinin-specific naive $CD4^+$ and $CD8^+$ T cells, which resulted in partial control of tumor growth. Using immune checkpoint therapy with anti-CTLA4 monoclonal antibody, mice were protected from tumor outgrowth but showed Purkinje neuron loss and neurological signs, such as reduced spontaneous locomotion and reduced motor activity.[109] Infiltration of antigen-specific T cells into the cerebellum upon CTLA4 blockade and the subsequent neuronal loss led to the assumption that $CD8^+$ T cells play an important role in disease development. Despite illustrating the importance of T cell activation and effect of

immune checkpoint therapy in paraneoplastic disorders, this model does not fully mimic PCD pathogenesis with the absence of onconeural antibodies.

Clinical features and diagnosis

PCD is charactierzed by cerebellar Purkinje neuron loss, severe ataxia, nystagmus and dysarthria[110]. Patients with anti-Yo associated PCD usually present with subacute development of the cerebellar deficits, over a period of weeks to months.[111] The disease affects women and the median age for onset is approximately 60 years.[62] An international panel supported by the Paraneoplastic Neurological Syndrome Euronetwork proposed a list of recommended diagnostic criteria for PNS.[17] Anti-Yo associated PCD falls under the category “classical” PNS, with a well-characterized antibody.

Treatment

As with other PNS, the neurological symptoms usually appear before the cancer has been identified. Antibody detection should therefore direct the search for an underlying neoplasm using computed tomography (CT) or with positron emission tomography (PET) scans if the CT is negative.[110-112] Treatment of the neoplasm to stabilize the neurological symptoms are the cornerstone of therapy in PCD.[103, 110] The use of immunotherapy, including rituximab, intravenous immunoglobulins and corticosteroids has rarely modified the neurological outcome.[110, 113]

2. AIMS OF THE STUDY

The principal goal throughout the course of this work was to determine the major Yo antibody target, investigate the precise subcellular location of CDR2L and CDR2, and address the possibility of including CDR2L as diagnostic marker in routine clinical tests.

Objectives:

Paper I: The aim of this study was to determine whether CDR2 or CDR2L is the major target of Yo antibodies.

Paper II: The aim of this study was to investigate the subcellular location of CDR2 and CDR2L in cancer cells and Purkinje neurons, as well as potential protein binding partners.

Paper III: The aim of this study was to address the value of including CDR2L as a diagnostic marker in routine clinical tests when diagnosing anti-Yo associated PCD.

3. MATERIALS AND METHODS

Primary antibodies included in this study were purchased from commercial companies. An overview is provided in Appendices, Table 9.1.

3.1 Patient samples and ethical considerations

Paper I and II

Five sex- and age-matched CSF and serum samples from patients with Yo antibodies and five without Yo antibodies and no neurological disease or underlying cancer (negative controls) were used to stain human and rat cerebellar tissues, rat Purkinje neuron cultures and cancer cells. The samples were obtained from the Neurological Research Laboratory, Haukeland University Hospital (Regional Committee for Medical and Health Research Ethics in Norway, REK #2013/1480).

Paper III

In the period 2017 to 2020, 9527 sera and CSF samples were screened for onconeural antibodies at the Neurological Research Laboratory. Twenty-four patients showed Yo reactive bands on the commercial PNS 14 Line Assay from Ravo Diagnostika and were included in the study. Patient records were obtained and anonymized prior to the study and PCD was diagnosed according to established criteria (Regional Committee for Health and Medical Research Ethics in Norway, REK #123524).

3.2 Cerebellar tissue

Cerebellar sections were cut from fresh frozen normal human tissue (REK, #2013/1503) or from paraformaldehyde (PFA)-perfused rat brains (FOTS 20135149/20157494/20170001). Heat-induced epitope retrieval was performed prior to immunostaining.

3.3 Cell cultures, recombinant DNA and transfection

Paper I and II

Cancer cell lines were maintained as a model system to investigate the subcellular location of proteins of interest and identify protein interaction partners by colocalization studies using STED microscopy and proximity ligation assay. OvCar3 (American Type Culture Collection (ATCC), #HTB-161) and HepG2 (ATCC, #HB-8065) cancer cell lines were maintained and subcultivated on poly-D-lysine-coated coverslips (Neuvitro, #GG-18-1.5-pdl) according to the manufacturer's protocol. Cells were washed twice with 0.1 M phosphate-buffered saline (PBS), fixed (15 minutes, 4% paraformaldehyde in PBS, Thermo Fisher Scientific, #28908), and quenched (5 minutes, 50 mM NH_4Cl, Sigma-Aldrich, #254134) prior to immunostaining.

OvCar3 and HepG2 cells were prone to transfection of full-length CDR2 (OriGene Technologies, Rockville, MD; #RG204900) and CDR2L (OriGene Technologies, #RC206909) ligated into a pCMV6-AC-GFP vector (OriGene Technologies, #PS100010). Following polymerase chain reaction, correct CDR2 and CDR2L vector sequences were confirmed using BioEdit v7.2.5. One Shot TOP10 Escherichia coli (Life Technologies, Carlsbad, CA; #C4040-10) were used for amplification, E.Z.N.A. Plasmid DNA Kit (Omega Bio-Tek, Norcross, GA, #D6942) for purification, and Lipofectamine 3000 (Thermo Fisher Scientific, #L3000008) for transfection.

Paper III

HEK293 cells were transfected with a plasmid for expression of Myc-DDK-tagged CDR2L (Origene, #RC206909) using Lipofectamine 3000 Reagent (Invitrogen, #L3000008). Cells were cultured in 8-well Nunc^{TM} Lab-Tec^{TM} II Chamber Slide^{TM} System (Thermo Fisher Scientific, #154534) in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (37 °C, 5% CO2). At 48 h after transfection, coverslips were washed with PBS and fixed with 4% PFA/4% glucose in PBS (20 min, room temperature). Demembranation with 0.1% Triton X-100 in PBS (7 min, room temperature) was followed by blocking with 10%

Sea Block blocking buffer (Thermo Fisher Scientific, #37527) in PBS (1 h, room temperature), prior to immunostaining.

3.4 Immunostaining

OvCar3 and HepG2 cells and cerebellar sections were permeabilized in 0.5% Triton X-100-PBS (Sigma-Aldrich, #11332481001, 5 minutes), washed in 0.5% gelatin-PBS (Sigma-Aldrich, #G7041, 5 minutes), blocked in 10% SEABLOCK (Thermo Fisher Scientific, #37527, 30 minutes) in PBS and incubated with primary antibodies overnight at 4 °C. Following incubations, cells and sections were washed in gelatin-PBS, incubated with secondary antibodies for 2 hours at room temperature, and mounted using ProLong Diamond with DAPI (Thermo Fisher Scientific, #P36962).

3.5 Immunoprecipitation

Immunoprecipitation was performed to evaluate the co-reactivity of CDR2L and Yo antibodies, to investigate potential protein interaction partners of CDR2 and CDR2L, as well as determining the specificity of numerous CDR2 and CDR2L antibodies, confirmed by mass spectrometry analysis. Proteins of interest were immunoprecipitated from OvCar3 and HepG2 cell lysates using Protein G Magnetic Beads (Dynabeads, Thermo Fisher Scientific, #1004D), as described in the Bio-Rad SureBeads immunoprecipitation protocol. The reaction products were detected by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), followed by western blot analysis. A negative control consisting of beads and cancer cell lysate was also included in each experiment.

3.6 Western blot analysis

Proteins of interest in whole cell lysates of immunoprecipitation products were subjected to SDS-PAGE separation on a 10% TGX gel (Bio-Rad, #456-1035), and transferred to a polyvinylidene difluoride membrane using the Trans-Blot Turbo

Transfer kit (Bio-Rad, #170-4274). The blots were blocked in 5% dry-milk (Bio-Rad, #170-6404) dissolved in 1x Tris-buffered saline with 0.1% Tween 20 (TBS-Tween 20), and incubated with serum/CSF samples from patients or antibodies, diluted in 3% BSA in TBS-Tween 20 (1:250 for sera and 1:100 for CSF, 4 °C, overnight). Antibody fixation was visualized using horseradish peroxidase anti-human IgG (Dako, #P0214, 1 h, room temperature).

3.7 Fluorescent immunoblotting

Cerebellar and cancer cell lysates were obtained using a Total Protein Extraction Kit (Millipore, Billerica, MA; #2140). Proteins were separated on a 10% TGX gel and transferred to a low autofluorescence PVDF membrane. G:Box (Syngene, Frederick, MA) was employed for visualization.

3.8 Mass spectrometry-based proteomics analysis

Mass spectrometry-based proteomics was used to evaluate the specificity of numerous CDR2 and CDR2L antibodies, and to screen for potential protein interaction partners. The proteins of interest were immunoprecipitated from OvCar3 and HepG2 cells and subjected to SDS-PAGE. Gel bands were cut into 1 mm^2 cubes and hydrated in MilliQ water. The remaining procedure was performed at the Proteomics Unit in Bergen (PROBE), University of Bergen, Norway.[114]

Proteins were identified after extracting the obtained mass spectra data with Proteome Discoverer (version 2.3.0.523, Thermo Fisher Scientific), followed by searching against human, reviewed protein sequences (SwissprotKB database, release 08-2018) with Sequest HT and MS Amanda search engines. In order to evaluate the likelihood of the predicted interactions, the following criteria were established: 1) nonspecific bindings were removed based on the negative control; 2) the number of recognized peptides was set to at least two; 3) proteins that were identified by more than one of the antibodies to CDR2L or CDR2 were considered as more likely partners; 4) the

likelihood of an interaction was evaluated based on the predicted cellular location of CDR2/CDR2L. Protein-protein interactions were analyzed using the STRING database.

3.9 Super-resolution microscopy

A Leica TCS SP8 Stimulated Emission Depletion (STED) 3X confocal microscope equipped with a 100x oil objective with a numerical aperture of 1.4 was used for imaging. The output of the excitation laser (up to 1.5 mW per line; pulsed) was kept between 1% and 20% and the STED laser (775 nm; up to 1.5 W) between 20% and 30%. Gating (between 1 and 6 ns) was applied for all channels as well as a minimum of three intensity averages. The lateral resolution was consistently measured to be between 40 and 50 nm. All experiments were run with negative controls, containing only secondary antibodies, simultaneously for laser intensity thresholding.

3.10 Proximity ligation assay

Potential interaction partners of CDR2 and CDR2L, identified by mass spectrometry analysis, were further evaluated by colocalization studies, including proximity ligation assay. The available Duolink kit from Sigma-Aldrich (#DUO92101) was used to identify interaction partners within a 40 nm range in OvCar3 cells. Fixed OvCar3 cells were permeabilized using 0.5% Triton X-100 diluted in PBS, blocked with 10% SEABLOCK in PBS and incubated with primary antibodies in blocking solution. Cells were washed with Wash buffer A, before and after incubation with probes (+ and -). Then, cells were incubated with ligation buffer (1:5) and ligase enzyme (1:40), followed by washes with Wash Buffer A. Amplification buffer (1:5) and the polymerase enzyme (1:80) were diluted in distilled water and applied to the cells for 100 minutes (37 °C, in the dark), followed by washes with Wash Buffer B (supplied with the kit). Prolong Diamond with DAPI was used to mount the coverslips (overnight, 4 °C). Mounted cells were stored at -20 °C.

3.11 Commercial line immunoassays

We used the commercial line immunoassays PNS 14 Line Assay (Ravo Diagnostika, #PNS14-003) and EUROLINE PNS 12 Ag (Euroimmun, #DL1111-1601-7-G) to detect onconeural antibodies in patients with suspected PCD. Patient samples that showed Yo reactive bands on the PNS 14 Line Assay, was further evaluated by EUROLINE PNS 12 Ag. Two independent investigators graded band intensities from + to +++, compared to a positive control sample (+++).

Serum and CSF from the patients with Yo reactive bands were also tested for anti-Yo using a commercial CBA (Purkinje Cell Mosaic 1, Euroimmun, #FA1113-1005-1) consisting of BIOCHIP Mosaics with four positions (Yo/CDR2-, Tr/DNER-, ITPR1- and CARP-transfected HEK293 cells). Aliquots of 30 µl serum (diluted 1:100) or of CSF (diluted 1:1) were applied to each reaction field on the BIOCHIP slide. After incubation (30 min, room temperature), the slide was washed with PBS-Tween 20 (5 min, room temperature), followed by incubation with secondary antibody (30 min, room temperature). The slide was rinsed with PBS-Tween 20, and mounted on a glass coverslip. The cut-off for Yo/CDR2 was set to 1:100, as advised by the manufacturer. Two independent investigators evaluated the results.

3.12 Imaging processing

Rat cerebellar sections and CBAs were imaged on a Leica Leitz DMRBE fluorescence microscope with CoolLED pE-300-W LED illumination. Images were evaluated by 2 independent investigators. ImageJ was used for background subtraction of microscopy images and evaluation of western blot results.

3.13 Methodological considerations

Patient cohort

The main limitation to the work presented in paper III, is the small patient cohort. Out of the 9,527 patient sera and CSF samples screened for onconeural antibodies at

Haukeland University Hospital, only six of these were diagnosed with anti-Yo associated PCD. Samples were selected on CDR2-positivity and we do not know whether testing for CDR2L antibodies alone would be sufficient. A follow up study including a larger patient cohort including PCD patients who test negative in commercial line immunoassays and patients with PNS caused by other paraneoplastic antibodies should be performed to evaluate the strength of our findings. However, despite the small cohort, our data demonstrate that including CDR2L as a diagnostic marker adds an important dimension to the accuracy of PCD testing.

Interpretation of immunofluorescence results

Immunofluorescence of rat cerebellar tissue is a suitable confirmatory test for PCD diagnosis but it is also a laborious approach that requires access to proper equipment. When interpreting immunofluorescence results, it is important to be aware that it is to some degree considered as a semi-quantitative method that cannot determine the absolute amount of protein available in the sample.[115] Thus, the relationship between staining intensity and antigen quantity is non-linear and a measure of the staining intensity will not simply reflect the severity of the disease. Furthemore, interpretation of the characteristic, granular staining pattern of Purkinje neurons must be handled with caution since false positive anti-Yo staining can be presented with a cytoplasmic but non-granular pattern, unrelated to PCD. To mitigate this limitation, we developed CBA for CDR2L as an alternative approach.

Antibody based methods

The majority of experimental techniques used in these projects depend on commercial antibodies. There has been an increasing focus on the limitations regarding the use of antibodies for research purposes, due to poor validation, unspecificity, low reproducibility and batch-to-batch variabilities.[116, 117]. It was therefore important to perform antibody validation of our available CDR2 and CDR2L antibodies, especially after observing considerable variations in staining patterns. For further discussion on the use of commercially available antibodies, see section 5.2.4.

4. RESULTS

4.1 Paper I: CDR2L Is the Major Yo Antibody Target in Paraneoplastic Cerebellar Degeneration

In paper I, we investigated whether CDR2 or CDR2L is the major target of Yo antibodies. CSF and serum from PCD patients, and CDR antibodies were used to stain human and rat cerebellar sections. CDR2L showed a cytoplasmic staining pattern in Purkinje neuron somas that overlapped with the Yo antibody staining from patient CSF and serum (Fig 4.1 A and B). An overlapping staining pattern of CDR2L and Yo was also observed in stellate and basket cells (Fig 4.1 C). CDR2 mainly stained the nucleus of Purkinje neurons and no overlap with Yo antibodies was detected (Fig 4.1 A, B and C). Fluorescent immunoblot of rat cerebellar lysate showed that CDR2L and Yo stain the same 55 kDa band, whereas CDR2 stains a 62 kDa band that does not overlap with Yo (Fig 4.1 D).

OvCar3 cells express CDR2L and CDR2 endogenously. Colocalization studies using STED microscopy showed the same pattern as observed in Purkinje neurons. CDR2L and Yo colocalized in OvCar3 cell cytoplasm, whereas CDR2 staining did not overlap with Yo. Fluorescent immunoblot and immunoprecipitation confirmed these results.

HepG2 cells, which express high levels of CDR2 endogenously, were transfected with recombinant, GFP linked CDR2 and CDR2L. We found that CDR2L and Yo antibodies colocalized in CDR2L-GFP transfected HepG2 cells, whereas the CDR2 antibody did not bind the recombinant CDR2L protein. Yo and CDR2 antibodies were not able to bind in untransfected cells. Only upon CDR2-GFP transfection was Yo and CDR2L antibodies able to bind recombinant CDR2.

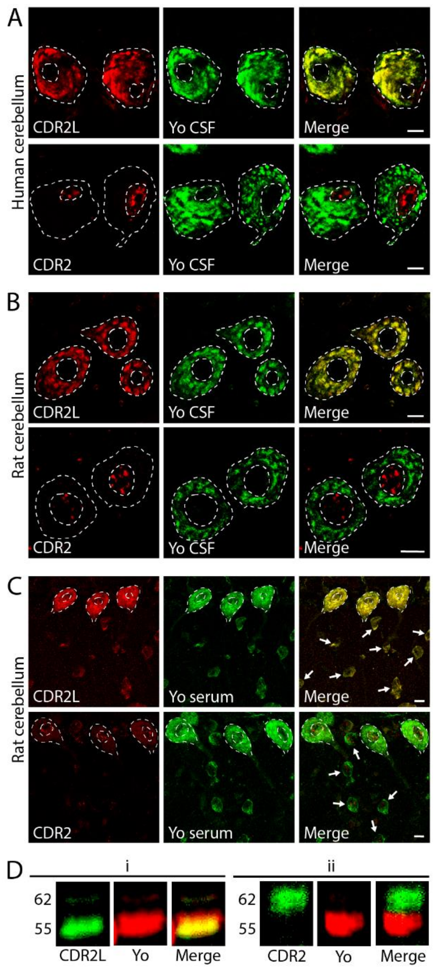
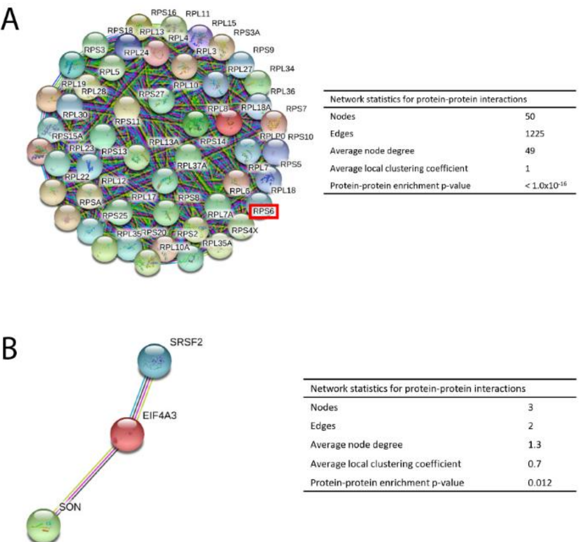


Figure 4.1: Yo antibodies bind to CDR2L, but not CDR2, in cerebellar Purkinje cells. Scale bars = 10µm. (A) Sections of fresh frozen human cerebellum. Upper row: Section stained with Yo (cerebrospinal fluid [CSF]; green) and anti-CDR2L (red); the antibodies colocalize in the cytoplasm (seen as yellow in the merge image). Lower row: Section stained with Yo (CSF; green) and anti-CDR2 (red); no colocalization is seen between Yo and CDR2. (B) Sections of paraformaldehyde (PFA)-perfused rat cerebellum. Upper row: Section stained with Yo (CSF; green) and anti-CDR2L (red); CDR2L colocalize with Yo. Lower row: Section stained with Yo (CSF; green) anti-CDR2 (red); no colocalization is seen. (C) Sections of PFA-perfused rat cerebellum. Upper row: Section stained with Yo (serum; green) and anti- CDR2L (red); Yo and CDR2L colocalize in the Purkinje cells (outlined) as well as in the stellate and basket cells (arrows). Lower row: Section stained with Yo (serum; green) and anti-CDR2 (red); no colocalization is seen between Yo and CDR2. These images are a z-stack merge, as not all stellate/basket cells were in the same focal plane as the Purkinje cells; thus, the cytoplasmic staining found over or under the nuclei may appear nuclear although it is not (eg, the Yo serum staining is not nuclear). (D) Fluorescent immunoblot of rat cerebellar lysate. Anti-CDR2L and Yo (CSF) stain the same band at 55kDa; anti-CDR2 does not. Secondary antibody controls were negative.[118]

4.2 Paper II: Localization of CDR2L and CDR2 in paraneoplastic cerebellar degeneration

To ensure antibody specificity, CDR2L and CDR2 were immunoprecipitated from OvCar3 and HepG2 cell lysates, respectively, and analyzed by mass spectrometry-based proteomics. We found that all of our commercially available antibodies were specific for their targets. Yo antibodies only precipitated CDR2L and confirmed our data from paper I, identifying CDR2L and the major Yo antibody target.

