

Brief Reports

Absence of Acanthocytosis in Huntington's Disease-like 2: A Prospective Comparison with Huntington's Disease

David G. Anderson^{1,2*}, Sergio Carmona³, Kubendran Naidoo³, Theresa L. Coetzer³, Jonathan Carr⁴, Dobrila D. Rudnicki^{5,6}, Ruth H. Walker^{7,8}, Russell L. Margolis⁹ & Amanda Krause^{2,10}

¹Department of Neurology, The University of the Witwatersrand Donald Gordon Medical Center, Johannesburg, South Africa, ²Division of Human Genetics, University of the Witwatersrand, Johannesburg, South Africa, ³Department of Molecular Medicine and Haematology, University of the Witwatersrand, Johannesburg, South Africa, ⁴Department of Neurology, University of Stellenbosch, Cape Town, South Africa, ⁵Office of Special Initiatives, National Center for Advancing Translational Sciences, NIH, Bethesda, MD, USA, ⁶Department of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, USA, ⁷Department of Neurology, James J. Peters Veterans Affairs Medical Center, New York, NY, USA, ⁸Department of Neurology, Mount Sinai School of Medicine, New York City, NY, USA, ⁹Departments of Psychiatry and Neurology, Johns Hopkins University School of Medicine, Baltimore, MD, USA, ¹⁰Division of Human Genetics, National Health Laboratory Service, Johannesburg, South Africa

Abstract

Background: Huntington's Disease-like 2 (HDL2) is classified as a neuroacanthocytosis; however, this remains unverified. We aim to determine if acanthocytes are present in HDL2 and whether acanthocytes can differentiate HDL2 from Huntington's disease (HD).

Methods: We prospectively compared 13 HD and 12 HDL2 cases against 21 unaffected controls in Johannesburg. Blood smears were prepared using international standards and reviewed by at least two blinded reviewers. An acanthocytosis rate of greater than 1.2% in the dry smear or greater than 3.7% in the wet smear was designated a priori as the threshold for clinical significance based on previously established standards. Flow cytometry was performed on all but four of the cases. Red cell membrane protein analysis was performed on all participants.

Results: There were 12 HDL2, 13 HD, and 21 controls enrolled. None of the HD or HDL2 participants had defined acanthocytosis or other morphological abnormalities. None of the HD or HDL2 cases had evidence of an abnormal band 3.

Discussion: Acanthocytosis was not identified in either HDL2 or HD in our patient population. Our results, based on the first prospective study of acanthocytes in HDL2 or HD, suggest that screening for acanthocytes will not help establish the diagnosis of HD or HDL2, nor differentiate between the two disorders and raises the question if HDL2 should be placed within the neuroacanthocytosis syndromes.

Keywords: Huntington's disease, Huntington's disease-like 2, acanthocytes, chorea

Citation: Anderson DG, Carmona S, Naidoo K, Coetzer TL, Carr J, Rudnicki DD, et al. Absence of acanthocytosis in Huntington's disease-like 2: a prospective comparison with Huntington's disease. Tremor Other Hyperkinet Mov. 2017; 7. doi: 10.7916/D81J9PDX

*To whom correspondence should be addressed. E-mail: david@neuro.joburg

Editor: Elan D. Louis, Yale University, USA

Received: September 25, 2017 **Accepted:** November 15, 2017 **Published:** December 5, 2017

Copyright: © 2017 Anderson et al. This is an open-access article distributed under the terms of the Creative Commons Attribution-Noncommercial-No Derivatives License, which permits the user to copy, distribute, and transmit the work provided that the original authors and source are credited; that no commercial use is made of the work; and that the work is not altered or transformed.

Funding: None.

Financial Disclosures: None.

Conflicts of Interest: The authors report no conflicts of interest.

Ethics Statement: This study was performed in accordance with the ethical standards detailed in the Declaration of Helsinki. The authors' institutional ethics committee has approved this study and all patients have provided written informed consent.

Introduction

Huntington's disease-like 2 (HDL2) is an autosomal dominant neurodegenerative disorder caused by a CTG/CAG expansion mutation in exon 2A of *junctophilin-3* (*JPH3*) on chromosome 16q24.3, which occurs in patients of African ancestry.¹ HDL2 is clinically and pathologically

similar to Huntington's disease (HD), with progressive dementia and movement abnormalities comprising the predominant clinical phenotype.² Anecdotal case reports suggested that, compared to HD, eye movement abnormalities are less common and parkinsonism more common in HDL2.³

Acanthocytes were documented in some of the early reported cases of HDL2, resulting in diagnostic confusion with chorea-acanthocytosis.^{4,5}

