RESEARCH NOTE

A polyalanine antibody for the diagnosis of oculopharyngeal muscular dystrophy and polyalanine-related diseases [version 1; peer review: 3 approved with reservations]

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Abstract
Eighteen severe human diseases have so far been associated with trinucleotide repeat expansions coding for either polyalanine (encoded by a GCN repeat tract) or polyglutamine (encoded by a CAG repeat tract). Among them, oculopharyngeal muscular dystrophy (OPMD), spinocerebellar ataxia type-3 (SCA3), and Huntington's disease (HD) are late-onset autosomal-dominant disorders characterized by the presence of intranuclear inclusions (INIs). We have previously identified the OPMD causative mutation as a small expansion (from 2 in normal to 7 in disease) of a GCG repeat tract in the PABPN1 gene. In addition, -1 ribosomal frameshifting has been reported to occur in expanded CAG repeat tracts in the ATXN3 (SCA3) and HTT (HD) genes, resulting in the translation of a hybrid CAG/GCA repeat tract and the production of a polyalanine-containing peptide. Previous studies on OPMD suggest that polyalanine-induced toxicity is very sensitive to the dosage and length of the alanine stretch. Here we report the characterization of a polyclonal antibody that selectively recognizes pathological expansions of polyalanine in PABPN1. Furthermore, our antibody also detects the presence of alanine proteins in INIs of SCA3 and HD patient samples.

Keywords
trinucleotide repeats, CAG, polyalanine, OPMD, SCA3, HD
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Competing interests: No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Introduction

Expansion of trinucleotide repeated sequences within the coding regions of distinct genes has been established to cause a number of severe human diseases [for reviews, see 1–4]. The expanded coding triplet sequences so far implicated in disease are either CAG repeats, which translate into polyglutamine tracts, or GCN repeats, which encode for polyalanine stretches. The former were shown to cause at least nine distinct adult-onset neurodegenerative conditions such as Huntington’s disease (HD), spinal bulbar muscular atrophy (SBMA), spinocerebellar ataxia (SCA) types 1, 2, 3, 6, 7 and 17 and dentatorubral-pallidoluysian atrophy (DRPLA)14; whereas polyalanine expansions have been implicated in oculopharyngeal muscular dystrophy (OPMD) and in numerous developmental disorders15.

The so-called “polyglutamine” diseases share a number of genetic and molecular events/features; among which are their mutation process (dynamic expansion of their respective CAG repeat), intergenerational repeat instability, anticipation, and a disease course that is progressive following a late onset (10 to 20 years)3. For these reasons, it has been proposed that expanded CAG repeat tract diseases also share, to some extent, a common pathogenic mechanism, whereas the phenotypic variability of each disease would reflect the intrinsic properties the protein in which the repeat resides and the cellular environment where the affected protein is expressed.

Mutant protein aggregation, often in the form of intranuclear inclusions (INIs), is a hallmark of these disorders and INIs were at first believed to be key contributors of the toxicity leading to the neurodegeneration associated with pathological repeat expansions. However, some evidence now suggests that the soluble form of these mutant proteins may be more toxic than their insoluble counterparts found in INIs5, and aggregation might actually protect cells from the toxic insults inherent to misfolded soluble forms of the mutant proteins6–9. Finally, for each of the polyglutamine diseases, the repeat tract expansion mutation affects specific populations of neuronal cells, despite ubiquitous expression of the mutant proteins [for a review see 4]. This could either be due to native properties of each protein, or could be explained by novel interactions of the mutant species with other cellular factors, specific for each cell type.

Fibrillar INIs have also been described in OPMD. OPMD is mainly a disease of skeletal muscle, with some reports suggesting partial neurological involvement10–12. The disease is caused by the expansion of a short polyalanine repeat in the polyadenylate binding protein nuclear 1 (PABPN1) gene13. The INIs can be typically found in the nuclei of affected muscle fibers, but an OPMD transgenic mouse model with expression of PABPN1 with a 13 alanine tract has INIs in muscle cells as well as neuronal cells of the spinal cord and cerebellum, which implies that the polyalanine expansion within PABPN1 can also be toxic to nervous tissues14. This finding was confirmed in post-mortem cerebellar samples of an OPMD patient14.