Based on mass spectrometry analysis, we also identified potential binding partners of CDR2L and CDR2. Numerous ribosomal proteins, including the ribosomal protein S6 (rpS6), were the most prominent CDR2L binding partners (Fig 4.2.1 A), whereas nuclear speckle proteins, SON, serine/arginine-rich splicing factor 2 (SRSF2) and eukaryotic initiation factor 4A-III (eIF4A3) were identified as potential binding partners of CDR2 (Fig. 4.2.1 B).



Network statistics for protein-protein interactions	
Nodes	50
Edges	1225
Average node degree	49
Average local clustering coefficient	1
Protein-protein enrichment p-value	$< 1.0 \times 10^{-16}$

Network statistics for protein-protein interactions	
Nodes	3
Edges	2
Average node degree	1.3
Average local clustering coefficient	0.7
Protein-protein enrichment p-value	0.012

Figure 4.2.1: Protein-protein interaction networks visualized by STRING. (A) CDR2L was predicted to interact with ribosomal proteins (rpS6, red box). The nodes indicate proteins, and the edges represent protein-protein associations. (B) Protein-protein interaction network of nuclear speckles proteins, SON, eIF4A3, and SRSF2, predicted to interact with CDR2. eIF4A3 (red) directly interacts with SON (light green) and SRSF2 (blue). Color-coded edges; light blue: curated databases, dark blue: gene co-occurrence, pink: experimentally determined, green: text mining. Interactions with a medium score of 0.400 or more are shown.[114]

Colocalization studies using STED microscopy, co-immunoprecipitation and proximity ligation assay confirmed rpS6 as a binding partner of CDR2L (Fig 4.2.2 A) and that CDR2 is in direct contact with eIF4A3 and SON (Fig 4.2.2 B). eIF4A3 can translocate from the nucleus to the cytoplasm to aid in mRNA binding to the 40S ribosomal subunit, in conjugation with other initiation factors.[119] We therefore proposed a model for CDR2 and CDR2L involvement in protein synthesis and that Yo antibody binding to CDR2L could result in altered or impaired functions of the ribosomal machinery or protein synthesis in patients with PCD.

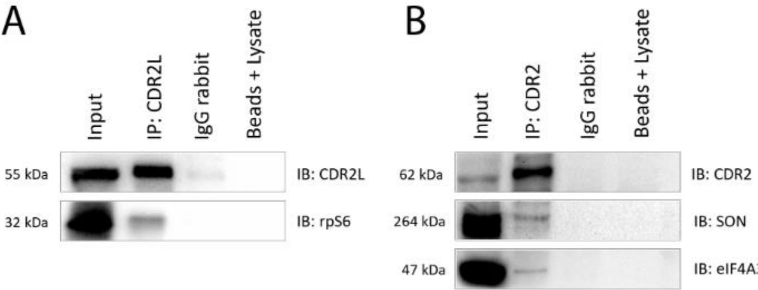


Figure 4.2.2: CDR2L co-immunoprecipitates with ribosomal protein rpS6, whereas CDR2 co-immunoprecipitates with nuclear speckle proteins SON and eIF4A3 in cancer cell lysates. (A) Immunoblot demonstrating the co-immunoprecipitation of CDR2L and rpS6 from OvCar3 cell lysates. (B) Immunoblot demonstrating the co-immunoprecipitation of CDR2, SON, and eIF4A3 from HepG2 cell lysates. Input = cancer cell lysates (OvCar3 or HepG2). Beads + lysate = samples that were not treated with primary antibody, and served as negative controls.[114]

4.3 Paper III: Paraneoplastic cerebellar degeneration: the importance of including CDR2L as a diagnostic marker

After identifying CDR2L as the major Yo antibody target and determining the subcellular location of the CDR proteins and binding partners, we aimed to evaluate the commercial tests used to diagnose patients with anti-Yo associated PCD. CDR2 was previously considered as the main Yo antigen and commercial line immunoassays and cell-based assays therefore use CDR2 as the target, but with low specificity.[44, 61]

From 2017 to 2020, we screened 9,527 patient sera and CSF for onconeural antibodies. Twenty-four patients showed Yo reactive bands (0.25%) on the commercial PNS 14 Line Assay from Ravo Diagnostika, and were included in the study. Positive samples

were tested with EUROLINE PNS 12 Ag, CBA for CDR2 and indirect immunofluorescence of rat cerebellar sections. Yo positive sera from six PCD patients showed granular, cytoplasmic staining in Purkinje neurons. In the group of 18 non-confirmed PCD cases, serum samples from two patients (7 and 8) stained Purkinje neurons but with no granular cytoplasmic staining; these patients were therefore interpreted as anti-Yo negative. The remaining 16 serum samples were negative.

We developed two in-house techniques for CDR2L detection: a CBA consisting of HEK293 cells transfected with a plasmid that expressed Myc-DDK-tagged CDR2L and a western blot based analysis of recombinant CDR2 and CDR2L proteins. To evaluate the specificity of the CDR2L CBA, transfected HEK239 cells were stained with anti-DDK, anti-CDR2L, or anti-CDR2 (Fig 4.3.1). No cross-reactivity was observed between CDR2 and CDR2L antibodies.

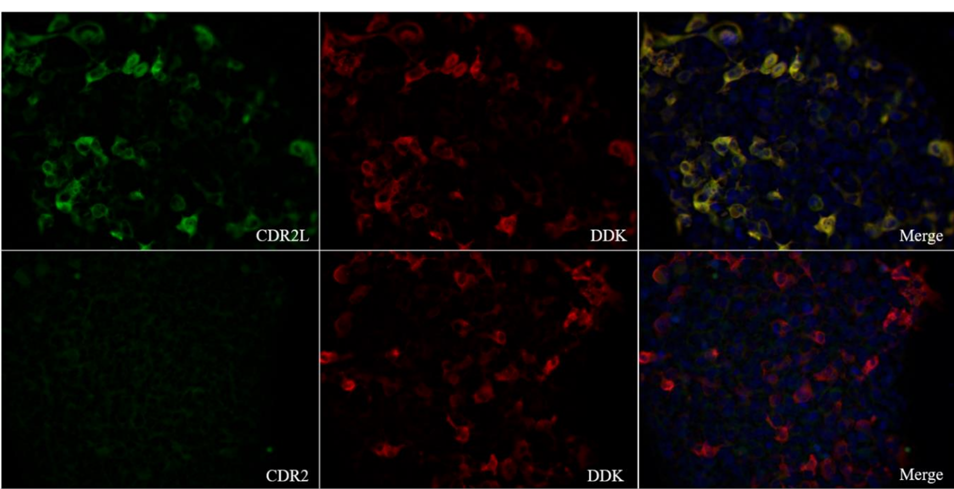


Figure 4.3.1: No cross-reactivity is observed between CDR2 antibodies and CDR2L in human embryonic kidney 293 cells that express Myc-DDK-tagged CDR2L. Upper row: cells stained with anti-CDR2L (green) and anti-DDK (red). Lower row: cells stained with anti-CDR2 (no reaction), and anti-DDK (red). Nuclei are stained with DAPI. Scale bar = 20 µm. CDR2 = cerebellar degeneration-related protein 2; CDR2L = cerebellar degeneration-related protein 2-like.[120]

Samples from the 6 confirmed PCD cases stained both CDR2L-transfected cells, commercial CBA for CDR2 and identified recombinant CDR2L (55 kDa) and CDR2 (62 kDa) in western blot analysis (Fig 4.3.2 A.a, A.b and A.c). The 7 patients with CBA CDR2-positive staining, but no PCD, were negative in the CBA for CDR2L (Fig 4.3.2

B.a and B.b). These results were confirmed by western blot analysis, which identified only recombinant CDR2 (62 kDa) in patient sera (Fig 4.3.2 B.c).

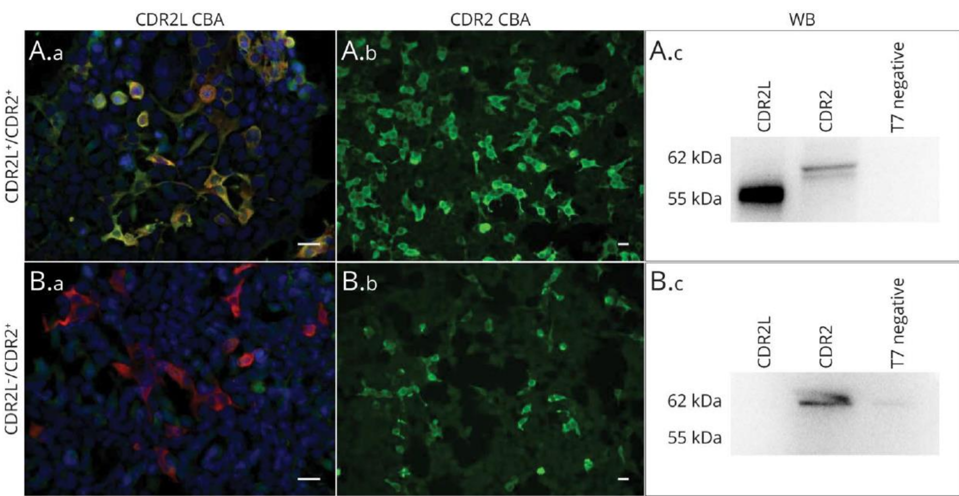


Figure 4.3.2: Representative images of patient sera (1:100) double positive for CDR2L and CDR2 (A.a–A.c), and single positive for CDR2 (B.a–B.c) in indirect immunofluorescence of CDR2L transfected human embryonic kidney 293 Cells (A.a, B.a), commercial CBA for CDR2 (A.b, B.b), and WB (A.c, B.c). A negative control containing reticulocyte lysate without recombinant protein was included in each experiment. Anti-CDR2/CDR2L, green; anti-DDK, red; merge, yellow. Scale bar = 20 µm. CBA, cell-based assay; CDR2, cerebellar degeneration-related protein 2; CDR2L, cerebellar degeneration-related protein 2-like; WB, western blot analysis.[120]

5. DISCUSSION

5.1 Paper I: CDR2L Is the Major Yo Antibody Target in Paraneoplastic Cerebellar Degeneration

We demonstrated that CDR2L is the major Yo antigen target in PCD, as Yo antibodies in patient CSF and serum consistently reacted with CDR2L in human and rat cerebellar tissue, and in cultured cancer cells.

Even though the CDR2L amino acid sequence was determined in 1996, it took about two decades before CDR2L was introduced as a new player in PCD.[75, 76] CDR2 was previously considered as the main antigen, partially based on the identification of only CDR2 mRNA expressed in PCD-associated tumors.[80] However, recent studies showed that both CDR2 and CDR2L are widely expressed in normal as well as PCD tumors, and that the CDR2L protein is overexpressed in the majority of Yo-PCD ovarian cancers.[89, 93] These findings and the fact that CDR2L staining of cerebellar tissue with commercial antibodies highly resembled that of anti-Yo, led to the speculation that CDR2L could be the major antigen in anti-Yo associated PCD.

Co-localization studies showed that CDR2L and Yo antibodies gave granular, cytoplasmic staining patterns that overlapped in human and rat Purkinje neurons, and in stellate and basket cells. A similar staining pattern was not found for CDR2, mainly localizing to the neuronal nuclei. These results were in line with previous experiments, in which Yo antibody reactivity in Purkinje neurons was abolished upon preabsorption with recombinant CDR2L, but only partially with recombinant CDR2.[75] Another study showed that recombinant CDR2 absorbed Yo antibodies from positive sera, thereby abolishing Purkinje neuron cytotoxicity.[102] There are potentially two main explanations for this discrepancy. First, the authors did not specify if anti-Yo was also absorbed with recombinant CDR2L. Second, there is a reactivity between recombinant CDR2 and Yo antibodies. The fact that recombinant CDR2 is used in routine diagnostic tests for PCD supports the latter. Therefore, we transfected HepG2 cells with CDR2 and CDR2L, and established that Yo antibodies can also bind recombinant CDR2.

The fact that CDR2 can be detected in a recombinant but not native form by Yo antibodies suggest that the epitope is inaccessible, most likely due to post-translational modification of CDR2 or by binding partners. Previous research suggests that CDR2 is phosphorylated by PKN and potentially targeted for degradation via ubiquitination.[83, 84] Furthermore, anti-Yo is recognized by recombinant CDR2 on commercial line immunoassays, and only patients with Yo antibodies towards both recombinant CDR2 and CDR2L develop PCD.[75, 120] These findings suggest that Yo antibodies harbor a paratope against a common epitope shared between CDR2 and CDR2L. Data from phage display immunoprecipitation and sequencing, used to create a high-resolution epitope profile from anti-Yo patients, show that there are no common linear epitopes between CDR2 and CDR2L.[94] It follows that any common epitope is most likely conformational, and more in depth knowledge on the 3D structure of the proteins is necessary to make any final conclusions.

Based on ours and recent findings we have shown that CDR2L is the major Yo antibody target but we cannot exclude an additional role of CDR2 in PCD.

5.2 Paper II: Localization of CDR2L and CDR2 in paraneoplastic cerebellar degeneration

With a few exceptions, our knowledge in terms of the biological functions of CDR2 and CDR2L is limited. Therefore, our goal was to establish the subcellular localization of CDR2 and CDR2L, and identify potential protein binding partners.

5.2.1 CDR2 expression and interaction with nuclear speckle proteins

It has become evident that several of the commercially available antibodies show varying staining patterns, localizing CDR2 to the cell cytoplasm and nucleus.[88, 118] Immunoprecipitation followed by mass spectrometry analysis confirmed that our antibodies are specific for their antigens. The discrepancy in the staining pattern may stem from the antibodies recognizing one of the five CDR2 isoforms (Appendices, Fig.

9.2) or from translocation of CDR2 between the cytoplasm and nuclei. Previous results imply that CDR2 translocate or facilitate transport of its proposed binding partners; c-myc, PKN, MRG15 and MRGX.[81, 82, 84, 85, 121] Another proposed mechanism is that phosphorylation of CDR2 impacts its subsequent degradation, initiated via APC/C-mediated ubiquitination in gynaecologic tumor cells. This is of importance as it is suggested that CDR2 in its unphosphorylated form during mitosis interacts with c-myc and regulates tumor cell growth. Inappropriate CDR2 expression might affect c-myc-dependent transcription during spindle formation and proliferation.[84]

The fact that numerous studies suggest both a cytoplasmic and nuclear localization of CDR2 in neuronal and cancer cells strongly support the hypothesis that CDR2 is tightly regulated via post-translational modification, exists in several isoforms and/or translocate based on the cellular milieu.

We identified three nuclear speckle proteins as potential binding partners of CDR2, by immunoprecipitation and mass spectrometry based proteomics; SON, SRSF2 and eIF4A3. Nuclear speckles are self-assembled clusters of around 200 proteins involved in pre-mRNA processing including splicing, surveillance, and RNA export. The speckles can vary in size and morphology within a single cell, but non-random organizations of proteins and RNAs occur, stabilized by intermolecular interactions.[122] SRSF2 and SON have domains enriched with arginine and serine repeats that are crucial for speckle core formation.[122, 123] Both proteins are also involved in mRNA splicing[124, 125] and interact with the ATP-dependent RNA helicase eIF4A3.[126] Based on STED microscopy, proximity ligation assay and co-immunoprecipitation we confirmed that CDR2 interacts directly with SON and eIF4A3.

Interestingly, SRSF2 can self-associate with SRSF1 (www.uniprot.org/Q01130), a protein that contributes to the oncogenic effect of myc.[127] SRSF1 cooperates with myc to increase eIF4E activation, and promotes formation of aggressive breast tumors in humans.[128] One can therefore speculate whether CDR2 could exert an oncogenic effect[84] through its involvement in this complex network of proteins that upon activation, overexpression or dysregulation contribute to tumorigenesis.

5.2.2 CDR2L expression and interaction with ribosomal proteins

We found that CDR2L specifically localizes to the cytoplasm of ovarian cancer cells, human and rat Purkinje neurons. Numerous ribosomal proteins were identified as potential binding partners of CDR2L based on immunoprecipitation-mass spectrometry data. Confirmatory tests showed that CDR2L co-immunoprecipitate and directly interacts with the ribosomal protein rps6. Rps6 is a component of the small (40S) subunit of ribosomes, and has mainly been studied due to rps6 being the first identified ribosomal protein to undergo phosphorylation.[129] The phosphorylation state of rps6 can be used to monitor neuronal activity, based on increased rps6 phosphorylation being reported during synaptic plasticity.[129]

Our findings are in line with previous analysis of PCD patient sera, localizing Yo antibodies to the cytoplasm in association with membrane-bound and free ribosomes.[91, 92] Here, CDR2 is referred to as the Yo antigen, but based on the results published in paper I and the fact that Yo antibodies only precipitated CDR2L and not CDR2 in immunoprecipitation-mass spectrometry analysis, we are confident that CDR2L is the major Yo antigen. This is also in line with a recent publication which suggests that CDR2L is the immunodominant antigen in PCD patients.[94]

5.2.3 CDR2 and CDR2L involvement in protein synthesis

We have shown that CDR2 interacts with eIF4A3 in nuclear speckles. eIF4A3 can translocate from the nucleus to cytoplasm, and facilitate mRNA binding to ribosomes, along with other initiation factors.[119] Affinity-capture mass spectrometry data imply that eIF4A3 interacts with rps6 in the cytoplasm.[130] Since CDR2L binds to rps6, we suggest that CDR2 and CDR2L are linked in protein synthesis via a common pathway (Fig 5.2.3), in which CDR2 is involved in transcription and CDR2L in translation.

