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ORIGINAL ARTICLE

A cerebellar degeneration-related protein 2-like cell-based assay for anti-Yo detection in patients with paraneoplastic cerebellar degeneration

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Abstract

Background and purpose: Commercially available tests for Yo antibody detection have low specificity for paraneoplastic cerebellar degeneration (PCD) because these assays use cerebellar degeneration-related protein 2 (CDR2) as the antigen, not CDR2-like (CDR2L). We aimed to test the hypothesis that use of a CDR2L cell-based assay (CBA), as an additional screening technique, would increase the accuracy of Yo-PCD diagnosis.

Methods: An in-house CBA to test for anti-CDR2L antibodies was developed and used to screen sera from 48 patients with confirmed anti-Yo-associated PCD. Fifteen non-Yo PCD patients, 22 patients with ovarian cancer without neurological syndromes, 50 healthy blood donors, 10 multiple sclerosis, 15 Parkinson's disease, and five non-paraneoplastic ataxic patients were included as controls. Sera were also tested by western blot analysis using recombinant CDR2 and CDR2L proteins developed in house, by the commercially available line immunoassays from Ravo Diagnostika and Euroimmun, and by the CDR2 CBA from Euroimmun.

Results: The CDR2L CBA identified all 48 patients with Yo-PCD. No CDR2L CBA reaction was observed in any of the control sera. The western blot technique had lower sensitivity and specificity as sera from eight and six of the 48 Yo-PCD patients did not react with recombinant CDR2 or CDR2L, respectively.

Conclusions: The CDR2L CBA is highly reliable for identification of Yo-PCD. Although our findings indicate that, currently, the combination of CDR2 and CDR2L yields the most reliable test results, it remains to be evaluated if a test for single anti-CDR2L positivity will serve as a sufficient biomarker for Yo-PCD diagnosis.

KEYWORDS
anti-Yo, cell-based assay, cerebellar degeneration-related proteins, paraneoplastic cerebellar degeneration

Kjell Inge Erikstad and Ida Herdlevær contributed equally to this work.

INTRODUCTION

Paraneoplastic cerebellar degeneration (PCD) is one of the most common forms of paraneoplastic neurological syndromes (PNS) [1–3]. PNS are rare disorders in which the immune system targets tumor antigens in cancer, and secondarily attacks cells in the central and/or peripheral nervous system expressing similar proteins. Paraneoplastic antibodies that are present in patient serum or cerebrospinal fluid serve as key diagnostic biomarkers for PNS [4]. The most frequently detected antibody associated with PCD in ovarian and breast cancer patients is anti-Yo [5]. Anti-Yo targets two antigens located in the nucleus and cytoplasm of Purkinje neurons, cerebellar degeneration-related protein 2 (CDR2) and CDR2-like (CDR2L), respectively. Experimental findings strongly suggest that CDR2L is the major Yo antibody target in PCD patients [6–8].

Rapid and precise diagnosis of PCD is important as treatment can improve patient outcome. Commercially available line immunoassays are widely used for PCD diagnosis as they enable rapid screening for paraneoplastic antibodies. However, previous findings show that the test specificity for anti-Yo is as low as 8%, resulting in numerous false-positive results that necessitate additional testing [9, 10]. Some laboratories use immunohistochemistry as the "gold standard" for initial antibody screening and thereby avoid false-positive results based on commercial line immunoassays. Nevertheless, these analyses are laborious and require skilled personnel to interpret the staining patterns.

In our previous study, we reported that inclusion of CDR2L as an additional Yo antibody target increases test specificity and therefore serves as an important diagnostic biomarker for Yo-PCD [8]. In this follow-up study, we further validated our CBA in comparison to western blot and line immunoassay techniques in a larger patient cohort and additional control groups.

METHODS

Study participants

Sera were collected retrospectively from patients with Yo-PCD: 34 patients from the Neurological Research Laboratory, Haukeland University Hospital, Bergen, Norway and 14 patients from the National Reference Center for PNS and Autoimmune Encephalitis, Lyon, France. All patients were diagnosed with Yo-associated PCD prior to inclusion, using line immunoassays, confirmed by immunohistochemistry, and supported by clinical findings. None of the Yo-PCD patients were included in our previous study [8]. Controls consisted of 15 patients from Lyon with PCD without Yo antibodies, as well as 22 patients with ovarian cancer without neurological syndrome, 50 healthy blood donors, 10 patients with multiple sclerosis, 15 patients with Parkinson's disease and five patients with non-paraneoplastic ataxia (mitochondrial disease with polymerase gamma [POLG] mutation), from Bergen.