A -1 base shift in reading frame within an expanded CAG repeat tract would lead to translation of protein polyalanine stretch from the GCA reading frame. Using (SCA3) as a model, we have previously postulated that (i) translational frameshifts in large CAG stretches result in a new reading frame with formation of a hybrid protein containing a mixed polyglutamine/polyalanine tract, (ii) the resultant polyalanine polymers aggregate, and (iii) polyalanine-containing peptides are toxic to cells. We have demonstrated the presence of -1 frameshifting events in cells cultured in vitro, in transgenic Drosophila lines, in mouse organotypic cultures, as well as in pontine neurons from SCA3 human brain autopsy material15–17. In cell culture, switching from polyglutamine to polyalanine seems to be CAG length-dependent and to occur during translation17. More importantly, we have established a direct correlation between the -1 translational frameshifting events (which we will henceforth refer to as frameshifting) and cellular toxicity using a stably transfected cell model. In addition, treating cells with specific antibiotics that are known to either enhance (e.g. sparsomycin) or inhibit (e.g. anisomycin) frameshifting can modulate the frequency of frameshifting events and the associated toxicity. Sparsomycin favours frameshifting by slowing the peptidyl transfer, allowing time for transfer RNA (tRNA) realignment, whereas anisomycin inhibits the accommodation of the frameshifted tRNA to the codon in the -1 frame18,19. Finally, the substitution of the expanded CAG repeat in the ATXN3 cDNA by an expanded CAA repeat of similar length (which also encodes a polyglutamine stretch in the main frame but will not produce polyalanine-containing peptides if a frameshift occurs) abolishes the toxicity of the transgene20–22. These findings suggest a pathogenic role for the -1 frameshifted protein species in SCA3, and possibly in other expanded CAG repeat tract diseases. Frameshifting has recently been shown to occur within the CAG repeats of the huntingtin gene (HTT)23,24, but a clear link has not been established between these events and toxic outcomes in vivo. The question thus remains as to the biological relevance of frameshifting within large CAG repeats of HD patients.

Polyalanine toxicity may underlie a number of severe human disorders. It would therefore be useful to develop a screening tool that would allow the detection of alanine polymers at a size above pathological threshold. A similar tool was developed for the polyglutamine expansion diseases in the form of an antibody directed against polyglutamine25, as well as for the expanded GGGGCC repeat in C9orf72 (amyotrophic lateral sclerosis and frontotemporal dementia; ALS/FTD) in the form of antibodies generated against the dipeptide products which were observed to arise from the pathological expansion of the GGGGCC hexanucleotide, antiC9RANT26. Here we report the characterization of a polyclonal polyalanine-targeting antibody, antibody 4340 (Ab4340), that selectively recognizes pathological expansions of the protein PABPN1 implicated in OPMD, as well as alanine-containing INIs in SCA3 and HD patient samples.

Methods

All the methods used for the work described herein were carried out in accordance with approved guidelines of McGill University (https://www.mcgill.ca/research/researchers/policies). The experimental protocols for the use of animals were approved by Montreal Neurological Institute Animal Care Committee at McGill University.
Production of polyalanine antibody

A 19 GCA repeat sequence was cloned into the multiple cloning site of pGEX-4T1 (Sigma #GE28-9545-49, BamHI and XhoI ligation). The recombinant pGEX-4T1 vector was transformed in E. coli BL-21 to produce GST-polyalanine fusion proteins. The same 19 GCA repeat sequence was cloned in the multiple cloning site of pMAL-c2X vector (NEB #E8200, BamHI and SalI ligation); this second recombinant vector was also transformed into E. coli BL21 to produce MBP-polyalanine fusion proteins. The presence and orientation of the 19 GCA inserts of the two vectors was confirmed using restriction enzymes, agarose gel electrophoresis and Sanger sequencing. Expression vectors from the bacterial clones confirmed to correctly express the two fusion proteins were transformed into E. coli DH5α to be expanded for the affinity purification of GST-polyalanine and MBP-polyalanine through glutathione-Sepharose and amylose resin columns, respectively.