Dysregulation of protein synthesis is associated with cancer and several neurological disorders.[131] We have therefore proposed a model for CDR2 and CDR2L involvement in anti-Yo associated PCD pathogenesis, in which binding of anti-Yo to CDR2L in Purkinje neurons interferes with the ribosomal machinery, resulting in disrupted

mRNA translation and protein synthesis. These events should be tracked in real-time to further address the dynamic interaction between the CDR proteins and their function in Purkinje neuron deterioration.

Based on immunoprecipitation-mass spectrometry analysis, CDR2 and CDR2L are not cross-reactive, suggesting that the antibody responses to each protein are independent.[94] This is in line with our findings in paper I, demonstrating that there is no cross-talk between the proteins in their native forms.

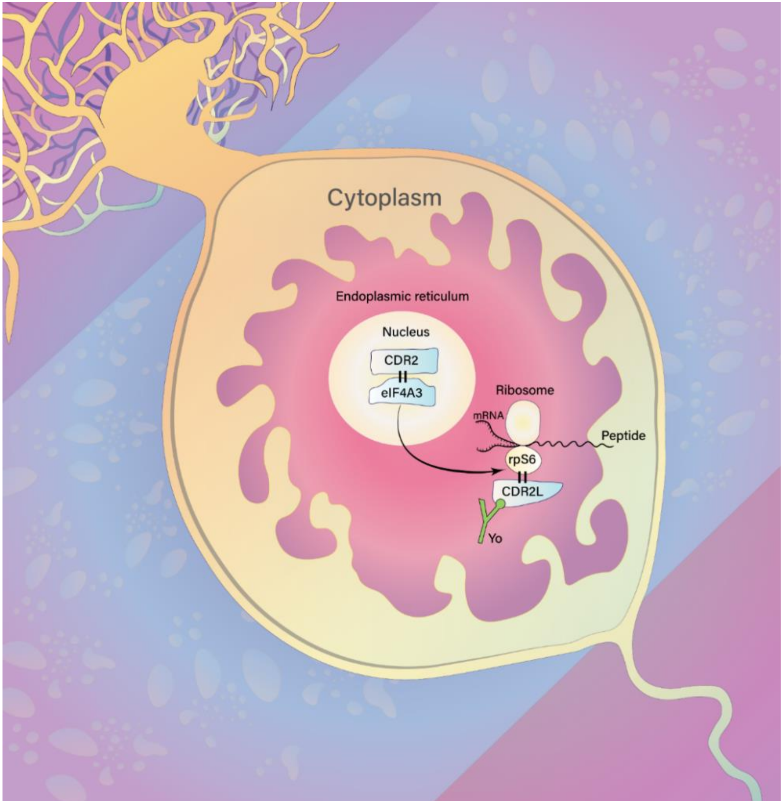


Figure 5.2.3: Hypothesis of CDR2L and CDR2 involvement in protein synthesis in Purkinje neurons. CDR2 localizes to the nucleus and directly interacts with nuclear speckle protein eIF4A3. eIF4A3, in conjugation with other cytoplasmic initiation factors, facilitates mRNA binding to the 40S ribosomal subunit. This event is important for mRNA maturation and translation, ultimately resulting in the synthesis of new proteins. CDR2L interacts with ribosomal subunit protein rpS6; therefore, we propose that CDR2L and CDR2 are both involved in the process of protein synthesis. Furthermore, Yo antibody (green) binding to CDR2L in Purkinje neurons of PCD patients may, therefore, interfere with the function of the ribosomal machinery, resulting in disrupted mRNA translation and/or protein synthesis.[114]

5.2.4 Antibodies: friend or foe?

We have found that commercial antibodies against CDR2 and CDR2L give divergent results amongst various experimental techniques. Therefore, we performed immunoprecipitation followed by mass spectrometry analysis to evaluate the specificity of several CDR2 and CDR2L antibodies produced to recognize the full-length protein or shorter sequences.

Antibodies are amongst the most frequently used tools in biology laboratories but proper antibody validation across research applications is lacking. There are a number of factors to consider when buying commercial antibodies for research purposes. Antibodies that perform well in one context may perform inadequate in others due to differences in protein conformation and target accessibility. Proteins are in near-native form in immunoprecipitation, but they are unfolded or denatured in western blot analysis.[116] Even small changes in sample preparation can have a large impact on how the antibody works.[117]

Data regarding antibody function provided by companies may stem from cell lines that have been engineered to express the protein at higher levels than under physiological conditions. Thus, researchers should ideally test if the antibody can detect the protein of interest at physiological levels in the desired technique and in the material they plan to use.[117] Off-target binding can occur if there is an antibody interest to proteins in the sample other than the target protein it was intended for. This holds even if the antibody`s affinity for other proteins is lower than it is for the target protein.[116]

Whether to use monoclonal or polyclonal antibodies will in part depend on the application. Polyclonal antibodies are particularly useful for immunoprecipitation as they recognize several antigen epitopes, thereby forming a large precipitating lattice.[39] A monoclonal antibody can then be used during confirmatory analysis, such as western blot, providing specificity for the protein of interest. Variabilities from batch-to-batch or lot-to-lot can occur, and happens to a much larger extent for polyclonal antibodies compared to monoclonal.[132]

Another growing concern is the high percentage (43%) of unidentifiable antibodies in published scientific articles.[117] This greatly impacts the ability to replicate already published work. An identification system has therefore been developed to uniquely identify antibodies, materials, reagents and tools with an ID called a Research Resource Identifier.[116, 133]

5.3 Paper III: Paraneoplastic cerebellar degeneration: the importance of including CDR2L as a diagnostic marker

The most established commercial tests for onconeural antibody detection, line immunoassay and CBA, use CDR2 as the Yo antibody target. However, recent studies pinpoint the low specificity of these tests, with a confirmation of approximately 6%.[44, 61] A potential reason for the high false positive rate is the use of CDR2, which is not the natural Yo antigen. Line immunoassays still detect Yo antibodies because anti-Yo bind recombinant CDR2, as shown in paper I. We found a discrepancy between the two commercial line immunoassays from Ravo Diagnostica and Euroimmun, most likely related to differences in use of CDR2 sequence length, and choice of cell line for production of recombinant CDR2. By combining results from the line immunoassays with CBA for CDR2, the number of false positive tests for PCD was reduced.

Rat cerebellar immunofluorescence is the most accurate amongst the well-established tests, based on our own experience. However, many clinical laboratories are not equipped to perform immunofluorescence assays and the granular cytoplasmic staining of Purkinje neurons, which is characteristic for anti-Yo, can be difficult to interpret. As stated in paper II, this granular staining most probably represents the ribosomal staining reflecting CDR2L binding to rpS6.[114]

We detected two samples from patients without PCD but with previous cancer that stained Purkinje neurons in a non-granular pattern. These samples can easily be detected as false positives for an untrained eye and the cytoplasmic staining must therefore be interpreted with caution, because it can be unrelated to PCD. Moreover, clinical features should also be taken into account to prevent misdiagnosis, unnecessary

testing and incorrect treatment. The number of false positive samples can also be reduced by performing immunohistochemistry as the initial screening, instead of line immunoassay or CBA. However, these analyses are laborious and require trained personnel to interpret staining patterns.

We developed two in-house techniques, CBA for CDR2L and western blot analysis with recombinant CDR2 and CDR2L proteins, to evaluate the advantage of including CDR2L as a diagnostic marker for anti-Yo associated PCD. CBA for CDR2L was the only technique that could identify the PCD patients with 100% accuracy. Western blot analysis was not as accurate as CDR2L CBA and this discrepancy could be differently expressed epitopes of CDR2L detected in each of the assays. However, the western blot analysis detected fewer false positive samples compared to the line immunoassays.

Although our study cohort is small our data demonstrate that detection of CDR2L is important to increase the diagnostic accuracy for Yo antibody detection. We do not know whether testing for CDR2L antibodies alone would be sufficient for diagnosis of PCD because our cohort was selected based on anti-CDR2 positivity. This question will require larger patient cohorts including PCD patients who test negative in commercial line immunoassays and patients who have PNS caused by other paraneoplastic antibodies.

5.4 General discussion of PCD

The main cornerstones in understanding PCD pathogenesis is to unravel the mechanisms underlying immune cell trafficking into the CNS and the potential pathogenic effect of paraneoplastic antibodies and immune cells on Purkinje neuron death.

The brain was previously viewed as an immune-privileged site protected by the BBB, in which infiltration of immune cells was considered forbidden and immune activation solely detrimental.[134] These assumptions regarding the immune-privileged status of the CNS have been discussed, especially in connection with technological advances and an increased understanding of the complexity of the immune system.[134] Identification

of a glymphatic system, and that activated T cells can cross the BBB in the absence of neuroinflammation suggest that trafficking across the BBB is a highly regulated process.[26]

Despite these advances, development of animal models to study PCD pathogenesis have proven difficult and most probably reflect the complexity of this disease. Organotypic slice cultures of rat brain tissue and Purkinje neuron cultures from rats lack the complexity of the BBB but serve as alternative model systems.[102, 104, 135]

Cumulative evidence highlights a key role of CD8 T cells in PCD. An important finding in the elucidation of an antigen-specific cytotoxic effect of CD8 T cells on Purkinje neuron loss has been the identification of up-regulated MHC class I molecule expression on Purkinje neurons during inflammation.[109, 136] This provides an opportunity for CD8 T cells to recognize intracellular antigens expressed by Purkinje neurons. Still, definitive evidence for a direct cytotoxic T cell effect is lacking.

The direct role of paraneoplastic antibodies in PCD pathogenesis is also unclear. The fact that anti-Yo targets intracellular antigens and failed attempts to produce animal models upon IgG treatment have led to the assumption that Yo antibodies unlikely contributes to the neuronal loss.[137] However, a direct cytotoxic effect on Purkinje neurons has been documented in rat organotypic slice cultures upon incubation with anti-Yo positive patient sera and CSF.[100, 102] Similar slice cultures have also been used to elucidate the mechanistic effect of anti-CDR2/CDR2L internalization causing altered calcium buffering capacity due to calbindin malfunction and subsequent Purkinje neuron death.[101, 104]

In line with these observations, a recent study presents a mechanism for intracellular localization of systemic lupus erythematosus (SLE) autoantibodies.[138] In the setting of endocytosis-mediated transport, the antibody binds to FcR or other binding partners and internalizes it into an endocytic vesicle that can be targeted for recycling, endo-lysosomal- or proteasomal degradation. Endosomal escape of the SLE-derived antibody is mediated by increased H^+ influx that results in enzyme activation and dissociation of the antibody from its binding partner (heparan sulfate proteoglycan).

The antibody can escape via a pore, formed by the increasing acidic milieu and subsequent membrane destabilization. Identification of IgG-FcγII receptors in Purkinje neurons and antibody uptake via Fcγ receptor endocytosis makes it tempting to hypothesize that anti-Yo can be internalized through similar mechanisms.[139, 140]

The fact that CDR2 and CDR2L are widely expressed in normal tissues and all ovarian cancers regardless of the presence of Yo antibodies, suggests that CDR2/CDR2L expression alone is insufficient to trigger autoimmunity.[89] The infrequent prevalence of anti-Yo associated PCD suggests that disease progression is most likely multifactorial, involving disruption of immune tolerance, genetic predisposition, mutations in the CDR2/CDR2L genes, B and T cell activation and trafficking into the CNS, a direct cytotoxic effect of Yo antibodies and/or as a side-effect of therapy with ICIs.[56, 60, 93, 102, 109, 141] Further studies are necessary to unravel the in depth mechanisms involved in pathogenesis of anti-Yo associated PCD.

6. ADVANCES AND FUTURE ASPECTS

Establishing that CDR2L is the major Yo antibody target in PCD is of great importance, as future research should be focused on this antigen, rather than CDR2. The role of humoral and cellular mechanisms in PCD is still unknown. A discrepancy in findings related to the presence of CDR2 specific cytotoxic T cells has been observed.[51, 52, 96, 98, 99] It follows that it would be interesting to explore if a CDR2L specific cytotoxic T cell response is involved in PCD pathogenesis. Regarding Yo antibodies, future research should be focused on possible endocytic or FcR mediated transport of the antibodies into Purkinje neurons.

The role of immune cells in anti-Yo associated PCD pathogenesis is not well established. The extensive loss of Purkinje neurons is proposed to be caused by a direct damaging effect of anti-Yo, infiltration of cytotoxic $CD8^+$ T cells and activation of microglia. The development of imaging mass cytometry (IMC) enables simultaneous detection of over 40 markers on tissue sections.[142] This technique couples traditional immunostaining approaches with metal-tagged antibodies and laser ablation. IMC can be used to study the presence, distribution, function and activation of immune cells in complex tissues, such as the human brain, and map cellular interactions. Including material from several brain regions would enable the study of the local and potentially systemic effect of immune responses in PCD pathogenesis.

We have unraveled the subcellular localization of CDR2 and CDR2L. However, the in depth cellular functions of the proteins remain largely unknown. Further investigation of the proposed model for CDR2 and CDR2L involvement in protein synthesis will be important when elucidating the specific mechanisms causing Purkinje neuron loss in PCD. CRISPR/Cas9 or RNA interference technologies can be applied to knock out or knock down the target genes to study the functions of CDR2 and CDR2L and to further validate the commercially available antibodies.

Development of a protocol for rat Purkinje neuron culture yields opportunities to further characterize PCD pathogenesis.[135] These cultures are suitable for live-cell imaging of fluorescently tagged CDR2 and CDR2L, thus allowing investigation of

activity dependent CDR expression in depth, and their effect on Purkinje neuron physiology in the presence or absence of anti-Yo.

7. CONCLUSIONS

Paper I: Establishing that CDR2L is the major Yo antibody target allows us to reconsider our view on the pathogenic mechanisms involved in PCD. The fact that anti-Yo still binds CDR2 in its recombinant form suggests that CDR2 might play a vital role in PCD pathogenesis, but the exact biological functions and molecular mechanisms remain unresolved.

Paper II: Previous research suggests that the Yo antibody target is localized in the cell cytoplasm in close association with ribosomes. We are confident that the observed staining pattern reflects the subcellular localization of CDR2L, being the major antigen found in sera and CSF of anti-Yo associated PCD patients. A proposed model for the involvement of CDR2 and CDR2L in protein synthesis is made based on the identification of protein binding partners. CDR2 is localized to the nuclear speckles in contact with eIF4A3, which can translocate to the cytoplasm and in cooperation with other initiation factors aid in mRNA binding to the ribosomes via its interaction with rps6. Since rps6 interacts with CDR2L, this provides a direct link between CDR2 and CDR2L. Whether these findings fully reflect the true nature of the CDR proteins and their functions remain to be resolved.

Paper III: We developed two in-house techniques, CBA for CDR2L and western blot analysis using recombinant CDR2 and CDR2L, to demonstrate the importance of including CDR2L as a diagnostic marker. Commercially available tests for Yo antibody detection have low specificity because these assays use CDR2 as target. We show that the accuracy of PCD diagnosis is greatly improved by adding a test using CDR2L as the antibody target.

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9. APPENDICES

9.1 Commercial antibodies

Table 10.1: Overview of antibodies used in scientific experiments in paper I-III.

Paper	Antibody	Company	Species	Catalog nr.	Method
Paper II, III	CDR2	Sigma-Aldrich	rb	HPA023870	IP-MS, CBA
Paper I, II	CDR2	Sigma-Aldrich	rb	HPA018151	ICC, WB, IP-MS
Paper II	CDR2	Santa Cruz	m	Sc-100320	IP-MS
Paper I, II	CDR2	LSBio	m	C181958	ICC, WB, IP-MS
Paper I, II, III	CDR2L	Proteintech	rb	14563-1-AP	ICC, IP, WB, IP-MS, CBA
Paper II	CDR2L	Proteintech	m	66791-1-Ig	IP-MS
Paper II	CDR2L	Sigma-Aldrich	rb	HPA022015	IP-MS
Paper III	DDK	Origene	m	TA50011-100	CBA
Paper II	eIF4A3	Abcam	rb	32485	WB
Paper II	eIF4A3	Santa Cruz	m	Sc-365549	ICC, WB
Paper II	Hsp60	Biotechnology	c	CPCA-HSP60	PLA
Paper II	rpS6	Cell Signaling	m	2317	ICC
Paper II	rpS6	Santa Cruz	m	Sc-74459	ICC, WB
Paper II	SON	Santa Cruz	m	Sc-398508	ICC, WB
Paper II	SRSF2	Abcam	m	Ab11826	ICC

CBA, cell-based assay; ICC, immunocytochemistry; IP-MS, immunoprecipitation-mass spectrometry analysis; PLA, proximity ligation assay; WB, western blot; c, chicken; m, mouse; rb, rabbit.

9.2 CDR2 amino acid sequence

MLAENLVEEFEMKEDEPWYDHQDLQQDLQLAAELGKTLLDRNTELEDSVQ
QMYTTNQEQLQEIEYLTKQVELLRQMNEQHAKVYEQLDVTARELEETNQKL
VADSKASQQKILSLTETIECLQTNIDHLQSQVEELKSSGQGRRSPGKCDQEKP
APSFACLKELYDLRQHFVYDHVFAEKITSLQGQPSPDEEENEHLKKTVTMLQ
AQLSLERQKRVTMEEEYGLVLKENSELEQQLGATGAYRARALELEAEVAEM
RQMLQSEHPFVNGVEKLVPDSLYVPFKEPSQSLLEEMFLTVPESHRKPLKRSS
SETILSSLAGSDIVKGHEETCIRRAKAVKQRGISLLHEVDTQYSALKVKYEELL
KKCQEEQDSLSHKAVQTSRAAAKDLTGVNAQSEPVASGWELASVNPEPVSS
PTTPPEYKALFKEIFSCIKKTKQEIDEQRTKYRSLSSHS
(RefSeq: NP_001793.1, human, gene ID: 1039, 454 amino acids)

Table 10.2: CDR2 antibody immunogen sequences

Target	Source/Supplier	Cat. no.	AA seq.	Species
CDR2	Sigma-Aldrich	HPA018151	270-392	rb
CDR2	Sigma-Aldrich	HPA023870	112-234	rb
CDR2	Santa Cruz	Sc-100320	296-405	m
CDR2	LS Bio	C181958	Full length	m

CDR2 isoforms

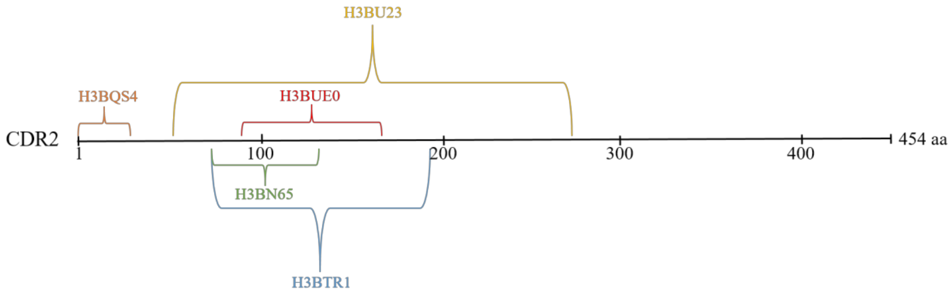

Figure 10.2: Predicted CDR2 isoform sequences (www.uniprot.org). H3BQS4, organge; H3BU23, yellow; H3BTR1, blue; H3BN65, green; H3BUE0, red.