Acanthocytes are erythrocytes with spiky deformations of their membranes, and when found in high numbers are associated with many diseases. The source of acanthocytes in chorea-acanthocytosis, McLeod syndrome, and pantothenate kinase-associated neurodegeneration (and possibly also aceruloplasminemia) appears to be complex. Abnormal phosphorylation of the proteins connecting the membrane and cytoskeleton of erythrocytes, erythrocyte metabolism, the involvement of lipids in underlying signaling pathways, changes in lipid composition, and signal transduction have all been implicated.⁶ The relationships of these potential mechanisms to neurodegeneration are unclear.⁷

A systematic review of all reported HDL2 patients showed acanthocytes were detected in four of 13 cases; however, the hematological findings in most HDL2 cases are unknown or unreported.³ In three of these four cases, the affected individuals were members of the same pedigree, and one individual had a Fragile X mutation (660 triplet repeats), while the two affected relatives had repeats in the Fragile X pre-mutation range (57 and 56 repeats). Peripheral smears and electron microscopy revealed that 30–35% of the red blood cells were acanthocytes and red blood cell membrane extract showed an abnormal breakdown of band 3 proteins.⁴ The fourth case was from a Mexican pedigree of likely African descent. Only one of the three family members with HDL2 had acanthocytes of 35% on peripheral smear, but cell membrane extracts did not show an abnormal band 3 protein.⁵ These findings have led to the suggestion that HDL2 be classified as one of the neuroacanthocytosis syndromes, and that the presence of acanthocytes might provide important insight into HDL2 pathogenesis, with implications for understanding HD and the neuroacanthocytoses. To our knowledge there have been no systematic efforts to document acanthocytes in HD.

We therefore designed a prospective systematic investigation to study acanthocytosis in HD and HDL2 patients of the same ethnicity, taking advantage of our finding that the area around Johannesburg, South Africa, contains the world's highest known concentration of HDL2 patients.⁸

Methods

HD and HDL2 patients with African or mixed ancestry were identified over 30 months at the Charlotte Maxeke Johannesburg Academic Hospital Huntington Disease Clinic as part of a larger HDL2 phenotype study.³ Ethics clearance was obtained from the Human Research Ethics Committee of The University of the Witwatersrand (M140872) and informed consent was obtained from all study participants. Diagnoses were confirmed by mutation testing for the specific triplet repeat expansion in the genes causing HD and HDL2 at the Division of Human Genetics, National Health Laboratory Service. Control cases, also of African or mixed ancestry, were genetically unrelated family members or friends of the affected patients.

Blood samples were collected from both the control and either the HD or the HDL2 subject at the same study visit. Two 5-ml blood samples were collected, one in Ethylenediaminetetraacetic Acid (EDTA) and the other in Acid Citrate Dextrose (ACD) (BD Vacutainer Systems, UK). Each tube was labeled using a non-identifying study number and was processed within 20 minutes of the venipuncture. The laboratory

was blinded to the diagnosis. Smears were prepared using the same techniques.

Methods for wet and fixed smears were performed according to previously established protocols.⁹ In brief, an aliquot (~5 μ L) of whole blood, collected in EDTA tubes, was used to prepare a fixed smear on a glass slide and stained using the Rapidiff Stain (Diagnostic Media Products, South Africa). In parallel, unfixed wet smears were prepared within an hour of the blood draw. A minimum of 10 fields of each preparation was examined with a Zeiss Axiostar (Carl Zeiss Jena GmbH, Germany) microscope under oil immersion at 1,000 \times magnification. Blood was obtained from an unaffected control at the same time and smears were prepared using the same techniques. An acanthocytosis rate of greater than 1.2% in the dry smear or greater than 3.7% in the wet smear was designated a priori as the threshold for clinical significance based on previously established standards.⁹

As a third approach to detecting abnormal hematological features in HDL2, red cell membranes were prepared from blood samples drawn in ACD tubes as described by Coetzer and Palek.¹⁰ The protein concentration was determined using the Coomassie Plus Protein Assay Reagent (Pierce Biotechnology Incorporated, USA). Aliquots of solubilized erythrocyte membrane proteins were prepared by boiling for one minute in 10 mM Tris-HCl pH 8, 1 mM EDTA, 1% SDS, 5% sucrose, 2% β -mercaptoethanol, and 20–50 μ g of protein was resolved on a 12% Laemmli SDS-polyacrylamide gel and a 4–17% Fairbanks gradient gel. Gels were stained with 0.05% Coomassie Blue (BDH Laboratory Supplies, UK) in 25% isopropanol, 10% acetic acid) and the amount of band 3 was quantitated using a Hoefer GS300 transmittance/reflectance scanning densitometer and GS365W software (Hoefer Scientific Instruments, USA).