Ethical considerations

The study was approved by The Regional Committee for Health and Medical Research Ethics in Norway, REK #123524 and conforms to the World Medical Association Declaration of Helsinki. The collection declaration (DC-2020-3919) and authorization to transfer samples (AC-2020-39,128) from the French Ministry of Research was approved. Informed patient consent was obtained and the patient data were anonymized prior to the start of the study. PCD was diagnosed according to established criteria [11]. We also included control sera from patients with multiple sclerosis, Parkinson's disease and mitochondrial disease with POLG mutation.

Commercial line immunoassays for detection of autoantibodies

The PNS 14 Line Assay (Ravo Diagnostika, #PNS14-03) and the EUROLINE PNS 12 assay (Euroimmun, #DL1111-1601-7-G) were used to test all serum and/or cerebrospinal fluid samples. The Ravo Diagnostika assay is used to test for antibodies to the following proteins: GAD65, HuD, Yo, Ri, CV2/CRMP5, amphiphysin, Ma1, Ma2, SOX1, Tr/DNER, Zic4, titin, recoverin, and protein kinase C γ. The Euroimmun assay is used to test for antibodies to the following proteins: amphiphysin, CV2/CRMP5, Ma2, Ri, Yo, Hu, recoverin, SOX1, titin, Zic4, GAD65, and Tr/DNER. The assays were performed in accordance with the manufacturers' instructions. Two independent investigators graded the line immunoassay band intensities on a scale ranging from + to +++ compared to the positive control (+++).

Commercial cell-based assay for anti-CDR2 detection

The Mosaic 1 CBA from Euroimmun (#FA1113-1005-1) contains transfected HEK293 cells with antigens for anti-CDR2, anti-DNER, anti-ITPR1, and anti-CARP. Serum was diluted 1:100, as recommended by the manufacturer, and applied to each field on the slide. After 30-min incubation at room temperature, the slides were washed with phosphate-buffered saline (PBS) containing 0.2% Tween 20 for 5 min at room temperature and were then incubated with goat anti-human IgG secondary antibody conjugated to Alexa Fluor 488 (#A-11013; Thermo Fisher Scientific, Waltham, MA, USA) in PBS-Tween 20 (1:500) for 30 min at room temperature. The slides were rinsed with PBS-Tween 20, followed by mounting of a glass coverslip.

Cell-based assay for anti-CDR2L detection

HEK293 cells were cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-Glutamine (Thermo Fischer Scientific, #25030081) in an 8-well Nunc Lab-Tec II CC2 Chamber Slide System (Thermo Fischer

Scientific, #54739PK) for 24 h. Cells were then transfected using Lipofectamine 3000 Reagent (Invitrogen, #L3000008) with plasmid for expression of Myc-DDK-tagged CDR2L (Origene, #RC206909). At 48 h after the transfection, cells were fixed with 4% paraformaldehyde for 20 min at room temperature, washed three times with PBS, and demembranized using 0.1% Triton X-100 in PBS for 10 min at room temperature. The cells were washed with PBS and quenched to minimize autofluorescence by incubation in 50 mM NH_4Cl for 5 min at room temperature. Blocking was performed by incubation for 1 h at room temperature with PBS-Tween 20 containing 10% Fish Serum Blocking Buffer (SEA BLOCK; Thermo Fisher Scientific, #37527). Coverslips were incubated with patient serum (1:10 and 1:100), mouse anti-DDK tag (Origene, #TA50011-100, 1:1000), anti-CDR2L (Protein Technology, #14563-1-AP, 1:400), and anti-CDR2 (Sigma-Aldrich, #HPA023870) in SEA BLOCK. Coverslips were incubated with secondary antibodies in SEA BLOCK for 1 h at room temperature and were washed twice with PBS-Tween 20 and twice with PBS. The slides were mounted with glass coverslips and ProLong Diamond Antifade Mountant with DAPI (Thermo Fischer Scientific, #P36962).