9.3 CDR2L amino acid sequence

MRRAAGMEDFSAEEEESWYDQQDLEQDLHLAAELGKTLLERNKELEGSLQQ
MYSTNEEQVQEIEYLTKQLDTLRHVNEQHAKVYEQLDLTARDLELTNHRLV
LESKAAQQKIHGLTETIERLQAQVEELQAQVEQLRGLEQLRVLREKRERRRTI
HTFPCLKELCTSPRCKDAFRLHSSSLELGPRPLEQENERLQTLVGALRSQVSQ
ERQRKERAEREYTAVLQEYSELERQLCEMEACRLRVQELEAELLELQQMKQ
AKTYLLGPDDHLAEALLAPLTQAPEADDPQPGRGDDLGAQDGVSSPAASPG
HVVRKSCSDTALNAIVAKDPASRHAGNLTLHANSVRKRGMSILREVDEQYH
ALLEKYEELLSKCRQHGAGVRHAGVQTSRPISRDSSWRDLRGGEEGQGEVK
AGEKSLSQHVEAVDKRLEQSQPEYKALFKEIFSRIQKTKADINATKVKTHSSK
(RefSeq: NP_055418.2, human, gene ID: 30850, 465 amino acids)

Table 10.3: CDR2L antibody immunogen sequences

Target	Source/Supplier	Cat. no.	AA seq.	Species
CDR2L	Sigma-Aldrich	HPA022015	395-464	rb
CDR2L	Proteintech	14563-1-AP	116-465	rb
CDR2L	Proteintech	66791-1-Ig	116-465	m

9.4 Aligned CDR2 and CDR2L amino acid sequences

CDR2: GREEN
CDR2L: RED

MLAENLVEEFEMKEDEPWYDHQDLQQDLQLAAELGKTLLDRNTELEDSVQQ
MRRAAGMEDFSAEEEESWYDQQDLEQDLHLAAELGKTLLERNKELEGSLQQ
MYTTNQEQLQEIEYLTKQVELLRQMNEQHAKVYEQLDVTARELEETNQKLV
MYSTNEEQVQEIEYLTKQLDTLRHVNEQHAKVYEQLDLTARDLELTNHRLVL
ADSKASQQKILSLTETIECLQTNIDHLQSQVEELKSSGQGRRSPGKCDQEKPAP
ESKAAQQKIHGLTETIERLQAQVEELQAQVEQLRGLEQLRVLREKRERRRTIH
SFACLKELYDLRQHFVYDHVFAEKITSLQGQPSPDEEENEHLKKTVTMLQAQL
TFPCLKELCTSPRCKDAFRLHSSSLELGPRPLEQENERLQTLVGALRSQVSQER
SLERQKRVTMEEEYGLVLKENSELEQQLGATGAYRARALELEAEVAEMRQM
QRKERAEREYTAVLQEYSELERQLCEMEACRLRVQELEAELLELQQMKQAKT
LQSEHPFVNGVEKLVPDSLYVPFKEPSQSLLEEMFLTVPESHRKPLKRSSSETIL
YLLGPDDHLAEALLAPLTQAPEADDPQPGRGDDLGAQDGVSSPAASPGHVVR
SSLAGSDIVKGHEETCIRRAKAVKQRGISLLHEVDTQYSALKVKYEELLKKCQ
KSCSDTALNAIVAKDPASRHAGNLTLHANSVRKRGMSILREVDEQYHALLEK
EEQDSLSHKAVQTSRAAAKDLTGVNAQSEPVASGWELASVNPEPVSSPTTP--
YEELLSKCRQHGAGVRHAGVQTSRPISRDSSWRDLRGGEEGQGEVKAGEKSL
------------------------------PEYKALFKEIFSCIKKTKQEIDEQRTKYRSLSSHS
SQHVEAVDKRLEQSQPEYKALFKEIFSRIQKTKADINATKVKTHSSK

Only three identical epitopes between CDR2 and CDR2L that are over six amino acids in length exist:

1. LAAELGKTLL (10)
2. NEQHAKVYEQLD (12)
3. PEYKALFKEIFS (12)

ORIGINAL PUBLICATIONS

CDR2L Is the Major Yo Antibody Target in Paraneoplastic Cerebellar Degeneration

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The pathogenesis of Yo-mediated paraneoplastic cerebellar degeneration (PCD) is unclear. We applied cerebrospinal fluid and serum from PCD patients as well as CDR2 and CDR2L antibodies to neuronal tissue, cancer cell lines, and cells transfected with recombinant CDR2 and CDR2L to elucidate which is the major antigen of Yo antibodies. We found that Yo antibodies bound endogenous CDR2L, but not endogenous CDR2. However, Yo antibodies can bind the recombinant CDR2 protein used in routine clinical testing for these antibodies. Because Yo antibodies only bind endogenous CDR2L, we conclude that CDR2L is the major antigen of Yo antibodies in PCD.

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Paraneoplastic cerebellar degeneration (PCD) is one of the most common paraneoplastic neurological syndromes.[1] In PCD patients, the immune system targets a tumor antigen that is also expressed endogenously in the nervous system.[2] Among the most frequently detected onconeural antibodies in PCD patients are Yo antibodies.[3] Yo reactivity with cerebellar degeneration-related (CDR) proteins present in Purkinje cells is associated with Purkinje cell death[4] and severe cerebellar degeneration.[5]

Yo antibodies react with 2 proteins, CDR2 (RefSeq NP_001793.1) and CDR2-like (CDR2L; RefSeq NP_055418.2), that have 45% sequence identity.[6] CDR2 has previously been considered as the main Yo antigen.[6–9] This assumption is based in part on the finding that only the *CDR2* gene is expressed in tumors obtained from PCD patients.[6] However, recent studies have demonstrated that both CDR2 and CDR2L are widely expressed in normal as well as in malignant tissues[10,11] and that the CDR2L protein, but not CDR2, is highly expressed in PCD tumors.[12] Furthermore, CDR2L protein deposits are detected in germinal centers of all Yo-mediated PCD tumors with tertiary lymphoid structures,[12] suggesting an ongoing local immune response against CDR2L. In line with this, we have shown that preabsorption with CDR2L abolishes Yo antibody staining of human Purkinje cells completely, whereas preabsorption with CDR2 does not.[13]

To determine which onconeural antigen is the major target of Yo antibodies, we studied the reactivity of Yo antibodies toward both native and recombinant CDR2 and CDR2L proteins. Our findings show that Yo antibodies react only to native CDR2L, and not to CDR2, suggesting that CDR2L is the major target of these antibodies in vivo.

Materials and Methods

Patient Samples

Five sex- and age-matched cerebrospinal fluid (CSF)/serum patient samples with Yo antibodies (PCD patients) and 5 without Yo antibodies (controls) were obtained from the Neurological Research Laboratory, Haukeland University Hospital (Regional Committees for Medical and Health Research Ethics (REK), #2013/1480).[10]

Cerebellar Tissue

Cerebellar sections were cut from fresh frozen normal human tissue (REK, #2013/1503) or paraformaldehyde (PFA)-perfused rat brains (The Norwegian regulation of the use of animals in research, #20157494) that required additional heat-induced epitope retrieval prior to immunostaining.[14]

Cell Cultures

The OvCar3 (American Type Culture Collection [ATCC], #HTB-161) and the HepG2 (ATCC, #HB-8065) cancer cell lines were maintained and subcultivated on poly-D-lysine–coated coverslips (Neuvitro, Vancouver, WA; #GG-18-1.5-pdl) according to the manufacturer's protocol. Cells were washed (2 × 0.1M phosphate-buffered saline [PBS]), fixed (15 minutes, 4% PFA-PBS; Thermo Fisher Scientific, Waltham, MA; #28908), and quenched (5 minutes, 50mM NH_4Cl; Sigma-Aldrich, St Louis, MO; #254134) prior to immunostaining.

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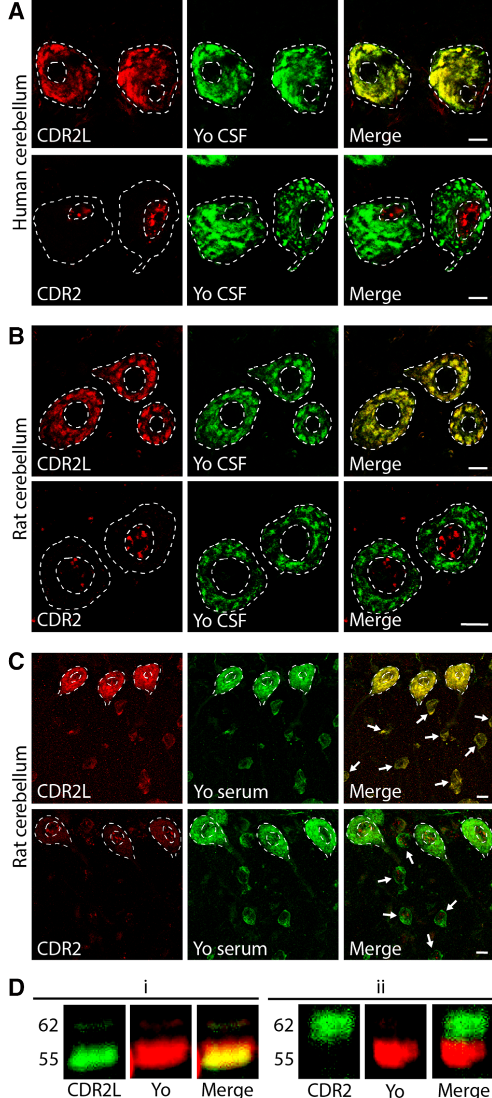

FIGURE 1: Yo antibodies bind to CDR2L, but not CDR2, in cerebellar Purkinje cells. Scale bars = 10µm. (A) Sections of fresh frozen human cerebellum. Upper row: Section stained with Yo (cerebrospinal fluid [CSF]; green) and anti-CDR2L (red); the antibodies colocalize in the cytoplasm (seen as yellow in the merge image). Lower row: Section stained with Yo (CSF; green) and anti-CDR2 (red); no colocalization is seen between Yo and CDR2. (B) Sections of paraformaldehyde (PFA)-perfused rat cerebellum. Upper row: Section stained with Yo (CSF; green) and anti-CDR2L (red); CDR2L colocalize with Yo. Lower row: Section stained with Yo (CSF; green) anti-CDR2 (red); no colocalization is seen. (C) Sections of PFA-perfused rat cerebellum. Upper row: Section stained with Yo (serum; green) and anti-CDR2L (red); Yo and CDR2L colocalize in the Purkinje cells (outlined) as well as in the stellate and basket cells (arrows). Lower row: Section stained with Yo (serum; green) and anti-CDR2 (red); no colocalization is seen between Yo and CDR2. These images are a z-stack merge, as not all stellate/basket cells were in the same focal plane as the Purkinje cells; thus, the cytoplasmic staining found over or under the nuclei may appear nuclear although it is not (eg, the Yo serum staining is not nuclear). (D) Fluorescent immunoblot of rat cerebellar lysate. Anti-CDR2L and Yo (CSF) stain the same band at 55kDa; anti-CDR2 does not. Secondary antibody controls were negative.

Immunochemistry

Cancer cells and cerebellar sections were permeabilized (5 minutes, 0.5% Triton X-100-PBS; Sigma-Aldrich, #11332481001), washed (3 × 15 minutes, 0.5% gelatin-PBS; Sigma-Aldrich, #G7041), blocked (30 minutes, 10% SEABLOCK; Thermo Fisher, #37527), incubated with primary antibodies (overnight, 4°C), washed, incubated with secondary antibodies (2 hours, room temperature), and mounted (ProLong Diamond with DAPI; Thermo Fisher Scientific, #P36962). Antibodies consisted of rabbit anti-CDR2 (AA270-392; Sigma-Aldrich, #HPA018151; cerebellar sections and HepG2 cells), mouse anti-CDR2 (full-length; LSBio, Seattle, WA; #C181958; OvCar3 cells), rabbit anti-CDR2L (AA116-465; Protein Technology, Wuhan, Hubei, P.R.C #14563-1-AP), antihuman Alexa Fluor 488/594 (Thermo Fisher Scientific, #A-11013/#A-11014), antirabbit Alexa Fluor 488/594 (Thermo Fisher Scientific, #R37116/#R37117), antirabbit STAR635P (Sigma-Aldrich, #53399-500UG), and antimouse Alexa Fluor 488/594 (Thermo Fisher Scientific, #R37120/#R37121). A Leica (Wetzlar, Germany) SP8 STED 3X confocal microscope equipped with a × 100 1.4 numerical aperture oil objective was used for imaging.

Immunoprecipitation

Following the Bio-Rad SureBeads immunoprecipitation protocol, the proteins were immunoprecipitated from OvCar3 cell lysate by using Protein G Magnetic Beads (Bio-Rad Laboratories, Hercules, CA; #161-4023). Immunoprecipitated proteins were separated on a 10% TGX gel (Bio-Rad, #456-1035) and transferred to a polyvinylidene difluoride (PVDF) membrane using the Trans-Blot Turbo Transfer kit (Bio-Rad, #170-4274). Western blot analysis was performed to detect the immunoprecipitated target proteins. Antibodies consisted of rabbit anti-CDR2L, mouse anti-CDR2, Yo-CSF, TidyBlot (Bio-Rad, #STAR209PA), and horseradish peroxidase antimouse IgG (Dako, Carpinteria, CA; #P0260).

Fluorescent Immunoblotting

The cerebellar and cancer cell lysates were obtained using a Total Protein Extraction Kit (Millipore, Billerica, MA; #2140). Proteins were separated on a 10% TGX gel and transferred to a low-autofluorescence PVDF membrane. Antibodies consisted of rabbit anti-CDR2L, rabbit anti-CDR2, Yo-CSF, antirabbit Alexa Fluor 488, and antihuman Alexa Fluor 647 (Thermo Fisher Scientific, #A-21445). G:Box (Syngene, Frederick, MA) was employed for visualization.

Recombinant DNA and Transfection

Full-length CDR2 (OriGene Technologies, Rockville, MD; #RG204900) and CDR2L (OriGene Technologies, #RC206909) were ligated into a pCMV6-AC-GFP vector (OriGene Technologies,

#PS100010). Following polymerase chain reaction, correct CDR2 and CDR2L vector sequences were confirmed using BioEdit v7.2.5. One Shot TOP10 *Escherichia coli* (Life Technologies, Carlsbad, CA; #C4040-10) were used for amplification, E.Z.N.A. Plasmid DNA Kit (Omega Bio-Tek, Norcross, GA, #D6942) for purification, and Lipofectamine 3000 (Thermo Fisher Scientific, #L3000008) for transfection.

Results

CDR2L and Yo Staining Overlap

In sections of human and rat cerebellum, CDR2L showed a cytoplasmic staining pattern in Purkinje cell somas that overlapped completely with the Yo antibody staining from both CSF and serum (Fig 1). CDR2L and Yo also colocalized in the stellate and basket cells. In contrast, CDR2 primarily stained the nuclei of these neurons and gave no overlap with the Yo antibodies. Under denaturing conditions, immunofluorescence blots of rat cerebellar lysate showed that CDR2L and Yo were recognized at 55kDa, whereas CDR2 was only visible at 62kDa.

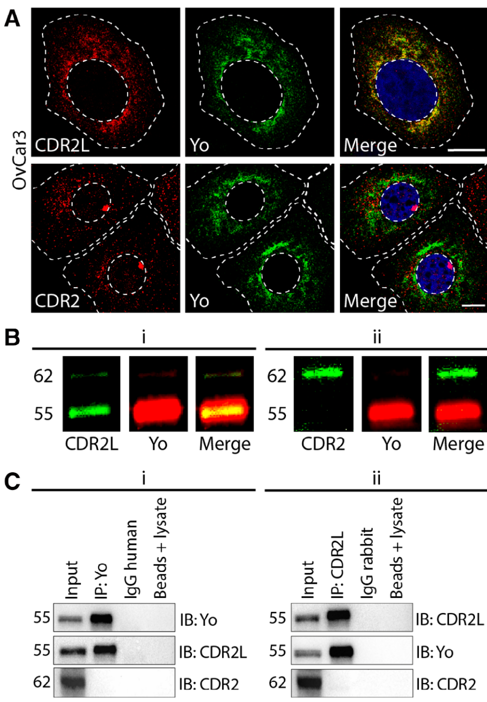

FIGURE 2: Yo antibodies bind to CDR2L, but not CDR2, in OvCar3 cells. Scale bars = 10µm. (A) Upper row: OvCar3 cells stained with Yo (cerebrospinal fluid [CSF]; green) and anti-CDR2L (red); Yo and CDR2L colocalize, giving the same granular, cytoplasmic staining pattern (seen as yellow in the merge image). Lower row: OvCar3 cells stained with Yo (CSF; green) and anti-CDR2 (red); Yo does not colocalize with CDR2. (B) Fluorescent immunoblot (IB) of OvCar3 lysate. Anti-CDR2L and Yo (CSF) stain the same 55kDa band; anti-CDR2 does not. Secondary antibody controls were negative. (C) Immunoblot of proteins immunoprecipitated (IP) from the OvCar3 lysate by Yo (CSF) or CDR2L. The protein precipitated by Yo antibodies was recognized by the CDR2L antibody on Western blot (i) and vice versa (ii); no relationship was observed between Yo or CDR2L and CDR2.

CDR2L and Yo Colocalize in Ovarian Cancer Cells

In OvCar3 cells, which express both CDR2 and CDR2L endogenously, we found that CDR2L and Yo colocalized in the cytoplasm, whereas CDR2 showed no colocalization with Yo (Fig 2A). The coreactivity of the CDR2L and Yo

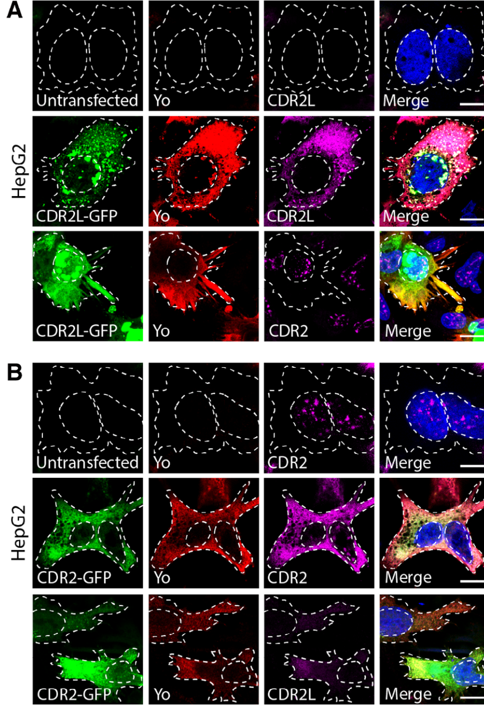

FIGURE 3: HepG2 cells, with a high endogenous level of CDR2, are not stained by Yo antibodies. However, Yo antibodies are able to bind recombinant CDR2. Scale bars = 10µm. (A) Untransfected HepG2 cells (first row) and HepG2 cells transfected with a vector expressing recombinant CDR2L-GFP (second and third row; green) were incubated with Yo (cerebrospinal fluid [CSF]; red), anti-CDR2L (first and second row; magenta), and anti-CDR2 (third row; magenta). Nuclei were stained with DAPI. Only upon expression of recombinant CDR2L was Yo and CDR2L antibody staining observed. The CDR2 antibody did not bind the recombinant CDR2L protein. (B) Untransfected HepG2 cells (first row) and HepG2 cells transfected with a vector expressing CDR2-GFP (second and third row; green) were incubated with Yo (CSF; red), anti-CDR2 (first and second row; magenta), and anti-CDR2L (third row; magenta). Native CDR2 is present in untransfected HepG2 cells, but no Yo staining was found. When recombinant CDR2-GFP was present, both the Yo and CDR2L antibody were able to bind.

antibodies was confirmed by both fluorescent Western blotting and immunoprecipitation (see Fig 2B, C).