The Fisher exact test was used to assess the relationship between categorized acanthocyte screening test results and groups. Data analysis was carried out using SAS version 9.4 for Windows. The 5% significance level was used.

Results

All HDL2 or HD cases enrolled had African or mixed ancestry. Control cases were genetically unrelated family members or acquaintances with African or mixed ancestry of the affected patients. The HD group included 11 separate pedigrees; two pedigrees each contributed two individuals to the study. The HDL2 group consisted of 12 unrelated individuals. Two HDL2 patients were also human immunodeficiency virus positive and receiving antiretroviral treatment. The results are presented in Table 1.

The acanthocyte counts remained below the defined normal thresholds in all of the HD, HDL2 and control cases in multiple biological replicates for both wet and dry smears. There were no other abnormal erythrocyte features noted on blood smear. The rest of the assessed flow cytometry for each group can be seen in Table 1 and all were within normal ranges. The flow cytometry evaluation was not available in four cases where there was a blood smear available. These were in three controls and one HD case. The amount of band 3 in the red cell

Table 1. Comparative Results between HDL2, HD, and Control Groups

	HDL2	HD	Control
Number of participants	12	13	21
Age (years)			
Mean age (SD)	49.2 (± 9.5)	46.8 (± 13.2)	49.5 (± 14.9)
Median age	49	47	52
Range	32–66	32–68	21–79
Disease duration (years)			
Mean duration of disease (SD)	5.9 (± 4.3)	8.2 (± 4.7)	–
Median duration of disease	6	6	–
Range	1–18	4–18	–
Triplet repeat length			
Mean abnormal repeat length (SD)	46.3 (± 3.3)	44.3 (± 4.5)	–
Median abnormal repeat length	45	46	–
Range	43–53	40–49	–
Hematology results			
Smear results			
Wet smear (defined as >3.7% erythrocytes being acanthocytes)			
Positive (number of patients)	0	0	0
Dry smear (defined as >1.2% erythrocytes being acanthocytes)			
Positive (number of patients)	0	0	0
Median % of acanthocytes (IQR)	0.21 (0.06–0.44)	0.06 (0.00–0.11)	0.06 (0.02–0.14)
Erythrocyte analysis (SD)			
Hemoglobin (g/dL)	14.62 (± 1.40)	14.28 (± 2.05)	15.56 (± 3.99)
Red cell count ($10^{12}/L$)	5.01 (± 0.63)	4.91 (± 0.47)	5.08 (± 0.56)
MCV (fL)	88.1 (± 4.2)	87.1 (± 5.6)	87.4 (± 4.9)
MCH (pg)	29.5 (± 2.0)	29.1 (± 2.6)	29.1 (± 2.0)
MCHC (g/dL)	33.3 (± 1.5)	33.3 (± 1.5)	33.3 (± 1.2)
RDW (%)	13.3 (± 0.9)	13.7 (± 1.4)	13.6 (± 0.8)

Abbreviations: IQR, Interquartile Range; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentrate; MCV, Mean Corpuscular Volume; RDW, Red cell Distribution Width; SD, Standard Deviation.

membranes of all the HD, HDL2, and control cases was normal, which correlates with the normal red cell morphology.

Discussion

There was no evidence of acanthocytosis in any of the HDL2 or HD patients. Assessments were performed using three different measures, and compared with a normal control group. We therefore conclude that acanthocytosis is not a significant component of HDL2 or HD in our patient population.

The presence of acanthocytosis in four previous cases of HDL2 remains intriguing. In the first three cases the technique for obtaining the smear described not stressing the erythrocytes with saline or aging them and that up to 5% acanthocytes/echinocytes was accepted as being within the normal range.⁵ Echinocytes are difficult to differentiate from acanthocytes on light microscopy and are usually from smear processing errors whereas acanthocytes are of pathological relevance. The method we employed for the detection of acanthocytes by Storch et al.⁹ was not published until 2005. This method is now considered to be the gold standard in testing for acanthocytes in neuroacanthocyte syndromes and reduces echinocytic stress.⁹ The method used to detect acanthocytes in the original four cases where acanthocytes were seen was not described in sufficient detail to duplicate and therefore the Storch method was used by our group.

Acanthocytes may be an infrequent feature of HDL2, or may only develop in the presence of other genetic or environmental modifiers. Three of the four HDL2 cases with acanthocytes were related, and all carried either the Fragile X mutation or premutation. With hindsight it may have been useful to have had a Fragile X control group; however, acanthocytosis has not been reported in either Fragile X or FXTAS patients. The fourth case did not have abnormal band 3 proteins, and had HDL2-affected siblings without acanthocytes. All four cases have subsequently died and are therefore unfortunately not available for further study.