1 mg of purified GST-polyala was injected subcutaneously in two rabbits (following the collection of a pre-immune blood samples to have a pre-immune antisera). Four immune response boosts were subsequently made (4 weeks apart) using the same dose. Bleeds were collected every month for 6 months (8.5 ml/kg). Animals were to be euthanized if the titer was insufficient 6 months after the initial immunization. Through the entire process care was taken to make sure the animals suffering was minimal. Animals were observed 15 minutes post-injections, and also on a daily for responses at the injection sites and signs of overall distress. When blood samples were collected the animals were tranquillized by injecting xylazine (5mg/kg) and ketamine (20–35 mg/kg) intramuscularly. At the end of the antiserum production, the antibodies were euthanized by exsanguination under general anaesthesia and the antisera were prepared for storage at -80°C. Animals were obtained from Charles River Laboratories and maintained (one per cage) at the Comparative Medicine and Animal Resources Center (McIntyre, McGill University).

The specific and further purification of the antisera against polyalanine was established using Western blots loaded with MBP-polyalanine fusion proteins transferred on PVDF membranes (Sigma #P2563). The use of this PVDF transfer and immobilization approach insured two aspects: First, the antisera were detecting the polyalanine region of the fusion peptide used for immunization and not the GST region. Second, the region of the PVDF membranes corresponding to the polyalanine signal could be cut out to elute antibodies that specifically recognized the polyalanine. Aliquots of PVDF eluted polyalanine antibodies were used in the immunodetections of material expressing polyalanine (and control).

Transgenic Drosophila lines

Stocks used in this study were previously described. Full-length ATXN3 cDNAs bearing CAG14 exp, CAG16 exp, CAA9, STOP-CAG14 or STOP-CAA9 repeats were subcloned in pUAST (some vectors have a STOP codon upstream of the repeat). Epitope tags were added to each reading frame: Myc for main-frame, HA for −1 frame and His for +1 frame. Vectors sequenced before injection into w1118 Drosophila eggs; a step followed by selection of positive transformants, mapping and balancing (Genetic Services, Inc.). Flies bearing transgenic constructs in a homozygous state were maintained at 25°C. Adult males were crossed to virgin gmr-GAL4 flies to obtain lines expressing transgenes in developing eyes (exp-GAL4, −16-GAL4, +96-GAL4, CAG/ gmr-GAL4, STOP-CAG/ gmr-GAL4 and STOP-CAA/ gmr- GAL4 genotypes). To obtain isogenic control flies, w1118 males were crossed with virgin gmr-GAL4.

Cell culture and transfections

Epstein-Barr Virus (EBV) immortalized lymphoblastoid cell lines (LCL) were prepared from patient and control individuals in the laboratory of Dr Rouleau. All subjects signed an informed consent, which is part of the experimental protocol approved by the Research Ethical Board (REB) of McGill University Health Research Centre (#NEU-14-051, June 11 2015). LCL and HeLa cells were cultured at 37°C in a humid atmosphere enriched with 5% CO2. HeLa cells (ATCC #ATCC® CCL-2™) cat were grown in Dulbecco’s Modified Eagle Medium (Gibco), supplemented with 10% fetal bovine serum (Gibco) and 1% Penicillin/Streptomycin/Glutamine (Gibco), while the lymphoblastoid cells were grown in Iscove’s Modified Dulbecco’s Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% Penicillin/Streptomycin/Glutamine (Gibco) and Fungizone antifungal (Gibco).

For transient transfections, HeLa cells were transfected at 70% confluence for 48 hours with GFP-tagged hPABPN1 plasmid DNA containing various length alanine expansions (0, 10, 13, 17, 30, and 40) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. These constructs were graciously provided by Dr. Bernard Brais (McGill University), and previously described.

Western blots

48 hours post transfection, HeLa cells were collected in ice-cold phosphate buffered saline (PBS), and lysed in radioimmunoprecipitation (RIPA) buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS)] supplemented with protease inhibitors (Roche), and sonicated for five 1 sec pulses. For OPMD patient and control individual lymphoblastoid cell lines, protein extractions were performed using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific) according to the manufacturer’s instructions.