Yo Antibodies Detect Recombinant CDR2 and CDR2L

Yo and CDR2L staining was absent in untransfected HepG2 cells, whereas CDR2 was present in the nuclei of these cells (Fig 3). In HepG2 cells transfected with recombinant CDR2 or CDR2L linked to green fluorescent protein (CDR2-GFP and CDR2L-GFP), however, Yo antibodies colocalized with both CDR2L-GFP and CDR2-GFP. Similar results were obtained for all PCD samples tested.

Discussion

We demonstrate that Yo antibodies in the CSF and serum of PCD patients consistently react with CDR2L in human

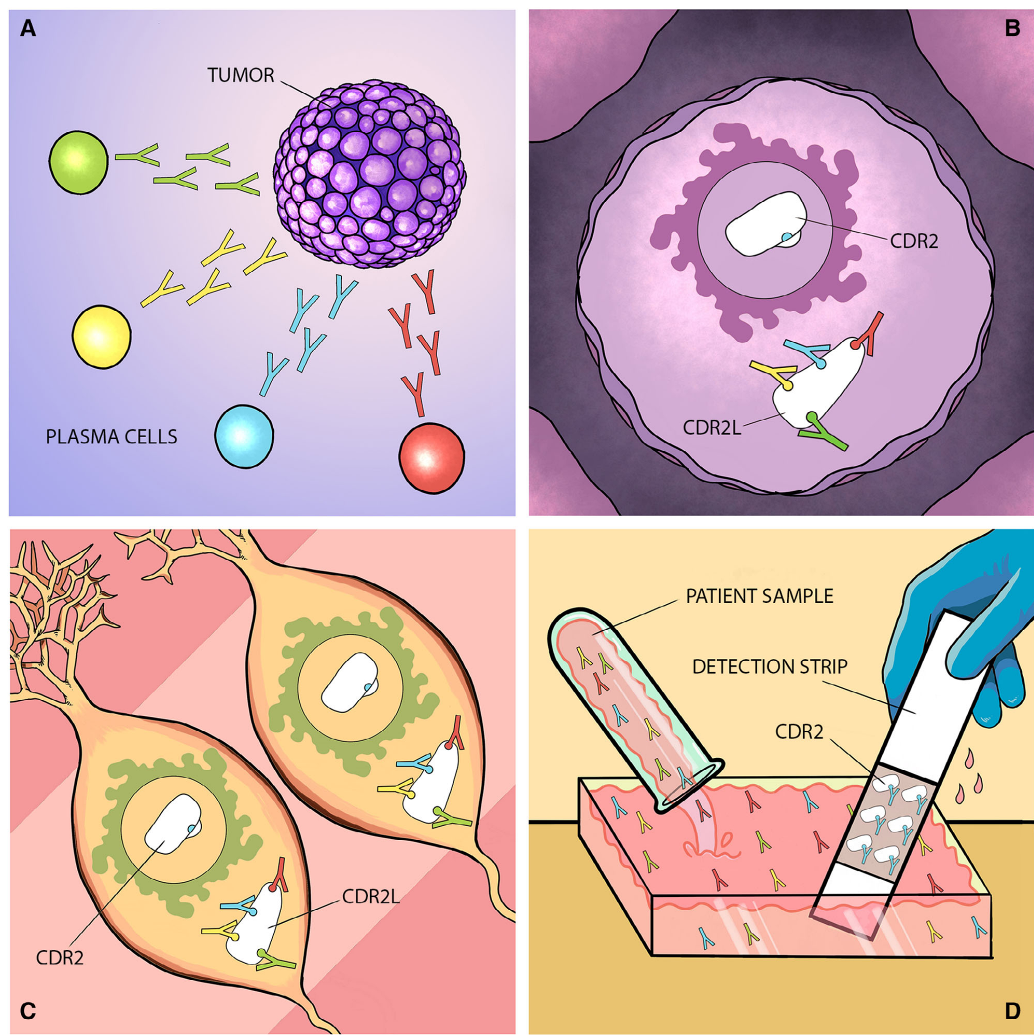

FIGURE 4: Proposed hypothesis of how Yo antibodies are able to bind both recombinant CDR2 and CDR2L, but only CDR2L under native conditions. (A) Illustration of the initial, polyclonal response of Yo antibodies toward CDR2L in the tumors of paraneoplastic cerebellar degeneration patients. (B) A tumor cell with the polyclonal Yo antibodies targeting the CDR2L protein; CDR2 is unaffected, as the epitope that is common to CDR2L (blue) is hidden by post-translational modifications or a partnering molecule (white fold covering the blue epitope). (C) The Yo antibodies also bind to CDR2L in cerebellar Purkinje cells; however, they do not bind CDR2, as the common epitope (blue) is hidden here as well (by modifications or partnering molecules; white fold). (D) When patient sera or cerebrospinal fluid is applied to a line blot with recombinant CDR2 attached, binding of the common epitope (blue) is possible, as it is not hidden by post-translational modifications or partnering molecules in the recombinant version.

and rat brain tissue as well as in cultured cancer cells. Despite sequence homology between CDR2 and CDR2L, Yo antibodies did not cross-react with endogenously expressed CDR2. These findings were confirmed by using HepG2 cells that express CDR2 endogenously, but not CDR2L; Yo antibodies were not able to bind the endogenous CDR2 in these cells either. We therefore conclude that CDR2L is the major antigen of Yo antibodies under native conditions. This result indicates that previous research on Yo-mediated PCD has focused on a protein that is not the major antigenic target of Yo antibodies.

CDR2L and Yo antibodies gave a granular, cytoplasmic staining pattern that colocalized in both human and rat Purkinje cells, as well as in stellate and basket cells. In contrast, CDR2 reactivity primarily occurred in the nuclei of these neuronal cells, where Yo antibody staining was absent. In the human cancer cell lines OvCar3 and HepG2, we found strong staining of CDR2 in the nuclei, as well as some cytoplasmic staining. Similar CDR2 staining has also been found in other cancer cell lines and tissues.[15]

We found that none of our PCD patient samples cross-reacted with endogenous CDR2. Thus, CDR2L-exclusive epitopes appear to be the major targets of Yo antibodies under native conditions. Furthermore, we observed competitive binding between the CDR2L and the Yo antibodies, whereas the CDR2 antibody staining was not affected by high Yo antibody concentrations (data not shown). This is in line with our previous results showing that the reactivity of Yo antibodies in the Purkinje cells disappears completely when preabsorbed with recombinant CDR2L protein, but only partially with recombinant CDR2.[13]

In routine clinical testing for onconeural antibodies, line blots and cell-based assays use recombinant CDR2 as the antigen target for Yo antibodies (Euroimmun, www.euroimmun.com; ravo Diagnostika, www.ravo.de). Because we did not find any reactivity of Yo antibodies toward native CDR2, we investigated this further by transfecting HepG2 cells with CDR2 and CDR2L linked to green fluorescent protein. Our results showed that Yo antibodies did bind recombinant CDR2, meaning that the protein can still be used for clinical diagnostic purposes. However, line blot and cell-based assays using CDR2L may be more sensitive for detecting Yo antibodies.

Whereas Yo antibodies are able to bind recombinant CDR2, they appear unable to access this epitope on endogenous CDR2, likely because it is hidden by post-translational modifications or by partnering molecules (Fig 4). A recent study did not find any common linear epitopes detected by Yo antibodies for CDR2 and CDR2L.[16] This suggests that any common epitope is likely conformational, a feature that can be elucidated once the 3-dimensional structures of these proteins are established.

Our present results strengthen the hypothesis that CDR2L is the major target of Yo antibodies. This is in line with the recent findings that CDR2L expression was detected in all samples of ovarian cancers from PCD patients, whereas CDR2 was only weakly expressed in 40% of the tumors.[12] Furthermore, CDR2L deposits were found in germinal centers of all Yo-mediated PCD tumors with tertiary lymphoid structures, suggesting a humoral immune response against CDR2L.[12] Thus, Yo antibodies targeting CDR2L in tumor cells, with binding of CDR2L in Purkinje cells as an unfortunate side effect, likely contributes to the development of PCD. CDR2L should therefore be included in future research into the pathogenesis of Yo-mediated PCD.

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Author Contributions

T.K., M.S., and C.A.V. contributed to the conception and design of the study; T.K., I.H., M.R., and M.H. contributed to the acquisition and analysis of data; T.K., M.S., and C.A.V. contributed to drafting the text and preparing figures.

Potential Conflicts of Interest

Nothing to report.

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RESEARCH ARTICLE

Localization of CDR2L and CDR2 in paraneoplastic cerebellar degeneration

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Abstract

Objective: Identify the subcellular location and potential binding partners of two cerebellar degeneration-related proteins, CDR2L and CDR2, associated with anti-Yo-mediated paraneoplastic cerebellar degeneration. **Methods:** Cancer cells, rat Purkinje neuron cultures, and human cerebellar sections were exposed to cerebrospinal fluid and serum from patients with paraneoplastic cerebellar degeneration with Yo antibodies and with several antibodies against CDR2L and CDR2. We used mass spectrometry-based proteomics, super-resolution microscopy, proximity ligation assay, and co-immunoprecipitation to verify the antibodies and to identify potential binding partners. **Results:** We confirmed the CDR2L specificity of Yo antibodies by mass spectrometry-based proteomics and found that CDR2L localized to the cytoplasm and CDR2 to the nucleus. CDR2L co-localized with the 40S ribosomal protein S6, while CDR2 co-localized with the nuclear speckle proteins SON, eukaryotic initiation factor 4A-III, and serine/arginine-rich splicing factor 2. **Interpretation:** We showed that Yo antibodies specifically bind to CDR2L in Purkinje neurons of PCD patients where they potentially interfere with the function of the ribosomal machinery resulting in disrupted mRNA translation and/or protein synthesis. Our findings demonstrating that CDR2L interacts with ribosomal proteins and CDR2 with nuclear speckle proteins is an important step toward understanding PCD pathogenesis.

Introduction

Paraneoplastic neurological syndromes are rare autoimmune-mediated diseases[1,2] characterized by the production of antibodies that target antigens expressed both by the tumor and endogenously in the central nervous system.[3,4] One of the most common forms of paraneoplastic neurological syndromes is paraneoplastic cerebellar degeneration (PCD).[5] In patients with PCD and breast or ovarian cancer, the dominant onconeural antibody, anti-Yo, is detected in both serum and cerebrospinal fluid (CSF).[6] Anti-Yo antibodies are directed against two proteins, cerebellar degeneration-related protein 2 (CDR2) and CDR2-like (CDR2L), which are endogenously expressed in Purkinje neurons of the cerebellum.[7] The interaction between anti-Yo and CDR proteins is thought to mediate Purkinje neuron dysfunction and death.[5] A two-step process has been proposed, with the internalization of Yo antibodies as the primary event,

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followed by the subsequent activation of cytotoxic T cells.[8,9] However, it has also been demonstrated that Yo antibodies can induce Purkinje neuron death in the absence of T lymphocytes.[8,10]

Previously we showed that CDR2L is the major Yo antibody target in PCD.[7] However, we cannot exclude a functional role for CDR2 in anti-Yo-mediated PCD pathogenesis. These proteins display a high degree of homology with approximately 45% sequence identity,[11,12] and both are widely expressed in normal as well as malignant tissues.[3,13] Ovarian malignancy is the most frequent cancer type found in Yo-mediated PCD, and both CDR2L and CDR2 are highly expressed in this type of cancer.[3,14]

Earlier studies have suggested that CDR2L and CDR2 are cytoplasmic proteins.[3,13] However, detailed subcellular localization using antigen-specific antibodies has not been performed. Current knowledge concerning the biologic function of CDR2L is limited. CDR2 has leucine zipper and zinc-finger DNA binding domains, characteristic of transcriptional regulatory proteins[11,15,16] and occurrence of these domains in the predicted open reading frame suggests that CDR2 has a role in regulating gene expression.[11,17] CDR2 interacts with the serine/threonine protein kinase PKN and cell cycle-related proteins MRG15 and MRGX; all involved in signal transduction or gene transcription.[15,18,19]

In this study, we examined the subcellular locations of CDR2L and CDR2 and their protein-protein interactions. Our findings suggest that CDR2L and CDR2 have different roles: CDR2L interacts with cytosolic ribosomes and appears to function in protein synthesis, while CDR2 associates with nuclear speckle proteins and appears to be involved in mRNA maturation.

Materials and Methods

Patient samples

Five sex- and age-matched CSF samples from patients with Yo antibodies (PCD patients) and five without Yo antibodies and no neurological disease or underlying cancer (negative controls) were obtained from the Neurological Research Laboratory, Haukeland University Hospital (Regional Committees for Medical and Health Research Ethics, 2013/1480).

Cell culture

OvCar3 (American Type Culture Collection (ATCC), #HTB-161) and HepG2 (ATCC, #HB-8065) cancer cell lines were maintained and subcultivated on poly-D-lysine-coated coverslips (Neuvitro, #GG-18-1.5-pdl) according to the manufacturer's protocol. Cells were washed twice with 0.1 M phosphate-buffered saline (PBS), fixed (15 min, 4% paraformaldehyde in PBS, Thermo Fisher Scientific, #28908), and quenched (5 min, 50 mmol/L NH_4Cl, Sigma-Aldrich, #254134) prior to immunostaining.

Cerebellar tissue preparation

Cerebellar sections were cut from fresh frozen normal human tissue (REK, #2013/1503). Heat-induced epitope retrieval was performed prior to immunostaining.

Rat Purkinje neuron cultures

All procedures were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals Norway (FOTS 20135149/20157494/20170001). Embryonic day 18 Wistar Hannover GLAST rat pups were used for neuronal culture preparation. The protocol has recently been described.[20]

Immunochemistry

Fixed OvCar3 cells and cerebellar sections were permeabilized in 0.5% Triton X-100-PBS (Sigma-Aldrich, #11332481001) for 5 min, washed in 0.5% gelatin-PBS (Sigma-Aldrich, #G7041) three times with 15 min each wash, blocked in 10% SEABLOCK (Thermo Fisher Scientific, #37527) in PBS for 30 min, and incubated with primary antibodies overnight at 4 °C. Following incubations, cells and sections were washed in gelatin-PBS, incubated with secondary antibodies for 2 h at room temperature, and mounted using ProLong Diamond with DAPI (Thermo Fisher Scientific, #P36962). The following antibodies were used: rabbit anti-CDR2 (Sigma-Aldrich, #HPA018151), rabbit anti-CDR2L (Protein Technology, #14563-1-AP), mouse anti-rpS6 (Cell Signaling, #2317/Santa Cruz #sc-74459), mouse anti-SON (Santa Cruz, #sc398508), mouse anti-eIF4A3 (Santa Cruz, #sc-365549), mouse anti-SRSF2 (Abcam, #ab11826), Alexa Fluor 488/594-labeled goat anti-human (Thermo Fisher Scientific, #A-11013/#A11014), Alexa Fluor 488/594-labeled goat anti-rabbit (Thermo Fisher Scientific, #R37116/#R37117), rabbit anti-STAR635P (Sigma-Aldrich, #53399-500UG), and Alexa Fluor 488/594-labeled goat anti-mouse (Thermo Fisher Scientific, #R37120/#R37121).

Super-resolution microscopy

A Leica TCS SP8 Stimulated Emission Depletion (STED) 3X confocal microscope equipped with a 100x oil objective with a numerical aperture of 1.4 was used for imaging. The output of the excitation laser (up to 1.5 mW per line; pulsed) was kept between 1% and 20% and the STED laser (775 nm; up to 1.5 W) between 20% and 30%. Gating (between 1 and

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6 ns) was applied for all channels as well as a minimum of three intensity averages. The lateral resolution was consistently measured to be between 40 and 50 nm.

Immunoprecipitation

OvCar3 and HepG2 cells were lysed in RIPA lysis buffer (Bioscience #786-490) containing protease inhibitor cocktail (Sigma-Aldrich #11873580001), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich #P7626), 1 mmol/L sodium fluoride (NaF, Sigma-Aldrich #S6776), and 1 mmol/L sodium orthovanadate (Na_3VO_4, Sigma-Aldrich #450243). The lysate was centrifuged (22,000*g*, 4°C, 15 min) and the supernatant was collected.

Following the Bio-Rad SureBeads immunoprecipitation protocol, the proteins were immunoprecipitated from OvCar3 and HepG2 cell lysates using Protein G Magnetic Beads (Dynabeads, Thermo Fischer Scientific, #1004D). Immunoprecipitated proteins were separated on a 10% TGX gel (Bio-Rad, #456-1035) and transferred to a polyvinylidene difluoride (PVDF) membrane using the Trans-Blot Turbo Transfer kit (Bio-Rad, #170-4274). Western blot analysis was performed to detect proteins of interest using the following primary antibodies: rabbit anti-CDR2L (Proteintech, #14563-1-AP), mouse anti-rpS6 (Santa Cruz #sc-74459), rabbit anti-CDR2 (Sigma-Aldrich, #018151), mouse anti-CDR2 (Santa Cruz, #sc100320) mouse anti-SON, mouse and rabbit anti-eIF4A3 (Abcam, #ab32485). The secondary antibodies used were TidyBlot (Bio-Rad, #STAR209PA) and horseradish peroxidase anti-mouse IgG and anti-rabbit IgG (Dako, #P0260 and #P0217). A negative control consisting of beads and cancer cell lysate was also included.

Proximity ligation assay

The proximity ligation assay was performed using the commercially available Duolink kit from Sigma-Aldrich (#DUO92101). Fixed OvCar3 cells were permeabilized for 5 min using 0.5% Triton X-100 diluted in PBS and blocked with 10% SEABLOCK in PBS. Primary antibodies against Hsp60 (EnCor Biotechnology, #CPCA-HSP60), CDR2 (Sigma-Aldrich, #018151), CDR2L (Proteintech, #14563-1-AP), SON, and SRSF2 were applied for 1 h (1:100 in blocking solution), followed by 3x 5-minute washes with Wash Buffer A supplied with the kit. Probes (+ and −) were diluted in blocking solution (1:5) and added to the cells for 1 h (37 °C). The cells were washed 3x for 5 min each with Wash Buffer A and incubated with ligation buffer (1:5) and ligase enzyme (1:40) for 30 min (37°C). After 2x 5-minute washes with Wash Buffer A amplification buffer (1:5) and the polymerase enzyme (1:80) were diluted in distilled water and applied to the cells for 100 min (37 °C, in the dark), followed by three 10-minute washes with Wash Buffer B (supplied with the kit). Prolong Diamond with DAPI was used to mount the coverslips (overnight, 4 °C). Mounted cells were stored at −20 °C.