Imaging

The CBAs were imaged on a Leica Leitz DM RBE fluorescence microscope and evaluated by two independent investigators. To merge images, ImageJ 1.50i bundled with Java 1.8.0_77 (64-bit) was used.

Western blot detection of anti-CDR2L and anti-CDR2

A reticulocyte lysate system (Promega, #L4610) was used for cell-free protein-expression of CDR2 and CDR2L. Purified plasmids for expression of Myc-DDK-tagged CDR2L and Myc-DDK-tagged CDR2 were incubated with transcription/translation lysate, T7 RNA polymerase promoter, reaction buffer, recombinant RNasin ribonuclease inhibitor, RNase free water, and amino acid mixture, in accordance with the instruction manual, at 30°C for 90 min. A negative control without plasmids was included in each experiment. Protein denaturation was performed in Laemmli buffer (Bio-Rad, #1610747) and 2.5% β-mercaptoethanol at 95°C for 5 min. Proteins were separated by SDS-PAGE on a 10% TGX gel (Bio-Rad, #456–1035) and transferred to a PVDF membrane using a transfer kit (Trans-Blot Turbo Transfer; Bio-Rad, #170–4274). The membrane was blocked in 5% dry milk in Tris-buffered saline with 0.1% Tween 20 (TBS-Tween 20), washed three times with TBS-Tween 20, and incubated with serum diluted in 3% bovine serum albumin in TBS-Tween 20 (1:250). The membrane was washed three times in TBS-Tween 20 and incubated with horseradish peroxidase anti-human IgG (Dako, #P0214) for 1 h at room temperature. Proteins were visualized using a Pierce ECL Western Blotting Substrate (Thermo Fischer Scientific, #32106) in a Bio-Rad Gel Doc XR Imaging system. Image Lab version 6.0.0 build 25 Standard Edition was used to merge the images.

RESULTS

Screening of serum samples on commercially available line immunoassays and CDR2 cell-based assay

The 34 patient samples from the Neurological Research Laboratory, Bergen, had previously been identified as anti-Yo-positive by RAVO line immunoassay and immunohistochemistry on rat cerebellar sections with clinical confirmation. Upon reevaluation for this study, all sera reacted strongly with the Yo reaction field on both RAVO and Euroimmun line immunoassays (Table 1). Sera from the remaining Yo-PCD patients from Lyon also tested positive on both line immunoassays. All 48 Yo-PCD patient sera were also tested with the commercially available CDR2 CBA. Forty-seven of the 48 Yo-PCD patients tested positive (Table 1), whereas no staining of the 15 non-Yo-PCD patients was observed.

CDR2L cell-based assay separates Yo-PCD from non-Yo-PCD

All 48 serum samples from the Yo-PCD patients tested positive in the CDR2L CBA (Table 1, Figure 1a). The CDR2L CBA was negative for the 15 non-Yo-PCD patients (Figure 1b), the 22 patients with ovarian cancer without neurological syndrome (Figure 1c) and the 50 healthy blood donors.

Western blot analysis partially separates Yo-PCD from non-Yo-PCD

Western blot analysis with CDR2 and CDR2L recombinant proteins was positive for 40 and 42 of the 48 Yo-PCD patients, respectively (Table 1). CDR2 positivity was indicated by a double band at 62 kDa, and CDR2L positivity by a 55-kDa band (Figure 2a). None of the 15 sera from the non-Yo-PCD patients or randomly selected sera from 15 of the patients with ovarian cancer without neurological syndrome or healthy blood donors were positive for CDR2 and CDR2L by western blotting (Figure 2b).

Screening of serum samples from patients with other neurological disorders

Sera from 10 multiple sclerosis, 15 Parkinson's disease, and five non-paraneoplastic ataxic patients tested negative in the line immunoassay from Ravo and our CDR2L CBA (Table S1).

DISCUSSION

We found that the CDR2L CBA identified all 48 patients with Yo-PCD, and was negative for the 15 non-PCD patients, 22 patients

TABLE 1 Anti-Yo reactivity in various assays compared to clinical diagnosis and associated cancer.