Membranes were blocked for 24 hours at 4°C in a PBS-T (0.1% Tween-20 in PBS) solution containing 5% milk (instant skim milk powder) and 5% bovine serum albumin (BSA; Fisher), and incubated overnight at 4°C in PBS-T (5% milk and 5% BSA) with one of the following primary antibodies: rabbit monoclonal anti-PABPN1 antibody (1:1,000; Abcam #ab75855); mouse monoclonal anti-GFP antibody (1:5,000; Clontech #632381); or the rabbit polyclonal antibody 4340 (1:500-3,000) developed by the laboratory of Dr McPherson. Membranes were then washed three times for 10 min in PBS-T, incubated for 2 hours at room temperature with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody [donkey anti-mouse IgG antibody
An earlier version of this article can be found as a thesis on the Université de Montréal Institutional Repository (https://papyrus.bib.umontreal.ca/xmlui/handle/1866/13553).

Results

Generation of a polyclonal antibody sensitive to polyalanine at the pathological threshold in OPMD

We generated an antibody (4340) against a 19-mer peptide composed of 18 alanines followed by a glycine. In order to evaluate the usefulness of this antibody, it was critical to determine the number of alanine repeats it could detect. Using OPMD as the disease model and Western blot immunodetection as a first assay, our analyses revealed that the antibody was able to produce a strong signal from whole protein lysates prepared from HeLa cells that transiently expressed a vector encoding a GFP-tagged hPABPN1 cDNA bearing alanine repeat lengths of 13, 17, 30, and 40 (Figure 1a). In contrast, only a weak signal was observed from lysates prepared from cells expressing the same cDNA if it encoded a 10-alanine repeat, and no signal could be observed from lysates prepared from cells that were either expressing a cDNA with no polyalanine tract (0-alanine) or that were untransfected (Figure 1a). To test whether the signals detected were the putative GFP-hPABPN1- alanine proteins, we probed the same samples with an antibody against GFP, and observed corresponding bands at ~75 kDa (Figure 1b). This suggests that the ~75 kDa bands detected by both antibodies correspond to the same protein, whereas the ~55 kDa bands detected by our antibody alone appear to be an unspecific contaminating signal.

HeLa cells that were transfected with the same expression vectors which were used for the Western blot analyses were also used to test the sensitivity of Ab4340 to polyalanine tracts through an in vitro immunofluorescence assay. The fusion of an N-terminal GFP-tag to each construct made it possible to visualize protein expression using fluorescence microscopy. Intranuclear expression with a strong GFP signal was observed across all constructs (Figure 1c to 1h). Using the 4340 antibody, we were able to specifically target the alanine-containing proteins and detect their expression in cells transfected with the expression vectors that encoded repeat lengths of 10, 13, 17, 30, and 40 alanines (Figure 1d to 1h). No alanine signal was detected following the expression of the 0-alanine construct (Figure 1c). The alanine-containing protein appears in aggregates, colocalizing with the GFP-expressing INIs (Figure 1d to 1h). These findings suggest that Ab4340 is more sensitive in detecting alanine expansions using an immunofluorescence assay (immunocytochemistry) than Western blot immunodetection.

Differentiation can be made between OPMD and control patient samples

The results from our Western blot immunodetections based on HeLa cells transiently expressing hPABPN1 cDNA with different polyalanine tracts demonstrate that the sensitivity of Ab4340 coincides with the pathological threshold known to cause OPMD. To determine whether or not it could be used to discriminate between samples obtained from OPMD patients and

Human immunohistochemistry

Formalin-fixed paraffin-embedded OPMD patient and control individual cerebellum samples were sectioned (5 μm) and placed on glass slides. The sections were deparaffinized, rehydrated, and incubated in an antigen retrieval solution (DAKO) at 85°C for 1 hour. Sections were cooled to room temperature, and washed three times in PBS. Immunohistochemical detection was carried out by permeabilizing sections in 0.2% Triton-X100 in PBS for 30 min, followed by blocking in PBS containing 10% normal goat serum (NGS; Gibco) for 1 hour, and incubating with primary antibodies overnight at room temperature [mouse monoclonal anti-ubiquitin antibody (1:1,000, Millipore), rabbit polyclonal antibody 4340 (1:500)]. Biotinylated secondary antibodies were used at a 1:500 dilution, and amplified using the ABC Elite kit (Vector). Reaction product was revealed using the DAB Substrate kit (Vector), mounted with VectaMount (Vector), and visualised on a Leica CTR6000 fluorescence microscope. All subjects signed an informed consent which is part of the experimental protocol approved by the Research Ethical Board (REB) of McGill University Health Research Centre (#NEU-14-051, June 11 2015).