The pathogenesis of HDL2 appears to be complex, with potential contributions from a toxic gain-of-function of abnormal *JPH3* RNA,¹¹ loss of *JPH3* expression,¹² and, potentially, expression of an antisense gene encoding expanded polyglutamine repeats.¹³ The present study, although a relatively small sample size, has nearly doubled the number of HDL2 patients tested for acanthocytes in the literature and for the first time examined acanthocytes in HD. Our results, based on the first prospective study of acanthocytes in HDL2 or HD, suggest that screening for acanthocytes will not help establish the diagnosis of HD or HDL2, nor differentiate between the two disorders. Further, the processes underlying acanthocyte formation are unlikely to substantially contribute to the pathogenesis of most cases of HDL2 and HD. The question is now raised as to whether HDL2 should be grouped with the neuroacanthocytosis syndromes. Finally, our results continue to demonstrate the remarkable phenotypic similarities between HD and HDL2.

Acknowledgements

The authors would like to thank all the patients and their families for their participation in this study and the South African Medical Research Council, National Health Laboratory Service (NHLS) and the ABCD Charitable Trust for financial support.

References

1. Holmes SE, O'Hearn E, Rosenblatt A, Callahan C, Hwang HS, Ingersoll-Ashworth RG, et al. A repeat expansion in the gene encoding junctophilin-3 is associated with Huntington disease-like 2. *Nat Genet* 2001;29:377–378. doi: 10.1038/ng760
2. Margolis RL, O'Hearn E, Rosenblatt A, Willour V, Holmes SE, Franz ML, et al. A disorder similar to Huntington's disease is associated with a novel CAG repeat expansion. *Ann Neurol* 2001;50:373–380. doi: 10.1002/ana.1312
3. Anderson DG, Walker RH, Connor M, Carr J, Margolis RL, Krause A, et al. A systematic review of the Huntington disease-like 2 phenotype. *J Huntington's Dis* 2017;6:37–46. doi: 10.3233/JHD-160232
4. Walker RH, Morgello S, Davidoff-Feldman B, Melnick A, Walsh MJ, Shashidharan P, et al. Autosomal dominant chorea-acanthocytosis with polyglutamine-containing neuronal inclusions. *Neurology* 2002;58:1031–1037. doi: 10.1212/WNL.58.7.1031
5. Walker RH, Rasmussen A, Rudnicki D, Holmes SE, Alonso E, Matsuura T, et al. Huntington's disease-like 2 can present as chorea-acanthocytosis. *Neurology* 2003;61:1002–1004. doi: 10.1212/01.WNL.0000085866.68470.6D
6. Adjobo-Hermans MJW, Cluitmans JCA, Bosman GJCGM. Neuroacanthocytosis: observations, theories and perspectives on the origin and significance of acanthocytes. *Tremor Other Hyperkinet Mov* 2015;5:328. doi: 10.7916/D8VH5N2M
7. Siegl C, Hamminger P, Jank H, Ahting U, Bader B, Danek A, et al. Alterations of red cell membrane properties in neuroacanthocytosis. *PLoS ONE* 2013;8:e76715. doi: 10.1371/journal.pone.0076715
8. Krause A, Mitchell C, Essop F, Tager S, Temlett J, Stevanin G, et al. Junctophilin 3 (JPH3) expansion mutations causing Huntington disease like 2 (HDL2) are common in South African patients with African ancestry and a Huntington disease phenotype. *Am J Med Genet B Neuropsychiatr Genet* 2015; 168:573–585. doi: 10.1002/ajmg.b.32332
9. Storch A, Kornhass M, Schwarz J. Testing for acanthocytosis. *J Neurol* 2005;252:84–90. doi: 10.1007/s00415-005-0616-3
10. Coetzer TL, Palek J. Partial spectrin deficiency in hereditary pyropoikilocytosis. *Blood* 1986;67:919–924.
11. Rudnicki DD, Holmes SE, Lin MW, Thornton CA, Ross CA, Margolis RL, et al. Huntington's disease-like 2 is associated with CUG repeat-containing RNA foci. *Ann Neurol* 2007;61:272–282. doi: 10.1002/ana.21081
12. Seixas AI, Holmes SE, Takeshima H, Pavlovich A, Sachs N, Pruitt JL, et al. Loss of junctophilin-3 contributes to Huntington disease-like 2 pathogenesis. *Ann Neurol* 2012;71:245–257. doi: 10.1002/ana.22598
13. Wilburn B, Rudnicki DD, Zhao J, Weitz TM, Cheng Y, Gu X, et al. An antisense CAG repeat transcript at JPH3 locus mediates expanded polyglutamine protein toxicity in Huntington's disease-like 2 mice. *Neuron* 2011; 70:427–440. doi: 10.1016/j.neuron.2011.03.021