Mass spectrometry-based proteomics analysis

Proteins of interest were immunoprecipitated from HepG2 or OvCar3 cell lysates using the antibodies listed in Table 1. A negative control consisting of beads and cancer cell lysate was also included. The samples were loaded on a 10% TGX gel and run approximately 1 cm into the resolving gel. Each lane was cut into cubes of approximately 1 mm^2 and hydrated in Milli-Q water (20 min, room temperature). Detergents (i.e. sodium dodecyl sulfate) and salts were removed by washing the gel in 25 mmol/L ammonium bicarbonate (Sigma-Aldrich, #09830-500G) and 50% acetonitrile (VWR, #34967-2.5L). Cysteine reduction and alkylation were accomplished with a 45-minute incubation in 10 mmol/L dithiothreitol (Amersham Biosciences, #171318-02) at 56

Table 1. Antibody specificities determined by mass spectrometry analysis of CDR2L and CDR2 proteins immunoprecipitated from OvCar3 and HepG2 cell lysates.

Target	Source/Supplier	Cat. no.	AA seq.	Cell line	#Peptides	Interaction
Yo	Yo positive CSF			OvCar3	54	CDR2L
Yo	Yo positive CSF			HepG2	-	-
CDR2L	Sigma- Aldrich	HPA022015	395-464	OvCar3	56	CDR2L
CDR2L	Proteintech	14563-1-AP	116-465	OvCar3	68	CDR2L
CDR2L	Proteintech	66791-1-Ig	116-465	OvCar3	69	CDR2L
CDR2	Sigma-Aldrich	HPA018151	270-392	HepG2	49	CDR2
CDR2	Sigma-Aldrich	HPA023870	112-234	HepG2	41	CDR2
CDR2	Santa Cruz	Sc-100320	296-405	HepG2	57	CDR2
CDR2	LS Bio	C181958	Full length	HepG2	51	CDR2

AA seq., amino acid sequence.

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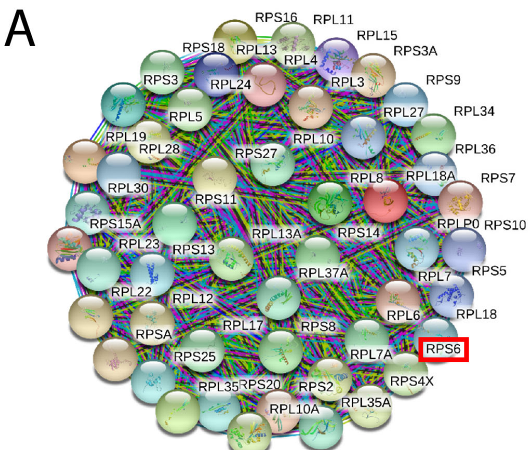
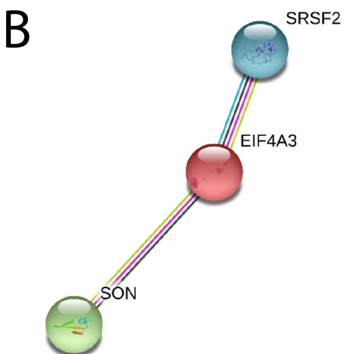
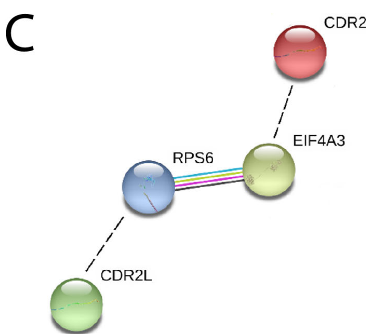


Network statistics for protein-protein interactions	
Nodes	50
Edges	1225
Average node degree	49
Average local clustering coefficient	1
Protein-protein enrichment p-value	$< 1.0 \times 10^{-16}$



Network statistics for protein-protein interactions	
Nodes	3
Edges	2
Average node degree	1.3
Average local clustering coefficient	0.7
Protein-protein enrichment p-value	0.012



Network statistics for protein-protein interactions	
Nodes	2
Edges	1
Average node degree	1
Average local clustering coefficient	1
Protein-protein enrichment p-value	0.168

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Figure 1. Protein-protein interaction networks visualized by STRING. (A) CDR2L was predicted to interact with ribosomal proteins (rpS6, red box). The nodes indicate proteins, and the edges represent protein-protein associations. (B) Protein-protein interaction network of nuclear speckles proteins, SON, eIF4A3, and SRSF2, predicted to interact with CDR2. eIF4A3 (red) directly interacts with SON (light green) and SRSF2 (blue). (C) eIF4A3 (yellow) interacts with rpS6 (blue), indicated by colored edges. Predicted binding partners, CDR2L (green) and CDR2 (red), are manually gated (black, dotted lines). Color-coded edges; light blue: curated databases, dark blue: gene co-occurrence, pink: experimentally determined, green: text mining. Interactions with a medium score of 0.400 or more are shown.

°C followed by a 30-minute incubation in 55 mmol/L iodoacetamide (VWR, #M216-30G) at room temperature in the dark. After washing in 25 mmol/L ammonium bicarbonate and 50% acetonitrile, dried gel pieces were hydrated on ice for 20 min with a minimum volume of 6 ng/µL trypsin (sequencing-grade modified, Promega, #V511A) in digestion buffer (20 mmol/L ammonium bicarbonate, 1 mmol/L calcium chloride (Sigma-Aldrich, #C7902)), then covered with digestion buffer and incubated for 16 h at 37°C. Trypsin activity was quenched by acidification with trifluoracetic acid (VWR, #1.08218.0050), and samples were desalted using StageTip C18 columns (Empore disk-C18, Agilent Life Sciences, #12145004) and the eluted peptides were dried and dissolved in 2% acetonitrile, 1% formic acid (VWR, #84865.260).[21]

About 0.5 µg tryptic peptides were loaded onto an Ultimate 3000 RSLC system (Thermo Fisher Scientific) connected online to a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific) equipped with EASY-spray nano-electrospray ion source (Thermo Fisher Scientific). All samples were loaded and desalted on a pre-column (Acclaim PepMap 100, 2 cm x 75 µm ID nanoViper column, packed with 3 µm C18 beads) at a flow rate of 5 µL/min with 0.1% trifluoracetic acid. Peptides were separated during a biphasic acetonitrile gradient (flow rate of 200 nL/minute) on a 50-cm analytical column (PepMap RSLC, 50 cm x 75 µm ID EASY-spray column, packed with 2 µm C18 beads). Solvent A and B were 0.1% formic acid in water and 100% acetonitrile, respectively. The gradient composition was 5% B during trapping (5 min) followed by 5–7% B over 0.5 min, 7–22% B for the next 59.5 min, 22–35% B over 22 min, and 35–80% B over 5 min. Elution of very hydrophobic peptides and conditioning of the column was performed during a 10-minute isocratic elution with 80% B and 15 min of isocratic conditioning with 5% B, respectively.

Charged peptides were analyzed by the Q-Exactive HF, operating in the data-dependent acquisition mode to automatically switch between full-scan MS and MS/MS acquisition. Mass spectra were acquired in the scan range 375–1500 m/z with a resolution of 60,000 at m/z 200 after an accumulation of 3,000,000 charges (maximum trap time set at 50 ms in the C-trap). The 12 peptides with the most intense signals above an intensity threshold of 50,000 counts and with charge states of 2 to 6 were sequentially isolated and accumulated to 100,000 charges (maximum trap time set at 110 ms) to a target value of 1×10^5 or a maximum trap time of 110 ms in the C-trap with isolation width maintained at 1.6 m/z (offset of 0.3 m/z) before fragmentation in the higher energy collision dissociation cell. Fragmentation was performed with a normalized collision energy of 32%, and fragments were detected in the Q-Exactive at a resolution of 60,000 at m/z 200 with first mass fixed at m/z 110. One MS/MS spectrum of a precursor mass was allowed before dynamic exclusion for 30 seconds with “exclude isotopes” on. Accurate mass measurements in MS mode were accomplished by enabling the lock-mass internal calibration of the polydimethylcyclosiloxane ions generated in the electrospray process from ambient air (m/z 445.12003).[22]

Database searching and criteria for protein identification

Tandem mass spectra data were extracted with Proteome Discoverer (version 2.3.0.523, Thermo Fisher Scientific) and were searched against human, reviewed protein sequences (SwissprotKB database, release 08-2018) with Sequest HT and MS Amanda search engines. The following search criteria were used: carbamidomethylation of cysteine (fixed modification), oxidation of methionine and acetyl of the protein N-terminus (variable modifications), a maximum of two missed trypsin cleavages, 0.02-Da fragment ion mass tolerance, and 10-ppm precursor ion tolerance. Search results from PD were loaded into Scaffold 4 (version 4.9.0, Proteome Software Inc.), and all spectra were searched with the X! Tandem search engine against identified proteins to identify nonspecific trypsin cleavages.

Peptide and protein identifications were filtered to achieve a false discovery rate $< 1.0\%$ (based on searching the reversed human database). Grouping of proteins sharing identical peptides was enabled. In order to evaluate the likelihood of the predicted interactions, the following criteria were established: (1) nonspecific bindings were removed based on the negative control (without primary antibodies); (2) the number of recognized peptides was set to at least two; (3) proteins that were identified by more than one of the antibodies to CDR2L or CDR2 were considered as more likely partners; (4) the likelihood of

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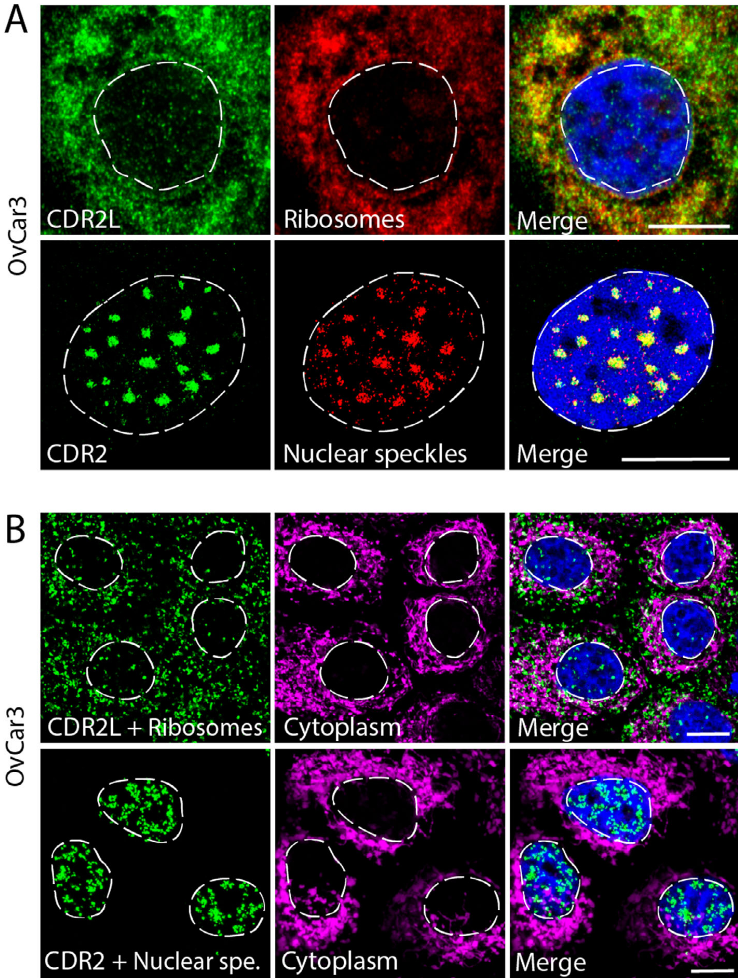

Figure 2. CDR2L co-localizes with ribosomes and CDR2 with nuclear speckles in OvCar3 cells as shown using proximity ligation assay. (A) Upper row: Co-localization of anti-CDR2L (green) and ribosomes (rpS6; red) in the cytoplasm (yellow; merged image). Lower row: Co-localization of anti-CDR2 (green) and nuclear speckles (SRSF2; red) in the nucleus (yellow; merged image). (B) Upper row: Positive Duolink (green) between CDR2L and ribosomes (rpS6) in the cytoplasm (hsp60 in magenta was used to show the extent of the cell cytoplasm; merged image). Lower row: Positive Duolink (green) between CDR2 and nuclear speckle marker (SRSF2) in the nuclei; no co-localization was observed with cytoplasmic marker hsp60 (magenta; merged image). DAPI was used as a marker for the nuclei (blue). Scare bars = 10 μm.

interaction was evaluated based on the predicted cellular location of each protein of interest. Protein-protein interactions were analyzed using the STRING database. STRING implements all publicly available sources of known and predicted protein-protein associations, together with computational analysis to evaluate potential connectivity networks.[23,24]

Results

Antibody specificity

To evaluate antibody specificity, we immunoprecipitated CDR2L and CDR2 from cancer cell lysates and analyzed the precipitates using mass spectrometry-based

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proteomics with the antibodies listed in Table 1. We found that the commercial antibodies raised against CDR2L and CDR2 were specific and recognized the expected antigens. Also, we confirmed our previous data showing that CDR2L is the major Yo antibody target. Analysis of lysates of OvCar3 cells, which expresses both CDR2L and CDR2, immunoprecipitated with Yo antibodies bound to magnetic beads showed that CDR2L, but not CDR2, was recognized by Yo antibodies. In similar experiments performed with a cell line that only expresses CDR2, HepG2 cells, Yo antibody did not precipitate CDR2.

CDR2L and CDR2 interaction partners identified by mass spectrometry analysis

Potential protein interaction partners were identified using mass spectrometry analysis of proteins immunoprecipitated with anti-CDR2L and anti-CDR2 antibodies from cancer cell lysates. Initially, several hundred hits were detected, and four criteria were established to determine the likelihood of the predicted interactions. Thereafter, we used the STRING database to evaluate the connectivity of the proteins that met our criteria. CDR2L was predicted to interact with 50 ribosomal proteins that were tightly connected (Fig. 1A). Of these 50 ribosomal proteins, 20 belong to the 40S subunit, and 30 belong to the 60S subunit. Proteins known to associate with nuclear speckles, eukaryotic initiation factor eIF4A3, SON, and the serine/arginine-rich splicing factor SRSF2, were identified as potential interaction partners of CDR2. According to the STRING analysis eIF4A3 interacts with SON and SRSF2 (Fig. 1B), as well as with the 40S ribosomal subunit factor rpS6 (Fig. 1C).

CDR2L Co-localizes with ribosomal proteins and CDR2 with nuclear speckle proteins in ovarian cancer cells

We used immunolabeling and proximity ligation assay to investigate the subcellular localization of CDR2L and CDR2. In OvCar3 cells, which express both CDR2L and CDR2, we found that CDR2L co-localizes with rpS6, whereas CDR2 co-localizes with nuclear speckle proteins SON, eIF4A3, and SRSF2 (Fig. 2A). These results were confirmed by proximity ligation assay in OvCar3 cells (Fig. 2B).

Co-Immunoprecipitation of CDR2L and CDR2 from OvCar3 cells confirms protein-protein interactions with ribosomal and nuclear speckle proteins

To analyze whether CDR2L directly interacts with rpS6, we performed co-immunoprecipitation assays from OvCar3 cell lysates. CDR2L specifically co-immunoprecipitated with rpS6, indicating that endogenous CDR2L forms a complex with rpS6 in cancer cells (Fig. 3A). Furthermore, we found that SON and eIF4A3 co-immunoprecipitated with CDR2 from HepG2 cells, thus indicating a strong and stable interaction between these proteins and CDR2 (Fig. 3B).

Co-localizations of CDR2L with ribosomal proteins and of CDR2 with nuclear speckle proteins occurs in Purkinje neurons in Human cerebellum sections and in Purkinje neuron cultures

In human cerebellum sections, CDR2L and Yo antibodies stained the cytoplasm in regions that overlapped with

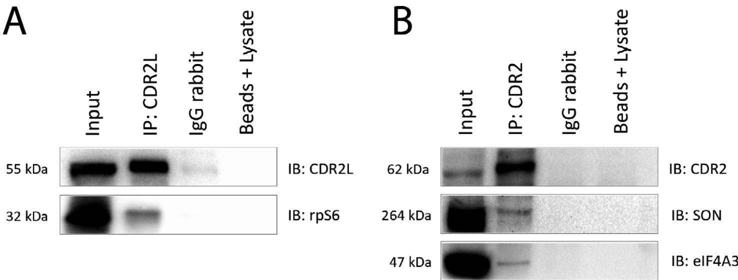

Figure 3. CDR2L co-immunoprecipitates with ribosomal protein rpS6, whereas CDR2 co-immunoprecipitates with nuclear speckle proteins SON and eIF4A3 in cancer cell lysates. (A) Immunoblot demonstrating the co-immunoprecipitation of CDR2L and rpS6 from OvCar3 cell lysates. (B) Immunoblot demonstrating the co-immunoprecipitation of CDR2, SON, and eIF4A3 from HepG2 cell lysates. Input = cancer cell lysates (OvCar3 or HepG2). Beads + lysate = samples that were not treated with primary antibody, and served as negative controls.

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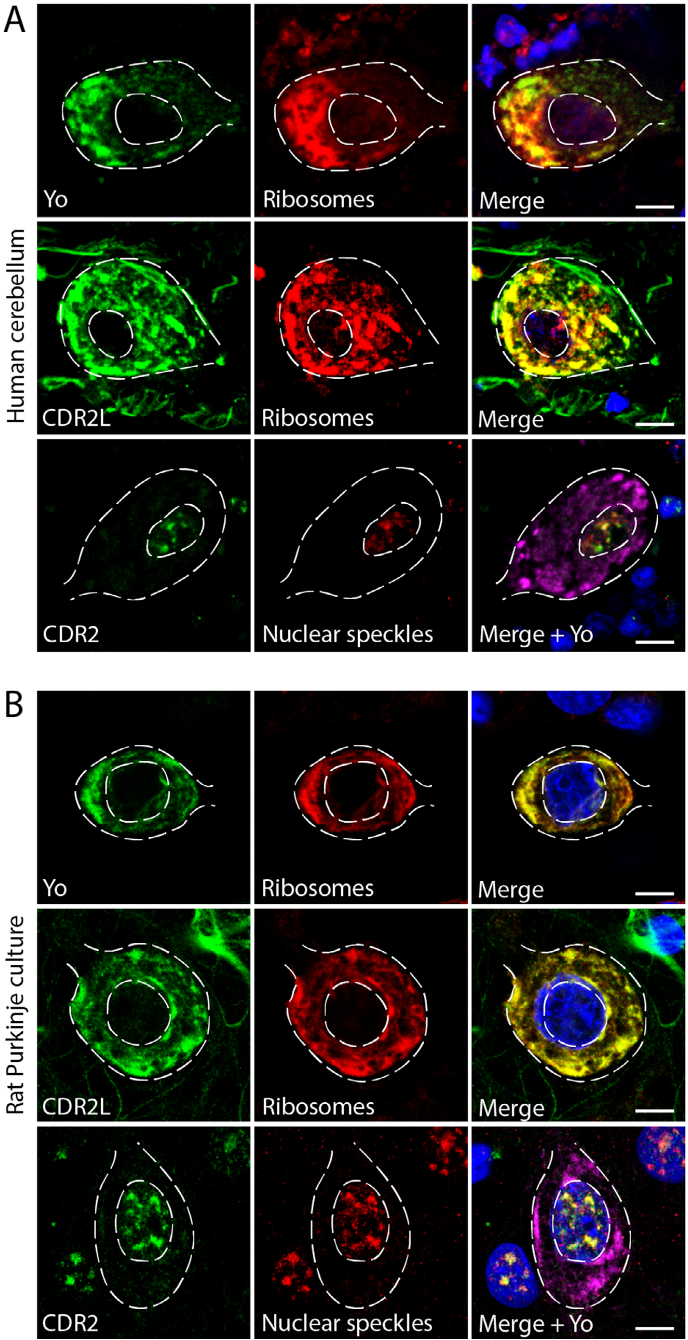

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Figure 4. CDR2L and Yo co-localize with ribosomal proteins and CDR2 co-localizes with nuclear speckle proteins in cerebellar Purkinje neurons as shown by super-resolution microscopy. (A) Upper row: Human cerebellar section stained with Yo-CSF (green) and anti-rpS6 (red); the proteins co-localize in the cytoplasm (yellow; merged image). Middle row: Human cerebellar section stained with anti-CDR2L (green) and ribosomal marker anti-rpS6 (red); the proteins co-localize in the cytoplasm (yellow; merged image). Lower row: Human cerebellar section stained with anti-CDR2 (green) and nuclear speckle marker anti-SRSF2 (red); the proteins co-localize in the nucleus. No co-localization was found with anti-Yo (magenta; merged image). (B) Upper row: Rat Purkinje neuron cultures stained with anti-Yo (CSF; green) and rpS6 (ribosomes; red); co-localization was observed in the cytoplasm (yellow; merged image). Middle row: Rat Purkinje neuron cultures stained with anti-CDR2L (green) and anti-rpS6 (red); co-localization was observed in the cytoplasm (yellow; merge image). Lower row: Rat Purkinje neurons stained with anti-CDR2 (green), nuclear speckle protein (red), and anti-Yo (magenta). CDR2 and the nuclear speckle protein co-localize in the cell nucleus (yellow; merged image), whereas Yo does not. Scale bars = 10 μm.

regions stained for the ribosomal marker rpS6, whereas CDR2 showed nuclear staining that overlapped with nuclear speckle markers (eIF4A3, SON, and SRSF2; Fig. 4A). These results were replicated in cultured rat Purkinje neurons (Fig. 4B).