LCL from HD and SCA3 patients, and control individuals were washed in PBS and deposited onto glass slides using a StatSpin Cytofuge 2 (Beckman Coulter) at 7,000 rpm for 4 min. Slides were dried for 30 min at room temperature, and fixed in 4% PFA for 20 min. Permeabilization, blocking, and incubation in primary antibody [rabbit polyclonal antibody 4340 (1:500)] was performed as described above. Cells were then incubated for 2 hours at room temperature in HRP-conjugated secondary antibody [donkey anti-rabbit IgG antibody (1:500; Jackson ImmunoResearch)]. Reaction product was revealed using the Vector VIP Substrate kit, mounted with VectaMount (Vector), and visualized on a Leica CTR6000 fluorescence microscope.

Drosophila immunohistochemistry

Adult flies were decapitated (3 days post eclosion), with heads immediately placed in Tissue-Tek (Sakura) and on dry ice to freeze. Ten micron sections were obtained by permeabilizing a Leica CM3050S cryostat, dried for 30 min at room temperature, and then fixed in 4% paraformaldehyde (PFA) for 15 min. Permeabilization, blocking, and incubation in primary antibodies [mouse monoclonal anti-SCA3 antibody (1:1,000, Chemicon), rabbit polyclonal antibody 4340 (1:500)] was performed as described above. Sections were then incubated with the appropriate fluorescent secondary antibodies for 1 hour (anti-mouse or anti-rabbit fluorescent tagged secondary antibodies, 1:500, AlexaFlour) and mounted with Mowiol. Visualization of immunofluorescence staining was carried out on a Leica CTR6000 fluorescence microscope.

(1:5,000; Jackson ImmunoResearch), or donkey anti-rabbit IgG antibody (1:2,500; Jackson ImmunoResearch # 111-035-144), followed by three 10 min washes in PBS-T. Immunodetection was performed using the enhanced chemiluminescence (ECL) system (Perkin Elmer), and membranes were exposed to HyBlot CL autoradiography film (Denville Scientific Inc.).
control individuals, we performed another series of Western blot immunodetections for which the protein lysates were prepared from lymphoblastoid cell lines (LCLs). Furthermore, we used our 4340 antibody in immunohistochemistry assays of cerebellar sections from OPMD patients and controls.

Western blots probed with Ab4340 reveal a strong signal at ~60 kDa in nuclear lysates prepared from OPMD patient material (Figure 2a, lanes 3–6), whereas no bands were detected in nuclear lysates prepared from unaffected individuals (Figure 2a, lanes 1 and 2). These same lysates were probed with an antibody directed against PABPN1, and a corresponding band at ~60 kDa was observed (Figure 2b). This indicates that the ~60 kDa bands detected by the two antibodies are the same predicted PABPN1-alanine protein. In contrast to the results obtained from HeLa cells, no unspecific contaminant signal was observed from patient lymphoblastoid cell lines.

Immunohistochemistry detections made using Ab4340 and an antibody directed against ubiquitin revealed strongly stained intranuclear structures in cerebellar neurons of the OPMD patient (Figure 2c and 2d). The ubiquitin-detecting antibody also revealed intranuclear signals in sections prepared using tissue sections of a control individual (Figure 2e); however, when Ab4340 was used on similar sections no intranuclear signal was observed (Figure 2f).
Figure 2. Testing the ability of Ab4340 to differentiate between OPMD patient and control individual samples. Western blot immunodetections of alanine (a) and PABPN1 (b) from nuclear extracts prepared from LCLs. Lanes 1 and 2: extracts from control individuals; and Lanes 3–6: extracts from OPMD patients. The 4340 antibody cleanly detected alanine-containing proteins exclusively from OPMD patient extracts [(a), Lanes 3–6], despite strong detection of PABPN1 in all patient extracts (b). Immunohistochemical detection of ubiquitin (c,e) and polyalanine (d,f) containing proteins in cerebellar neurons of an OPMD patient (c,d) and control individual (e,f). Both antibodies immunostained intranuclear structures in the OPMD patient's sample (c,d), whereas only ubiquitin immunostaining was achieved in the control patient's sample (e). Scale bar, 2.5 µm.
Alanine-containing proteins are detected in a transgenic Drosophila model of SCA3, and lymphoblastoid cells of SCA3 and HD patients.

