



CDR2L Is the Major Yo Antibody Target in Paraneoplastic Cerebellar Degeneration

Torbjørn Kråkenes, MSc ¹,
 Ida Herdlevær, MSc,^{1,2}
 Margrethe Raspotnig, MD,¹
 Mette Haugen, BSc,²
 Manja Schubert, ScD ² and
 Christian A. Vedeler, MD, PhD ^{1,2,3}

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.

ANN NEUROL 2019;86:316–321

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 death⁴ 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 NH₄Cl; Sigma-Aldrich, St Louis, MO; #254134) prior to immunostaining.

From the ¹Department of Clinical Medicine, University of Bergen, Bergen, Norway; ²Department of Neurology, Haukeland University Hospital, Bergen, Norway; and ³Neuro-SysMed - Centre of Excellence for Experimental Therapy in Neurology, Departments of Neurology and Clinical Medicine, Bergen, Norway

Address correspondence to Kråkenes, Department of Clinical Medicine, Faculty of Medicine, University of Bergen, Jonas Lies vei 87, Bergen, 5021, Norway. E-mail: torbjornkrakenes@gmail.com

Received Nov 26, 2018, and in revised form May 9, 2019. Accepted for publication May 27, 2019.

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.25511.

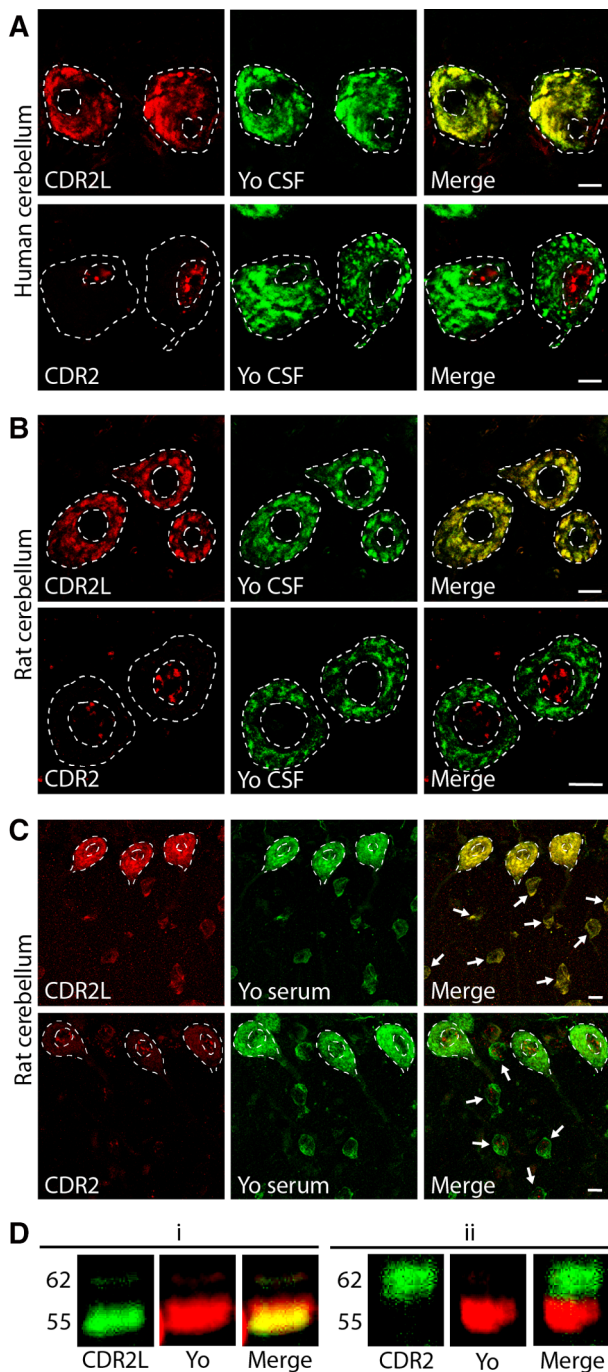


FIGURE 1: Yo antibodies bind to CDR2L, but not CDR2, in cerebellar Purkinje cells. Scale bars = 10 μ m. (A) Sections of fresh frozen human cerebellum. Upper row: Section stained with Yo (cerebrospinal fluid [CSF]; green) and anti-CDR2L (red); the antibodies colocalize in the cytoplasm (seen as yellow 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

Immunocytochemistry

Cancer cells and cerebellar sections were permeabilized (5 minutes, 0.5% Triton X-100-PBS; Sigma-Aldrich, #11332481001), washed (3 \times 15 minutes, 0.5% gelatin-PBS; Sigma-Aldrich, #G7041), blocked (30 minutes, 10% SEABLOCK; Thermo Fisher, #37527), incubated with primary antibodies (overnight, 4 $^{\circ}$ 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 \times 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,

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.