Discussion

The pathogenesis of Yo-mediated PCD remains incompletely understood, but it has been postulated that the Purkinje neuron loss is due to auto-reactive T cells and a direct damaging effect of Yo antibodies.[3,4,6,25] We demonstrated previously that CDR2L, not CDR2, is the major target of the Yo antibody[7]: Yo antibodies bind both endogenous and recombinant CDR2L, but only recombinant CDR2, not the native form. In this study, we confirmed the CDR2L specificity of Yo antibodies by mass spectrometry-based proteomics and showed that while CDR2L and CDR2 have differing localizations, it is possible to link their putative roles to ribosomal function.

The biological functions and precise subcellular localization of both CDR2L and CDR2 have been unresolved questions. Analysis of PCD patient sera has shown that Yo antibodies localize to the cytoplasm and associate with both membrane-bound and free ribosomes.[26,27] In these studies, the Yo antigen is referred to as "CDR2." However, based on our recent findings, we are confident that the main Yo antigen is indeed CDR2L. Here, we used available antibodies against CDR2L and CDR2, as well as anti-Yo, to characterize the cellular localization of these proteins and their potential binding partners.

Immunolabeling cells with commercially available anti-CDR2 antibodies result in various expression patterns, localizing CDR2 to both the cytoplasm and the nucleus.[7,13] Therefore, we first evaluated the specificity of the available CDR2L and CDR2 antibodies produced to recognize the full-length protein or shorter sequences. Immunoprecipitation followed by mass spectrometry analysis confirmed antibody specificity. The previously reported inconsistent results for CDR2 may either stem from the antibody recognition of one of the four CDR2 isoforms (www.uniprot.org) or from the translocation of CDR2 between the cytoplasm and nucleus. Furthermore, previous studies also identified PKN, MRG15, and MRGX as CDR2 binding partners. Since these proteins function both in the cytoplasm and nucleus, this raises the possibility that CDR2 might facilitate the transport of these proteins or translocate itself.[15,18,19,28] In addition, no CDR2L-CDR2 cross-talk was observed, which supports our finding that there is no cross-talk between CDR2L and CDR2 in their native forms. Furthermore, our immunoprecipitation-mass spectrometry results showed that Yo antibodies only precipitated CDR2L and not CDR2 from cancer cells. This is in line with recent work, which shows that Yo antibodies bind to the CDR2L regions of least homology with CDR2.[29]

In addition to confirming antibody specificity, the mass spectrometry analysis revealed potential interacting partners for CDR2L and CDR2. A number of ribosomal proteins, including rpS6, were identified as potential CDR2L binding partners. The most prominent CDR2 binding partners were three nuclear speckle proteins: SON, eIF4A3, and SRSF2. Next, we used super-resolution microscopy and proximity ligation assay to evaluate co-localization within a 40-nm range in cancer cells and Purkinje neurons. CDR2L was found to co-localize with rpS6, whereas CDR2 co-localized with nuclear speckle proteins eIF4A3, SON, and SRSF2. Co-immunoprecipitation analyses established that CDR2L directly interacts with rpS6 and that CDR2 directly interacts with eIF4A3 and SON.

Nuclear speckles are self-assembled organelles consisting of around 200 proteins involved in pre-mRNA processing including splicing, surveillance, and RNA export.[30] The speckles can vary in size and morphology within a single cell, but have been shown to be non-random organizations of proteins and RNAs stabilized by favorable intermolecular interactions.[30] SRSF2 and SON localize to the core region of the speckle; both proteins have domains enriched with arginine and serine repeats that are crucial for speckle core formation.[30,31] Both proteins are also involved in mRNA splicing[32,33] and interact with the ATP-dependent RNA helicase eIF4A3.[34] It has been suggested that eIF4A3 may provide a link between

splicing and translation in the cytoplasm through its connection to rpS6[34,35], which co-localizes with CDR2L.

Translation in eukaryotes relies on the assembly of the small (40S) and the large (60S) ribosomal subunit into the 80S ribosomes.[36] Each subunit is composed of ribosomal proteins and RNAs that work together to catalyze protein synthesis using mRNA as a template.[37,38] Ribosomal proteins often undergo post-translational modifications and rps6, the identified CDR2L binding partner, is regulated by phosphorylation.[39,40] Five phosphorylation sites have been identified and these phosphorylation events could participate in regulating the translation of specific subclasses of mRNA, synaptic plasticity and behavior.[41] Thus, rpS6 phosphorylation is often used to track neuronal activity.[40,41]

Our findings linking CDR2 to nuclear speckles and CDR2L to ribosomes allow us to speculate that these two proteins may participate in a common pathway (Fig. 5). First, we show that CDR2 interacts with eIF4A3 in the nucleus. Second, eIF4A3, along with other initiation factors, facilitates mRNA binding to ribosomes.[42] Furthermore, eIF4A3 and rpS6 have been shown to interact based on affinity-capture mass spectrometry analysis.[35] Third, we show that CDR2L interacts with the ribosomes through rpS6. These findings place CDR2 and CDR2L in the process of protein translation, one involved in mRNA maturation and the other directly with the synthesis of proteins.

Ensuring proper protein homeostasis is crucial to the cell.[36,38] We show that Yo antibodies specifically bind to

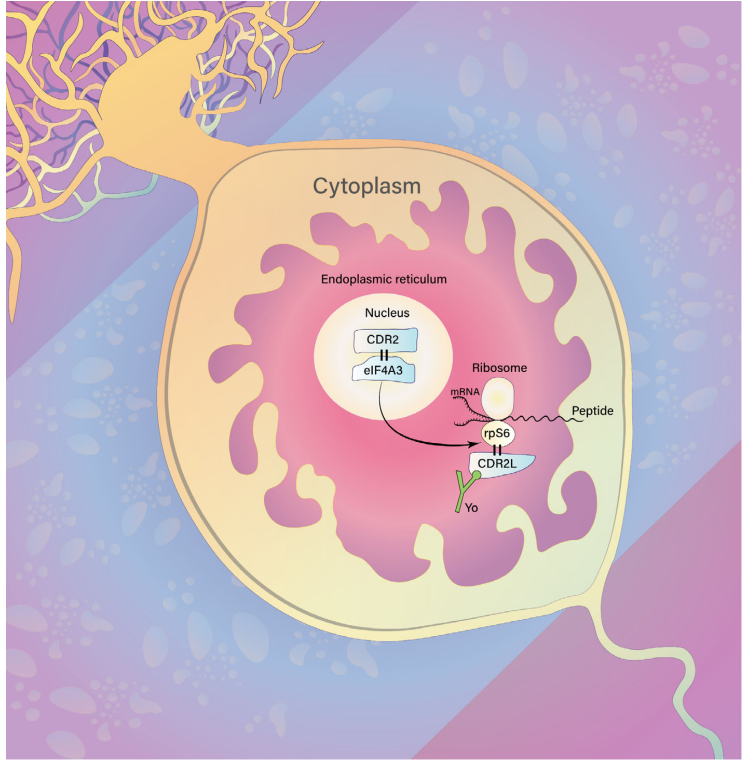

Figure 5. Hypothesis of CDR2L and CDR2 involvement in protein synthesis in Purkinje neurons. CDR2 localizes to the nucleus and directly interacts with nuclear speckle protein eIF4A3. eIF4A3, in conjugation with other cytoplasmic initiation factors, facilitates mRNA binding to the 40S ribosomal subunit. This event is important for mRNA maturation and translation, ultimately resulting in the synthesis of new proteins. CDR2L interacts with ribosomal subunit protein rpS6; therefore, we propose that CDR2L and CDR2 are both involved in the process of protein synthesis. Furthermore, Yo antibody (green) binding to CDR2L in Purkinje neurons of PCD patients may, therefore, interfere with the function of the ribosomal machinery, resulting in disrupted mRNA translation and/or protein synthesis.

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CDR2L in Purkinje neurons of PCD patients where they potentially interfere with the function of the ribosomal machinery resulting in disrupted mRNA translation and/or protein synthesis. Taken together, our findings that CDR2L interacts with ribosomal proteins and CDR2 with nuclear speckle proteins is an important step toward understanding PCD pathogenesis. Future studies are needed to track the subcellular events in real-time with the aim of addressing the dynamic interaction between the CDR2L and CDR2 molecules. This will be vital to understand whether there is a functional relationship between CDR2L and CDR2 in the Purkinje neuron deterioration that occurs in PCD.

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Author Contributions

All authors contributed to the conception and design of the study; I.H. and T.K. performed the acquisition, data analysis, and prepared figures; I.H., T.K., M.S., and C.V. drafted the manuscript.

Conflicts of Interest

The authors have no conflicts of interest to report.

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ARTICLE OPEN ACCESS CLASS OF EVIDENCE

Paraneoplastic Cerebellar Degeneration

The Importance of Including CDR2L as a Diagnostic Marker

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Abstract

Objective
Investigate the value of including cerebellar degeneration-related protein 2-like (CDR2L) as a marker in commercial diagnostic tests for anti-Yo–associated paraneoplastic cerebellar degeneration (PCD).

Methods
We included sera and CSF samples from 24 patients with suspected PCD (6 of whom had PCD with underlying gynecologic or breast cancer), who were positive for Yo antibodies using the commercially available, paraneoplastic neurologic syndromes (PNS) 14 Line Assay from Ravo Diagnostika. The samples were further evaluated using the EUROLINE PNS 12 Ag Line Assay and a cell-based assay (CBA) from Euroimmun. For confirmation of positive lineblot results, we used indirect immunofluorescence of rat cerebellar sections. We also tested all samples in 2 assays developed in-house: a CBA for CDR2L and a Western blot analysis using recombinant cerebellar degeneration-related protein 2 (CDR2) and CDR2L proteins.

Results
In PNS 14 and PNS 12 Ag Line Assays, anti-CDR2 reactivity was observed for 24 (100%) and 20 (83%) of the 24 samples, respectively. Thirteen of 24 subjects (54%) were also positive using the Euroimmun CBA. Rat cerebellar immunofluorescence was the best confirmatory test. In our in-house CBA for CDR2L and Western blot for CDR2 and CDR2L, only the 6 patients with confirmed PCD reacted with CDR2L.

Conclusions
Commercially available tests for Yo antibody detection have low specificity for PCD because these assays use CDR2 as antigen. By adding a test for CDR2L, which is the major Yo antigen, the accuracy of PCD diagnosis greatly improved.

Classification of Evidence
This study provides Class III evidence that a CBA for CDR2L accurately identifies patients with PCD.

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Glossary

BSA = bovine serum albumin; **CBA** = cell-based assay; **CDR2** = cerebellar degeneration-related protein 2; **CDR2L** = cerebellar degeneration-related protein 2-like; **HEK293** = human embryonic kidney 293; **PCD** = paraneoplastic cerebellar degeneration; **PFA** = paraformaldehyde; **PNS** = paraneoplastic neurologic syndromes; **SDS** = sodium dodecyl sulfate.

Paraneoplastic neurologic syndromes (PNS) are rare, immune-mediated diseases triggered by cancer that differ in clinical features, prognosis, and associated onconeural antibodies.[1–4] Paraneoplastic cerebellar degeneration (PCD) is one of the most common of these syndromes, observed in individuals with gynecologic or breast cancer. These patients usually have Yo antibodies in serum and CSF.[5–7] Evidence suggests that anti-Yo targets 2 intracellular antigens, cerebellar degeneration-related protein 2 (CDR2) and CDR2-like (CDR2L), expressed in the nucleus and cytoplasm of Purkinje neurons in the cerebellum, respectively.[8–10] The interaction between anti-Yo and CDR proteins is believed to mediate Purkinje neuron dysfunction and death, leaving the patients in a severely disabled state.[11,12]

Onconeural antibodies identified in the sera or CSF of patients are key diagnostic biomarkers for PCD.[3] Commercial line assays are available, but the diagnostic value of these tests has been questioned.[1,13] The specificity of anti-Yo is low, with less than 10% confirmation rate.[1] Because anti-Yo is associated with most PCD cases related to gynecologic and breast cancer, improved diagnostic tests for anti-Yo is important to ensure a correct clinical diagnosis and prevent unnecessary tests or inappropriate treatment.

Our recent studies have suggested that CDR2L, which shares 50% sequence homology with CDR2, is likely the main target of anti-Yo.[12] We postulate that the low specificity for detecting Yo antibodies seen with commercial immunoassays is that they use CDR2 as the Yo-antigen. Here, we assessed the value of including CDR2L as a diagnostic marker to increase the specificity for Yo antibody detection.

Methods

Patients

We performed a retrospective analysis of 9,527 sera and CSF samples from patients screened for onconeural antibodies at the Neurological Research Laboratory, Haukeland University Hospital, Bergen, from 2017 to 2020. We included the 24 patients with serum and/or CSF Yo reactive bands detected by the commercial PNS 14 Line Assay from Ravo Diagnostika (Freiburg im Breisgau, Germany) in the study. Positive samples were also tested with EUROLINE PNS 12 Ag and cell-based assay (CBA) from Euroimmun (Lübeck, Germany) and were further explored by indirect immunofluorescence of rat cerebellar section. Samples were also tested in 2 assays for CDR2L developed in-house.

Standard Protocol Approvals, Registration, and Patient Consents

Patient records for the 24 included cases were obtained and anonymized before the study. PCD was diagnosed according to the established criteria.[2] The study was approved by The Regional Committee for Health and Medical Research Ethics in Norway, REK #123524.

Commercial Line Immunoassays for Anti-CDR2 Detection

The PNS 14 Line Assay (Ravo Diagnostika, #PNS14-003) includes 14 different antigens for PNS: GAD65, HuD, Yo, Ri, CV2/CRMP5, amphiphysin, Ma1, Ma2, SOX1, Tr/DNER, Zic4, titin, recoverin, and Protein Kinase C γ. The EUROLINE PNS 12 Ag (Euroimmun, #DL1111-1601-7-G) includes 12 different antigens for PNS: amphiphysin, CV2/CRMP5, Ma2, Ri, Yo, Hu, recoverin, SOX1, titin, Zic4, GAD65, and Tr/DNER. Serum and CSF samples from 24 patients were analyzed in both immunoassays following the manufacturer's instructions. Two independent investigators graded band intensities from + to +++, compared to a positive control sample (+++).

The serum and CSF from the 24 patients were also tested for anti-Yo using a commercial CBA (Purkinje Cell Mosaic 1; Euroimmun, #FA1113-1005-1) consisting of BIOCHIP Mosaics with 4 positions (Yo/CDR2-, Tr/DNER-, ITPR1-, and CARP-transfected human embryonic kidney 293 [HEK293] cells), positive and negative controls. Briefly, aliquots of 30 µL serum (diluted 1:100) or of CSF (diluted 1:1) were applied to each reaction field on the BIOCHIP slide. After incubation (30 minutes, room temperature), the slide was washed with phosphate-buffered saline containing 0.2% Tween 20 (PBS-Tween 20; 5 minutes, room temperature), followed by incubation with goat anti-human IgG secondary antibody conjugated to Alexa Fluor 488 (Thermo Fisher Scientific, Waltham, MA, 1:500, #A-11013, 30 minutes, room temperature). The slide was rinsed with PBS-Tween 20 and mounted on a glass coverslip. The cutoff for Yo/CDR2 was set to 1:100, as advised by the manufacturer. Two independent investigators evaluated the results.