To test whether our antibody could detect polyalanine-containing proteins in polyglutamine diseases that have a propensity to present frameshifting, we investigated SCA3 and HD. Using expCAG92 and isogenic control flies from our previously reported transgenic ATXN3 Drosophila model\textsuperscript{16}, we made immunohistochemical detections with both our 4340 antibody and one directed against ataxin-3. Alanine-containing proteins (red) were observed exclusively within the eyes of expCAG92 flies (Figure 3a). In these same flies, ataxin-3 containing aggregates (green) were present throughout the eye, confirming transgene expression (Figure 3a). No ataxin-3 containing proteins were detected in the isogenic control flies (Figure 3b).

Immunocytochemical detections were also made with LCLs derived from SCA3 patients, HD patients, and control individuals. Ab4340 detected alanine-containing protein aggregates in LCLs from both SCA3 (Figure 3c, arrows) and HD (Figure 3d, arrows) patients, whereas no aggregates were observed in the control individual’s LCLs (Figure 3e). When comparing the number of cells presenting aggregates among the SCA3 and HD patients, their occurrence were observed more frequently in HD patient LCLs.

![Figure 3. Detection of polyalanine in a transgenic Drosophila model of SCA3, and lymphoblastoid cells of an SCA3 and HD patient.](image-url)
Discussion
Ab4340 was assessed for its ability to selectively detect alanine-containing proteins in disease models of OPMD, SCA3, and HD, while confirming that unaffected control individuals would not present significant levels of these same polyalanine peptides. We chose to test the antibody’s sensitivity using OPMD as a model since the protein underlying this pathology contained an expanded polyalanine tract, and this disease shares a number of similarities with polyglutamine expansion diseases: late-onset, autosomal-dominant, repeat expansion effects age of onset and severity, and the presence of aggregated proteins (INIs)\textsuperscript{12,13}. Importantly, of the nine severe human diseases that have been associated with expansions of the polyalanine tract, PABPN1 is the only gene that does not encode for a transcription factor that acts during early development phases\textsuperscript{4}. The results of Western blots prepared using lysates of HeLa cells expressing GFP-tagged hPABPN1 showed that the signal generated by Ab4340 was substantially stronger in lysates of cells where the length of the polyalanine tract was longer than what is found in the unaffected population (10 alanines) and within the pathological threshold (11 to 17 alanines). While fluorescent immunohistochemistry detections of these same HeLa cells did not show a corresponding profile (increased signal in cells expressing a pathological length polyalanine tract) the antibody could discriminate between biological materials of OPMD patients and control individuals; the antibody did so by both Western blots and immunohistochemistry detections. The discrepancy seen with the transient expression assays made using HeLa cells may be due to the combination of the strong cytomagalovirus promoter used and the high sensitivity of the confocal microscopy which could detect lower amounts of fluorescence-tagged proteins\textsuperscript{24}. Another explanation for this discrepancy may be the structural conformation of complexes formed during the aggregation of polyalanine expanded proteins. In vitro studies have shown that polyalanine proteins transition from α-helical monomers to macromolecular β-sheets as the number of alanine residues increase (7 to 15), whereas in vivo these same polyalanine proteins adopt mainly β-sheet conformations\textsuperscript{25–27}. Thus, the affinity of our antibody could be directed toward the α-helical/β-sheet transition complex of 10-alanine repeats found predominantly in HeLa cells transiently expressing them.

In support of earlier reports where we established the occurrences of frameshifting in SCA3 using cell culture, cerebellar and cortical organotypic slice culture, transgenic Drosophila, and patient tissue samples\textsuperscript{15–17}, we detected the expression of alanine-containing proteins in the expCAG\textsubscript{92} Drosophila line, as well as alanine-containing protein aggregates in the LCLs of SCA3 patients. Moreover the antibody could also detect alanine-positive aggregates in LCLs of HD patients; the morphology of these aggregates was similar to what was observed with SCA3. This result with HD LCLs is in agreement with the detection of frameshifted products in human huntingtin (HTT) stable transfectant cells, an HTT transgenic mouse model, and HD patient tissue samples\textsuperscript{19,20}.