#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

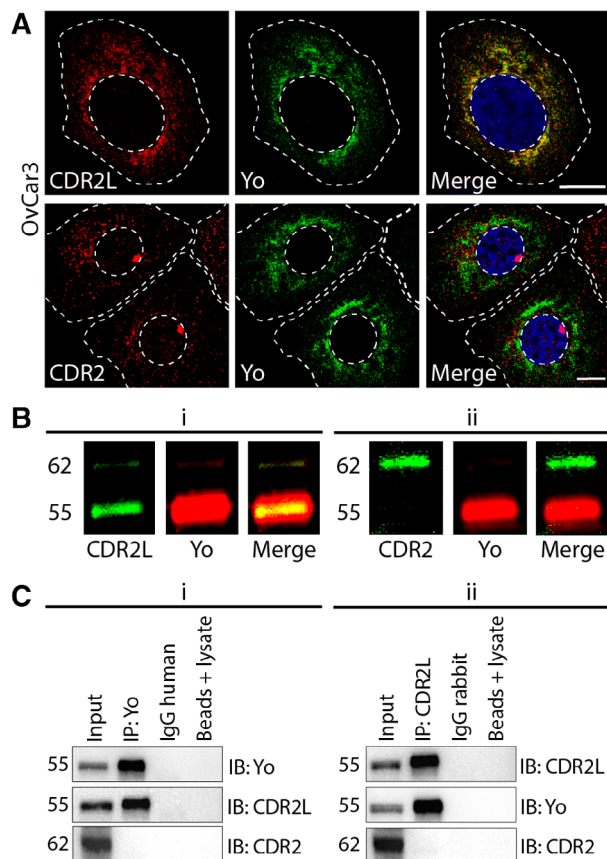


FIGURE 2: Yo antibodies bind to CDR2L, but not CDR2, in OvCar3 cells. Scale bars = 10µm. (A) Upper row: OvCar3 cells stained with Yo (cerebrospinal fluid [CSF]; green) and anti-CDR2L (red); Yo and CDR2L colocalize, giving the same granular, cytoplasmic staining pattern (seen as yellow in the merge image). 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.

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.

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

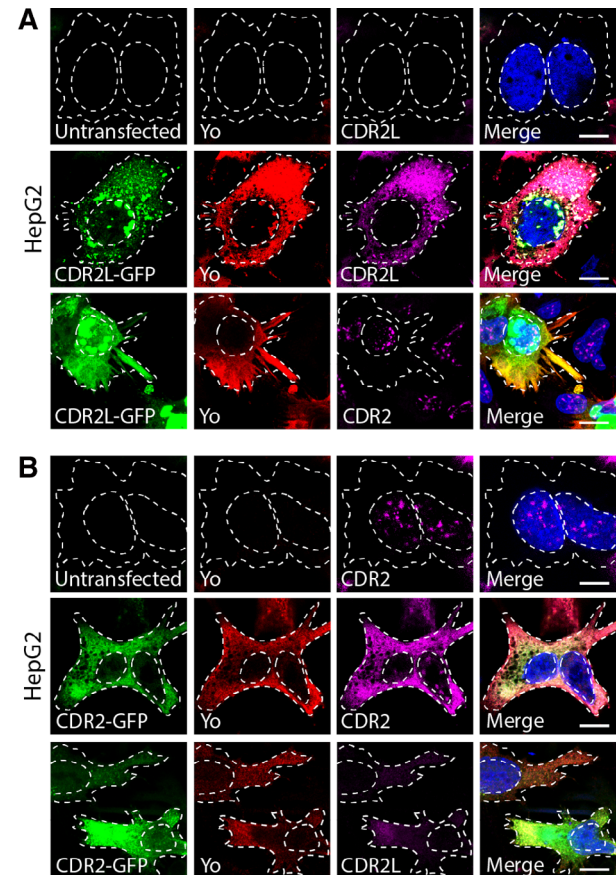


FIGURE 3: HepG2 cells, with a high endogenous level of CDR2, are not stained by Yo antibodies. However, Yo antibodies are able to bind recombinant CDR2. Scale bars = 10µm. (A) Untransfected HepG2 cells (first row) and HepG2 cells transfected with a vector expressing recombinant CDR2L-GFP (second and 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