Indirect Immunofluorescence

All procedures were performed according to the NIH Guidelines for the Care and Use of Laboratory Animals Norway (FOTS 20135149/20157494/20170001). Wistar Hannover GLAST rats were anesthetized and transcardially perfused with ice-cold 4% paraformaldehyde (PFA) in PBS. The brains were postfixed (24 hours, 4°C), incubated with 18% sucrose in PBS (72 hours, 4°C), snap-frozen, and cut on

Table Results of Commercial Line Immunoassays, Confirmatory Tests, Clinical Presentation, and Potential Cancer Found in 24 Patients Tested for Anti-Yo

Patient	F/M	Age	Ravo[a]	EUROLINE[a]	CBA CDR2	IIF	CBA CDR2L	WB (CDR2L/CDR2)	Clinical presentation	Cancer
1	F	37	Yo +++	Yo +++	+	+	+	+/+	PCD	Breast
2	F	63	Yo +++	Yo +++	+	+	+	+/+	PCD	Uterine
3	F	63	Yo +++	Yo +++	+	+	+	+/+	PCD	Uterine
4	F	74	Yo +++	Yo +++	+	+	+	+/+	PCD	Ovary
5[b]	F	77	Yo +++	Yo +++	+	+	+	+/+	PCD	Uterine
6	F	73	Yo +++	Yo +++	+	+	+	+/+	PCD	Ovary
7[c]	F	66	Yo +++	Yo +++	+	+	−	+/+	Headache	Breast
8	M	61	Yo ++	—	+	+	−	−/+	Polyneuropathy	Lung
9	F	20	Yo ++	Yo ++	+	−	−	−/+	Myalgia	No
10	F	34	Yo +	Yo ++	+	−	−	−/+	Myalgia	No
11	M	53	Yo ++	Yo +++	+	−	−	−/+	Polyneuropathy	No
12	M	47	Yo ++	Yo +++	+	−	−	−/+	Confusion	No
13	M	36	Yo +	Yo ++	+	−	−	−/+	Psychosis	No
14	F	70	Yo ++	Yo ++	−	−	−	−/+	Neuropathy	No
15	M	47	Yo +	Yo +	−	−	−	−/−	Epilepsy	No
16	M	71	Yo ++	Yo ++	−	−	−	−/+	Neuropathy	No
17	F	54	Yo +	Yo ++	−	−	−	−/−	Myalgia	No
18	F	69	Yo +	−	−	−	−	−/−	Encephalopathy	No
19	M	35	Yo +	Yo +	−	−	−	−/−	Encephalopathy	No
20	F	73	Yo +	−	−	−	−	−/−	Encephalopathy	No
21	M	31	Yo +	Yo ++	−	−	−	−/−	Psychosis	No
22	M	74	Yo +	−	−	−	−	−/−	Myalgia	No
23	F	42	Yo +	Yo +	−	−	−	−/−	Paresthesia	No
24	M	71	Yo +	Yo +	−	−	−	−/−	Diplopia	No

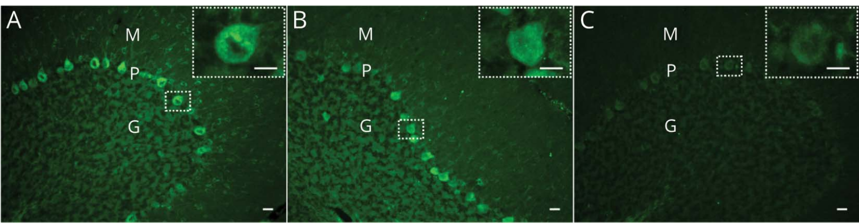
Abbreviations: CBA = cell-based assay; CDR2 = cerebellar degeneration-related protein 2; CDR2L = cerebellar degeneration-related protein 2-like; IIF = indirect immunofluorescence; PCD = paraneoplastic cerebellar degeneration; WB = Western blot analysis.
[a] Lineblots were graded by trained investigators as − (negative), + (weak positive), ++ (moderate positive), or +++ (strong positive) depending on the band intensity.
[b] All samples are sera except from patient 5, which was the CSF.
[c] All cancers were detected within 2 years of sampling except for patient 7 where cancer was diagnosed 19 years previously.

a cryostat to 10-μm parasagittal sections.[14] Heat-induced antigen retrieval was performed with a pressure cooker in Diva Decloaker buffer solution (Biocare Medical, Pacheco, CA; #DV2004MX). Sections were blocked in bovine serum albumin (BSA) and Triton X-100 in PBS (2 hours, room temperature), followed by incubation with patient samples (1:500 in blocking solution, overnight, 4°C). Finally, the sections were rinsed with PBS, incubated with secondary antibody (Alexa Fluor 488 goat anti-human IgG, 1:100, 90 minutes, room temperature), rinsed, and mounted with ProLong Diamond Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, #P36962).[8]

CBA for Anti-CDR2L Detection

HEK293 cells were cultured in 8-well Nunc Lab-Tec II Chamber Slide System (Thermo Fisher Scientific, #154534) in Eagle Minimum Essential Medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (37°C, 5% CO_2). Cells were transfected with a plasmid for expression of Myc-DDK-tagged CDR2L (Origene, Rockville, MD; #RC206909) using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA; #L3000008). At 48 hours after transfection, coverslips were washed with PBS and fixed with 4% PFA/4% glucose in PBS (20 minutes, room temperature). Demembranation with 0.1% triton X-100 in PBS (7 minutes, room temperature) was followed by blocking with

Figure 1 Representative Images of Rat Cerebellar Sections Incubated With Patient Samples



(A) Sera from confirmed paraneoplastic cerebellar degeneration (PCD) cases (cerebellar degeneration-related protein 2-like [CDR2L]+/cerebellar degeneration-related protein 2 [CDR2]+, patients 1–6) show granular, cytoplasmic staining of Purkinje neurons. (B) Sera from the 2 cases without PCD but with previously detected cancer (CDR2L−/CDR2+, patients 7 and 8) stain the cytoplasm of Purkinje neurons, but no granular staining is observed. (C) Sera from the remaining cases without PCD and without cancer (CDR2L−/CDR2+, patient 9–13) do not stain Purkinje neurons of rat cerebellar sections. CDR2L/CDR2 testing is based on line blots and cell-based assays. G = granular layer; M = molecular layer; P = Purkinje neuron layer. Scale bar = 20 μm; zoom in scale bar = 10 μm.

10% Sea Block blocking buffer (Thermo Fisher Scientific, #37527) in PBS (1 hour, room temperature). Coverslips were incubated with the serum (1:10 and 1:100) or CSF (1:10 and 1:100), mouse anti-DYKDDDDK tag or FLAG tag (DDK) (Origene, #TA50011-100, 1:1,000), anti-CDR2L (Protein Technology, Pencroft Way, Manchester, UK; #14563-1-AP), or anti-CDR2 (Sigma-Aldrich, St. Louis, MO; #HPA023870) in blocking solution (1 hour, room temperature). Finally, coverslips were washed with PBS, incubated with secondary antibody (Alexa Fluor 488 goat anti-human, Alexa Fluor 488 goat anti-rabbit, or Alexa Fluor 594 goat-anti-mouse, Thermo Fisher Scientific, #A-11013, #A-11008, #A-11005, respectively, 1 hour, room temperature) and mounted using ProLong Diamond Antifade Mountant with DAPI.

Western Blot for Anti-CDR2 and Anti-CDR2L Detection

The transcription/translation-coupled reticulocyte lysate system (Promega, Madison, WI; #L4610) was used for cell-free protein expression of CDR2L and CDR2. Purified plasmids encoding the 2 proteins (2.0 μg; Origene, RC204900 [CDR2] and #RC206909 [CDR2L]) were incubated with the transcription/translation lysate, T7 RNA polymerase promoter, reaction buffer, recombinant RNasin ribonuclease inhibitor (Promega, #N2511), and amino acid mixture (30°C, 1.5 hours). A negative control without plasmid was included in each experiment. The reaction products were evaluated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, followed by Western blot analysis.

The reticulocyte extract was denatured in Laemmli buffer (Bio-Rad, Hercules, CA; #1610747, 95°C, 5 minutes) and 2.5% β-mercaptoethanol subjected to SDS-polyacrylamide gel electrophoresis separation on a 10% TGX gel (Bio-Rad, #456-1035) and transferred to a polyvinylidene difluoride membrane using the Trans-Blot Turbo Transfer kit (Bio-Rad, #170-4274). The blots were blocked in 5% dry milk (Bio-Rad, #170-6404) dissolved in 1x Tris-buffered saline with 0.1% Tween 20 (TBS-Tween 20) and incubated with serum or CSF sample diluted in 3% BSA in TBS-Tween 20 (1:250/1:100, 4°C, overnight). Antibody fixation was visualized using horseradish peroxidase anti-human IgG (Dako, Carpinteria, CA; #P0214, 1 hour, room temperature).

Imaging

Rat cerebellar sections and CBAs were imaged on a Leica Leitz DM RBE fluorescence microscope with CoolLED pE-300-W LED illumination. Images were evaluated by 2 independent investigators. ImageJ was used for background subtraction of microscopy images and evaluation of Western blot results.

Data Availability

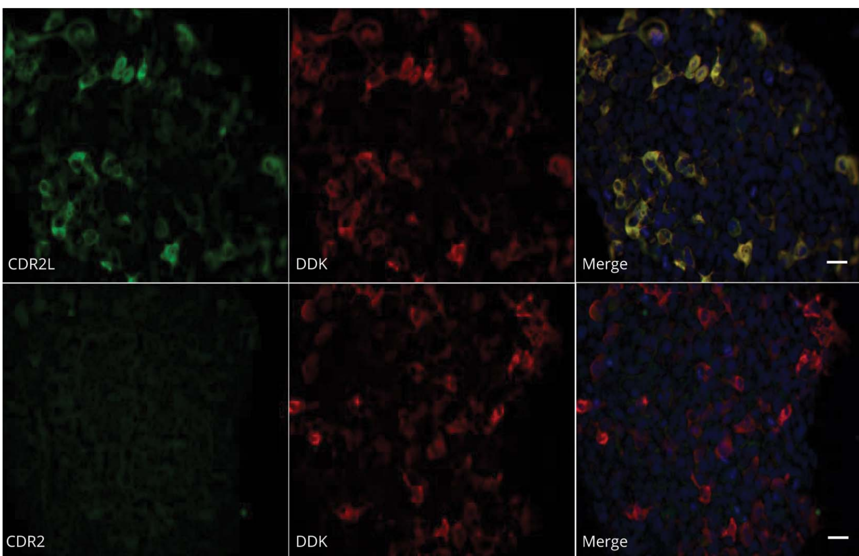
Data related to the current article are available from the corresponding authors on reasonable request.

Results

Between 2017 and 2020, 24 of 9,527 tested serum or CSF samples (0.25%) from patients with suspected PNS showed a reactive band for Yo antibodies using the PNS 14 Line Assay from Ravo Diagnostika and 20 (83%) showed a reactive band using EUROLINE PNS 12 Ag from Euroimmun (table). Thirteen of the 24 patients (54%) had a confirmed positive CBA CDR2 assay, whereas only 8 stained Purkinje cells in the immunofluorescence assay (table).

Using staining of rat cerebellar sections as a confirmatory test, we found that Yo positive sera from 6 PCD patients showed granular, cytoplasmic staining in Purkinje neurons (figure 1A). In the group of 18 nonconfirmed PCD cases, serum samples from 2 patients (7 and 8) stained Purkinje neurons but with no granular cytoplasmic staining; these patients were therefore interpreted as anti-Yo negative (figure 1B). The remaining 16 serum samples were negative (figure 1C). The commercial line immunoassays alone yielded a high number of false positive results (18/24 [75%] for the Ravo assay and 16/24 [67%] for the Euroimmun assay). Even when combined with the CBA CDR2 (Euroimmun), the false positive rate was high at 7 of 24 (29%). The best-established method for Yo antibody confirmation was careful interpretation of

Figure 2 No Cross-Reactivity Is Observed Between CDR2 Antibodies and CDR2L in Human Embryonic Kidney 293 Cells That Express Myc-DDK-Tagged CDR2L



Upper row: cells stained with anti-CDR2L (green) and anti-DDK (red). Lower row: cells stained with anti-CDR2 (no reaction), and anti-DDK (red). Nuclei are stained with DAPI. Scale bar = 20 μm. CDR2 = cerebellar degeneration-related protein 2; CDR2L = cerebellar degeneration-related protein 2-like.

immunofluorescent staining of Purkinje neurons in rat cerebellar sections.

Because we recently showed that the major target for Yo-antibodies is not CDR2, but CDR2L,[12] we developed an assay based on HEK293 cells transfected with a plasmid for expression of Myc-DDK-tagged CDR2L and stained these cells with patient sera or CSF. To evaluate the specificity of our in-house CDR2L CBA, HEK293 cells that express Myc-DDK-tagged CDR2L were stained with anti-DDK, anti-CDR2L, or anti-CDR2 (figure 2). There was complete overlap between CDR2L and DDK cytoplasmic staining. The absence of CDR2 antibody staining confirmed that there was no cross-reactivity between CDR2 and CDR2L antibodies.

Samples from the 6 confirmed PCD cases stained both CDR2L-transfected cells and commercial CBA for CDR2 (figure 3, A.a and A.b). However, the samples from the 7 patients with CBA CDR2-positive staining, but no PCD, did not show CBA staining for CDR2L (figure 3, B.a and B.b). These results were confirmed by Western blot analysis of recombinant CDR2 (62 kDa) and CDR2L (55 kDa) proteins (figure 3, A.c and B.c) with the exception of the sample from one patient without PCD (patient7) who tested positive for CDR2L in Western blot but not in CBA. We also found that Western blot analysis with CDR2 was negative for 9 and 6 patients who were weak-to-moderate positive on the Ravo and Euroimmun assays, respectively (table).

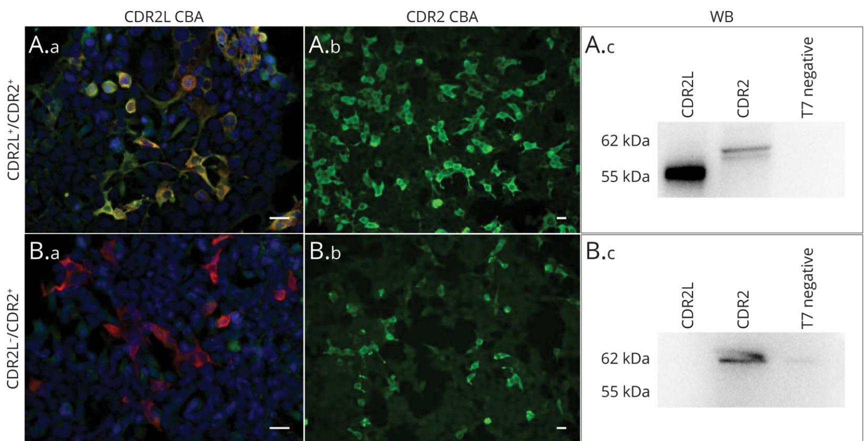
Discussion

Commercial line immunoassays enable simple and rapid detection of onconeural antibodies in patients with suspected PNS. In this study, we evaluated the diagnostic accuracy of Yo antibody testing by commercial line immunoassays and routine confirmatory tests. We found an approximate 70% false positivity rate using commercial assays alone, which is in line with recent studies.[1,13] The discrepancy between the 2 commercial assays is most likely related to the nature of the antigens: Both use recombinant CDR2 proteins, but the sequence length, and therefore protein structure, and the cell lines in which the recombinant CDR2 is produced differ. Band intensities were graded from + to +++ compared to a positive control. Overall, we observed that samples with intense reactive bands on the line immunoassays were more likely to be from patients with PCD than those with weaker reactive bands, as was also reported recently.[13]

The number of false positive tests for PCD was reduced by combining the results from the 2 line immunoassays with a CBA for CDR2. In agreement with another study,[1] we found several men among the CDR2-positive but PCD-negative patients, supporting the hypothesis that CDR2 is not the natural Yo antigen.

In our hands, the best confirmatory test among the established techniques were rat cerebellar immunofluorescence.

Figure 3 Representative Images of Patient Sera (1:100) Double Positive for CDR2L and CDR2 (A.a–A.c), and Single Positive for CDR2 (B.a–B.c) in Indirect Immunofluorescence of CDR2L Transfected Human Embryonic Kidney 293 Cells (A.a, B.a), Commercial CBA for CDR2 (A.b, B.b), and WB (A.c, B.c)



A negative control containing reticulocyte lysate without recombinant protein was included in each experiment. Anti-CDR2/CDR2L, green; anti-DDK, red; merge, yellow. Scale bar = 20 μm. CBA = cell-based assay; CDR2 = cerebellar degeneration-related protein 2; CDR2L = cerebellar degeneration-related protein 2-like; WB = Western blot analysis.

However, many clinical laboratories are not equipped to perform indirect immunofluorescence assays, and Purkinje cell staining can be difficult to interpret because only granular cytoplasmic staining is characteristic of anti-Yo.[8,12] This pattern probably represents ribosomal staining because it has been shown recently that CDR2L interacts with the ribosomal subunit protein rpS6.[10] Sera from 2 of our patients without PCD, but with previous cancer, also stained the rat Purkinje cell cytoplasm, but the cytoplasmic staining was not granular. The specific target for this staining is unknown, but such false positive anti-Yo staining must be interpreted with caution because it is unrelated to PCD.

In our cohort of 24 patients, all positive for Yo antibodies based on the commercial line immunoassays, only 6 had PCD. This means that routine testing using only line immunoassays must be performed with care and must be confirmed to prevent misdiagnosis, unnecessary testing, and incorrect treatment. Some laboratories use immunohistochemistry for initial screening, which may avoid false positive results based on commercial line immunoassays alone. However, these analyses are laborious and require skilled personnel to interpret the binding patterns.

We have previously shown that Yo antibodies bind both endogenous and recombinant CDR2L but only recombinant CDR2.[12] These findings imply that there are independent antibody responses to CDR2L and CDR2, supported by the fact that the most highly enriched regions of CDR2L are the most divergent regions between the 2 proteins.[15] Because CDR2 and CDR2L share common epitopes, this probably explains the frequent detection of false positive results, which are CDR2 restricted. This is supported by our recent findings that PCD-related Yo antibodies bind only endogenous CDR2L not endogenous CDR2.[12]

We hypothesized that the specificity of the routine commercial tests could be increased by including CDR2L as a target protein. In the present study, we developed 2 techniques for detection of CDR2L: a CBA consisting of HEK293 cells that express Myc-DDK-tagged CDR2L and a Western blot–based analysis of recombinant CDR2 and CDR2L proteins. Our CDR2L CBA identified all 6 patients with PCD and was negative for the 18 nonconfirmed cases. Western blot analysis with recombinant CDR2L identified the 6 PCD patients and one patient with no PCD but with a previous diagnosis of breast cancer. The apparent mismatch between our CBA and Western blot assays is unclear but may represent a differently expressed epitope of CDR2L detected by each of the 2 assays. Interestingly, patients with weak-to-moderate positive commercial line immunoassays were also found negative by the CDR2 Western blot analysis, again suggesting differences in the epitopes detected.

Although our study cohort is small, our data demonstrate that detection of CDR2L adds an important dimension to the diagnostic accuracy of PCD testing. Currently, we do not know whether testing for CDR2L antibodies alone would be sufficient for diagnosis of PCD because our cohort were selected based on anti-CDR2 positivity. This question will require larger patient cohorts including PCD patients who test negative in commercial line

immunoassays and patients who have PNS caused by other onconeural antibodies.

In conclusion, our results underline the importance of confirmatory tests when interpreting results from the currently commercially available anti-Yo detection assays. The high proportion of false positive results appears to be due to the use of CDR2 as antigen. Therefore, all positive samples tested by commercial line immunoassays must be confirmed by immunofluorescence or immunohistochemistry. However, our results support the thesis that CDR2L is the major Yo antigen, and we suggest that CDR2L should be included in the commercially available line immunoassays and CBAs for Yo antibody detection.

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Disclosure

I. Herdlevær, M. Haugen, K. Mazengia, C. Totland, and C. Vedeler report no disclosures relevant to the manuscript. Go to Neurology.org/NN for full disclosures.

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Mette Haugen, BSc	Department of Neurology, Haukeland University Hospital, Bergen, Norway	Designed and conceptualized the study, acquired the data, analyzed the data, and revised the manuscript for intellectual content
Kibret Mazengia, MSc	Department of Neurology, Haukeland University Hospital, Bergen, Norway	Acquired and revised the manuscript for intellectual content
Cecilie Totland, PhD	Department of Neurology, Haukeland University Hospital, Bergen, Norway	Designed and conceptualized the study; acquired; analyzed the data; revised the manuscript for intellectual content
Christian Vedeler, MD, PhD	Department of Neurology, Haukeland University Hospital, Bergen, Norway; Department of Clinical Medicine, University of Bergen, Norway; Departments of Neurology and Clinical Medicine, Neuro-SysMed—Centre of Excellence for Experimental Therapy in Neurology, Bergen, Norway	Designed and conceptualized the study, revised the manuscript for intellectual content, and study supervisor

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