It is important to note that Ab4340 did not detect alanine-containing proteins in any samples obtained from control individuals, and this is noteworthy as there are currently over 100 known human proteins to comprise a polyalanine tract of seven alanines or greater\textsuperscript{24}. Since the majority of these polyalanine-containing proteins are DNA binding transcription regulators, which often bind transcription factors, it is likely that they share a similar low level of expression that is below the detection threshold level of our antibody\textsuperscript{24,29}.

In summary, our experiments with Ab4340 demonstrate that it is a valuable tool for the detection of alanine-containing proteins in OPMD, SCA3, and HD. This antibody could be used to screen other “orphan” neurodegenerative or developmental diseases for the presence of expanded alanine tracts which may help uncover new polyalanine diseases. It could also help to further characterize the subcellular localization of proteins containing such polyalanine tracts.

Data availability
Uncropped western blots for Figure 1a and 1b, Figure 2a and 2b are available on Zenodo: http://doi.org/10.5281/zenodo.1068265\textsuperscript{30}

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Competing interests
No competing interests were declared.

Grant information
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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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The authors have identified a new potential method of detection of a rare adult onset neuromuscular disease, oculopharyngeal muscular dystrophy (OPMD) caused by a short, stable, GCN expansion mutation. They also identified a frameshift that produces the same protein in two CAG expansion mutation diseases, spinocerebellar ataxia (SCA) and Huntington disease (HD). The current state of diagnosis of these diseases relies heavily on expensive DNA testing to identify trinucleotide repeat mutations. The research findings were carefully elucidated and confirmed in the case of OPMD to detect only mutant protein lengths obtained from circulating lymphoblastoid cell lines (LCL’s).

The title suggests a clear diagnostic indication and is an exciting concept, potentially more quickly and less expensive that DNA testing methods. It does not identify the mutation length however, and this should be pointed out. In the background, the statement that the CAG repeat disorders are adult onset is not correct with several appearing in childhood. The discussion about soluble and insoluble aggregated proteins which requires further connection to the research findings. Thereafter the term fibrillar INI’s is noted, but without explanation as to how these relate (if at all) to soluble/insoluble aggregates or other points made in results and discussion. There is discussion about the CNS (cerebellar) involvement in OPMD but not how this information relates to the methods except that cerebellar sections were utilized rather than muscle. There is discussion about the role of antibiotics which influence frameshifting which is not further discussed. The background section generally should be tied more closely to the conclusions with clear sections on OPMD versus CAG disorders. Authors should cite prior antibody detection in OPMD by Vest et al. 2015 as seminal work (literature review incomplete).

References

Is the work clearly and accurately presented and does it cite the current literature?
Partly
Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 03 April 2018

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The authors describe an antibody that detects the Ala expanded proteins, and suggest it can be a relevant tool in protein aggregation research and diagnostics. The paper should be reconsidered for indexing after major revisions.

**General comments:**
The authors do not acknowledge previous publication of an antibody detecting Ala expanded PABPN1. The authors must cite that paper and discuss how their tool differ from the one previously published. The authors suggests a “pathological threshold” for Ala expanded proteins. However, how to define this threshold is not described. This term is unclear: is it Ala expansion length (Discussion, first paragraph) or protein accumulation?

**Specific comments:**
**Title:**
The term “polyalanine-related diseases” is unclear.

**Abstract**
The authors write: “from 2 in normal to 7 in disease” however, +1 and +8 Ala expansion were also
reported. (+8 from the same group).
The term causative mutation is unclear.
GCG should be changed to GCN.
Last sentence: should be alanine-expanded proteins.

Introduction is not clearly structured: the authors shift from polyGlu to PolyAla expansion mutations, which can confuse the reader.
The authors suggests: “Polyalanine toxicity may underlie a number of severe human disorders.” There is not sufficient evidence in the literature for this statement. This statement comes without a citation.