Patient #	F/M	Age	Ravo	EuroLine	CBA CDR2	CBA CDR2L	WB CDR2/ CDR2L	Clinical presentation	Cancer
1	F	60	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
2	F	57	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
3	F	59	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
4	F	57	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
5	F	64	Yo +++	Yo +++	+	+	+/+	PCD	Breast
6	F	39	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
7	F	44	Yo +++	Yo +++	+	+	+/+	PCD	Tubal
8	F	66	Yo +++	Yo +++	+	+	+/+	PCD	Tubal
9	F	55	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
10	F	56	Yo +++	Yo +++	+	+	+/+	PCD	Breast
11	F	52	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
12	F	78	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
13	F	70	Yo +++	Yo +++	+	+	+/+	PCD	Tubal
14	F	58	Yo +++	Yo +++	+	+	+/+	PCD	Tubal
15	F	65	Yo +++	Yo +++	+	+	+/+	PCD	Endometrial
16	F	56	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
17	F	70	Yo +++	Yo +++	+	+	+/+	PCD	Breast
18	F	54	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
19	F	72	Yo +++	Yo +++	+	+	+/+	PCD	Endometrial
20	F	58	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
21	F	64	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
22	F	79	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
23	F	63	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
24	F	51	Yo +++	Yo +++	+	+	−/−	PCD	Ovarian
25	F	57	Yo +++	Yo +++	+	+	−/+	PCD	Ovarian
26	F	57	Yo +++	Yo +++	+	+	−/−	PCD	Ovarian
27	F	54	Yo +++	Yo +++	+	+	−/+	PCD	Ovarian
28	F	65	Yo +++	Yo +++	+	+	−/−	PCD	Ovarian
29	F	69	Yo +++	Yo +++	+	+	−/−	PCD	Ovarian
30	F	72	Yo +++	Yo +++	+	+	−/−	PCD	Ovarian
31	F	71	Yo +++	Yo +++	+	+	−/−	PCD	Ovarian
32	F	81	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
33	F	72	Yo +++	Yo +++	+	+	+/+	PCD	Breast
34	F	57	Yo ++	Yo +++	+	+	+/+	PCD	Tubal
35	F	80	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
36	F	74	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
37	M	67	Yo +++	Yo +++	+	+	+/+	PCD	Unknown
38	F	64	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
39	F	60	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
40	F	81	Yo +++	Yo +++	+	+	+/+	PCD	Unknown
41	F	63	Yo +++	Yo +++	+	+	+/+	PCD	Unknown
42	F	52	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
43	F	66	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
44	F	71	Yo +++	Yo +++	+	+	+/+	PCD	Breast

TABLE 1 (Continued)

Patient #	F/M	Age	Ravo	EuroLine	CBA CDR2	CBA CDR2L	WB CDR2/ CDR2L	Clinical presentation	Cancer
45	F	64	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian
46	F	74	Yo +++	Yo ++	+	+	+/+	PCD	Endometrial
47	F	45	Yo ++	Yo ++	–	+	+/+	PCD	Breast
48	F	57	Yo +++	Yo +++	+	+	+/+	PCD	Ovarian

Note: Ravo and Euroimmun lineblots were graded by trained investigators as – (negative), + (weak positive) ++ (moderate positive), or +++ (strong positive) depending on the band intensity. CBA and WB were graded as + (positive) or – (negative).

Abbreviations: CBA, cell-based assay; CDR2, cerebellar degeneration-related protein 2; CDR2L, cerebellar degeneration-related protein 2-like; F, female; M, male; PCD, paraneoplastic cerebellar degeneration; WB, western blot.