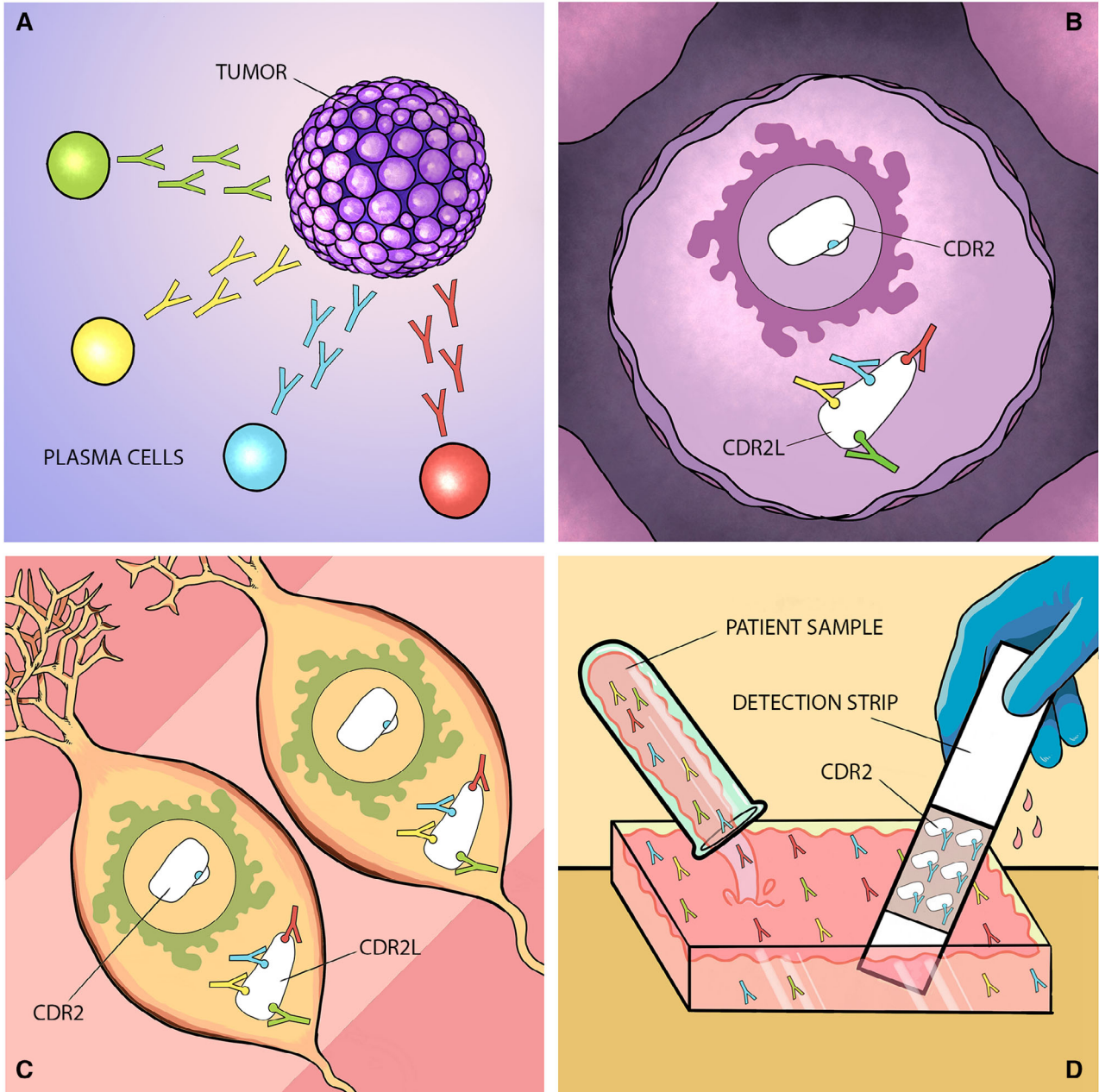


FIGURE 4: Proposed hypothesis of how Yo antibodies are able to bind both recombinant CDR2 and CDR2L, but only CDR2L under native conditions. (A) Illustration of the initial, polyclonal response of Yo antibodies toward CDR2L in the tumors of paraneoplastic cerebellar degeneration patients. (B) A tumor 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.