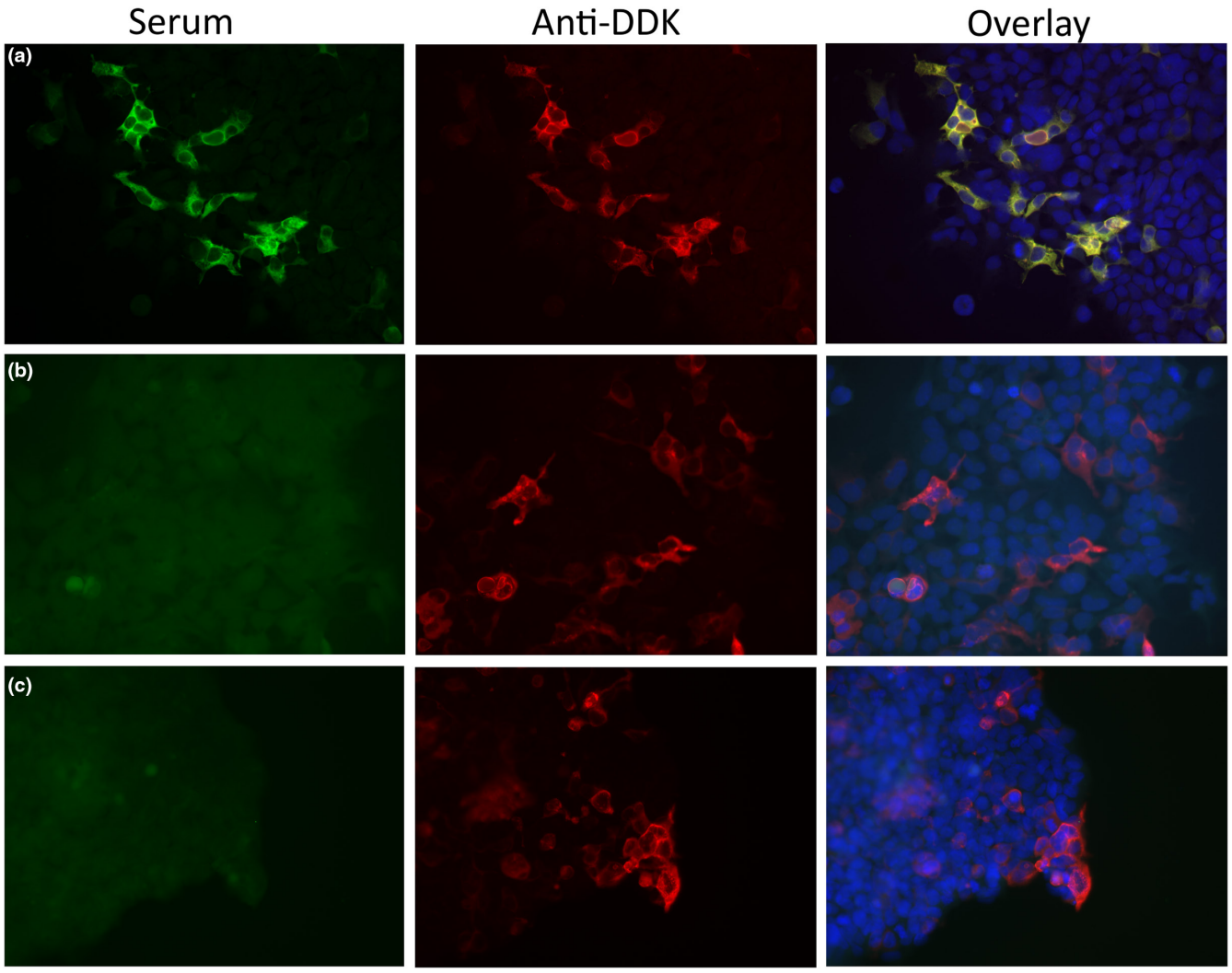

FIGURE 1 Only Yo-positive sera react with cerebellar degeneration-related protein 2-like (CDR2L) cell-based assay (CBA). (a) Serum from a representative Yo-positive patient (green) strongly stains myc-DDK-CDR2L-transfected HEK293 cells. Overlay is observed in yellow. (b) Serum from a representative paraneoplastic cerebellar degeneration (PCD) patient without Yo antibodies, and (c) a representative patient with ovarian cancer, did not stain myc-DDK-CDR2L-transfected cells (red). Anti-DDK (red) was used to confirm location of transfected cells. Nuclei are visualized with DAPI (blue).

with ovarian cancer without neurological syndromes and 50 healthy blood donors. In our previous study, 24 patient sera that tested positive for anti-Yo by line immunoassay were evaluated by CDR2L CBA and western blot analysis with recombinant CDR2 and CDR2L proteins [8]. Only the six true-positive anti-Yo PCD sera tested positive in the CDR2L CBA, rendering the 18 remaining sera false positive. In the present study, we also included 30 serum samples from patients with other neurological disorders and non-paraneoplastic