Acknowledgment

This work was supported by grants from Helse Vest, the Torbjørg Hauges Legacy, and the University of Bergen (PhD stipend #2016/7580-RAKA). The confocal imaging was performed at the Molecular Imaging Center and was thus supported by the Department of Biomedicine and the Faculty of Medicine at the University of Bergen and its partners.

We thank Dr L. Bindoff for valuable discussion of our paper.

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.

References

1. Rees JH. Paraneoplastic syndromes: when to suspect, how to confirm, and how to manage. *J Neurol Neurosurg Psychiatry* 2004;75(suppl 2): ii43–ii50.
2. Darnell RB, Posner JB. Paraneoplastic syndromes involving the nervous system. *N Engl J Med* 2003;349:1543–1554.
3. Storstein A, Monstad SE, Haugen M, et al. Onconeural antibodies: improved detection and clinical correlations. *J Neuroimmunol* 2011; 232:166–170.
4. Storstein A, Krossnes BK, Vedeler CA. Morphological and immunohistochemical characterization of paraneoplastic cerebellar degeneration associated with Yo antibodies. *Acta Neurol Scand* 2009; 120:64–67.
5. Greenlee JE, Brashear HR. Antibodies to cerebellar Purkinje cells in patients with paraneoplastic cerebellar degeneration and ovarian carcinoma. *Ann Neurol* 1983;14:609–613.
6. Corradi JP, Yang C, Darnell JC, et al. A post-transcriptional regulatory mechanism restricts expression of the paraneoplastic cerebellar degeneration antigen cdr2 to immune privileged tissues. *J Neurosci* 1997;17:1406–1415.

7. Darnell JC, Albert ML, Darnell RB. Cdr2, a target antigen of naturally occurring human tumor immunity, is widely expressed in gynecological tumors. *Cancer Res* 2000;60:2136–2139.
8. Okano HJ, Park WY, Corradi JP, Darnell RB. The cytoplasmic Purkinje onconeural antigen cdr2 down-regulates c-Myc function: implications for neuronal and tumor cell survival. *Genes Dev* 1999;13:2087–2097.
9. Albert ML, Darnell JC, Bender A, et al. Tumor-specific killer cells in paraneoplastic cerebellar degeneration. *Nat Med* 1998;4:1321–1324.
10. Raspotnig M, Haugen M, Thorsteinsdottir M, et al. Cerebellar degeneration-related proteins 2 and 2-like are present in ovarian cancer in patients with and without Yo antibodies. *Cancer Immunol Immunother* 2017;66:1463–1471.
11. Totland C, Ying M, Haugen M, et al. Avidity of onconeural antibodies is of clinical relevance. *Cancer Immunol Immunother* 2013;62:1393–1396.
12. Small M, Treilleux I, Couillault C, et al. Genetic alterations and tumor immune attack in Yo paraneoplastic cerebellar degeneration. *Acta Neuropathol* 2018;135:569–579.
13. Eichler TW, Totland C, Haugen M, et al. CDR2L antibodies: a new player in paraneoplastic cerebellar degeneration. *PLoS One* 2013;8:e66002.
14. Schubert M, Panja D, Haugen M, et al. Paraneoplastic CDR2 and CDR2L antibodies affect Purkinje cell calcium homeostasis. *Acta Neuropathol* 2014;128:835–852.
15. Totland C, Aarskog NK, Eichler TW, et al. CDR2 antigen and Yo antibodies. *Cancer Immunol Immunother* 2011;60:283–289.
16. O'Donovan BD, Mandel-Brehm C, Vazquez SE, et al. Exploration of anti-Yo and anti-Hu paraneoplastic neurological disorders by PhIP-Seq reveals a highly restricted pattern of antibody epitopes. *bioRxiv* 2018;502187.