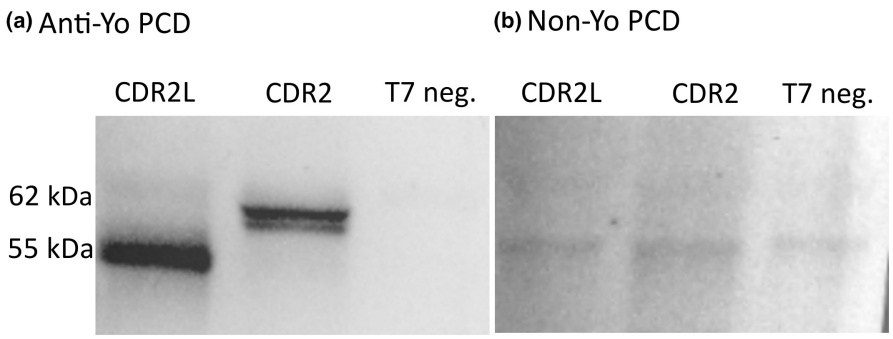

FIGURE 2 (a) Western blot analysis of serum from a representative patient with Yo-associated paraneoplastic cerebellar degeneration (PCD). Note the intense cerebellar degeneration-related protein 2-like (CDR2L) band at 55 kDa, and a weaker cerebellar degeneration-related protein 2 (CDR2) double band at 62 kDa. (b) Western blot of serum from an anti-Yo-negative PCD patient. No bands corresponding to CDR2 and CDR2L were observed.

ataxia, which tested negative in the CDR2L CBA and line immunoassay from Ravo. Taken together, the results show that the CDR2L CBA is 100% specific and sensitive for Yo-PCD.

The western blot analysis for PCD had lower sensitivity than the CBA. This could be explained by the fact that the recombinant proteins were produced in a rabbit reticulocyte lysate, which may contain additional proteins that mask the CDR2/CDR2L reaction. Varying degrees of background staining may also interfere with interpretation of the western blot results [12]. That the system for protein expression and purification is responsible for the low sensitivity is supported by the fact that all sera reacted strongly with CDR2 on line immunoassays from both Ravo Diagnostika and Euroimmun, which employ baculovirus or *Escherichia coli* for protein expression. This highlights the need for a cut-off value when evaluating the positivity of a western blot analysis and for careful optimization for the protein of interest.

We found that ovarian or breast cancer was most commonly associated with Yo-PCD [13]. However, tubal and endometrial cancer were also observed with Yo-PCD, as described previously [6, 14, 15]. One man with PCD and unknown cancer tested positive for anti-Yo by all techniques. Yo antibodies are rarely reported in male patients but are in these cases usually associated with adenocarcinomas of the gastrointestinal tract [16]. In our previous study, male sera that tested positive for anti-Yo in the line immunoassay were not confirmed positive by CDR2L CBA, and did not present with PCD [8]. However, since this man tested positive in all assays and showed symptoms of PCD, we must consider this to be a true positive.

We previously found that detection of anti-CDR2L antibodies increases accuracy of diagnosis of anti-Yo-associated PCD [8]. The present study including a larger population of Yo-PCD and controls supports this finding. In contrast, commercially available line immunoassays using CDR2 as antigen show that the test specificity for anti-Yo is as low as 8%, resulting in numerous false-positive results that necessitate additional testing [9, 10]. In our previous study, we found that false-positive anti-Yo by line immunoassays were negative by CDR2L CBA [8]. It is therefore necessary to include CDR2L as another antigen on these tests to increase their specificity for anti-Yo. This is further supported by the results of a PhIP-Seq library where enrichment for both CDR2L and CDR2 peptides was only observed in 83% of anti-Yo-positive patients, whereas an enrichment in CDR2L peptides was found in all patients [17]. Taken together, although our findings indicate that the CDR2L CBAs yield the most reliable test results, it remains to be evaluated if a test only for anti-CDR2L will serve as a sufficient biomarker for PCD diagnosis.

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CONFLICT OF INTEREST STATEMENT

None.

DATA AVAILABILITY STATEMENT

Upon reasonable request, the data from the experiments in this article are available from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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