Results
Figure 1. It would be useful to add anti-PABPN1 and loading control. It is unclear whether the antibody recognizes Ala13 and Ala17 better than Ala30 and Ala40, or that is due to loading differences. This point is also noticeable in the immunofluorescence: the higher polyAla signal is found in the highest overexpressing cells, regardless of alanine expansion length. Moreover, a strong signal is found in the 10Ala expressing cells. The authors suggests that the polyAla antibody recognizes GFP-containing INIs, but the assay to confirm that (1M KCl or 1% Triton) was not employed. Nearly every anti-PABPN1 antibody detects the protein in immunofluorescence better than in Western blot, because the signal is concentrated to the nucleus (small volume), this is not specific to the polyAla antibody.
Figure 2. Loading control is missing in 2A and 2B. The size of PABPN1 in a Western blot is 50-52 kDa. According to Fig. 2A and2b it is >60 kDa. Another protein ~140kDa is recognized by the polyAla antibody, can you please comment on it? Immunostaining in cerebellar neurons: should we assume that samples were taken from three OPMD patients and three controls (are those healthy controls, what is the age, gender?) This information must be added for both controls and OPMD patients. If the data is from only one patient, it should be clearly indicated, and the conclusions must be tuned down. The authors claim that staining is of intranuclear structures, but the nucleus is not clearly visible. A nuclear staining must be included.
Figure 3. a-b. There is quite some auto fluorescence in the red channel in the a panel, and the overlap with the GFP signal is not specific. It is unclear why there is no auto fluorescence from the in the b panel? From the low magnification image of human samples it is clearly appreciated that there is more signal in cells from patients compared with control. But the foci the authors pointed to in the higher magnification are also seen in control, in the low magnification image. Also in that presentation, the descriptions of patients and control must be included. If the authors wish to specify intranuclear staining, a nuclear staining must be added. The structure of foci in those patients’ cells, seems very different from those in OPMD. Can the authors comment on it?

Discussion:
Since the results from SCA3 and HD are from only 1 patient per condition, the conclusions must be tuned down.
The authors show aggregates in cerebellar neurons in OPMD. Do this/those patients have neurological defects? There is one sentence in the Introduction about neurological defects. However, the observations in Figure 2 should be brought into a clinical perspective.

Literature list: misses citation of recent publications. For example:
1- “OPMD is mainly a disease of skeletal muscle, with some reports suggesting partial neurological involvement.” There are more recent publications showing neurological defects also in heterozygous OPMD patients, in the homozygous it is more evident. If the authors wish to describe that neurological defects in OPMD they should cite also the recent publications.
2- The authors suggests the antibody could be a relevant tool to study translational frameshift in polyGlu expansion disorders. It would have been more convincing if the authors include more citations, rather than their own work.
References

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
No

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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In the manuscript entitled « A polyalanine antibody for the diagnosis of oculopharyngeal muscular dystrophy and polyalanine-related diseases », Stochmanski et al describe the validation of a polyclonal antibody ab4340 that selectively recognizes pathological expansions of polyalanine proteins in OPMD, SCA3, and HD. Using a GST-tagged alanine peptide they immunized rabbits and obtained an antibody
that detect a whole range of alanine residues (from 13 to 40). Overall I support the publication of this work in MNI Open Research. However, I do have several comments that should be addressed:

- Vest et al published in 2015 the description of a comparable antibody in J Neuromuscul Dis\(^1\). This reference must be added and discussed.
- The genotyping of the OPMD patients (samples used for histology and cell lines) is not mentioned and should be added.
- There is a discrepancy between the Methods and Results sections: the authors should clarify if their antibody was made with a 19GCA (= 19ala) peptide or 18ala+1gly? If a glycine was used this needs to be explained. Could the authors better justify the choice of this number of alanine for the GST construct?
- While the observation of INIs in the cerebellar neurons of an OPMD patient is of great interest, an immunostaining using the ab4340 antibody (with and without KCl 1M treatment) on a muscle section would be also informative.
- In figure 2a-b the band observed appear very high compared to classical western-blot on PABPN1. In figure 2d, could the authors perform a KCl treatment to confirm that the staining observed are insoluble intranuclear inclusions? A control staining against wtPABPN1 (using Abcam ab75855 for example) would also be useful to compare both staining.
- There is a mistake in the abstract: the authors wrote “from 2 in normal to 7 in disease” when it should read “from 2 to 7 in disease”. Besides, it should be “from 1 to 8 in disease” with the addition of references Jouan et al Can J Neurol Sci. 2014\(^2\) (publication from the authors) and Richard et al J Neuromuscul Dis. 2015\(^3\).
- Reference 12 is not correctly cited. The reference should be mentioned for the first sentence of the paragraph and not the second one regarding neurological involvement.

References

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable
Are all the source data underlying the results available to ensure full reproducibility? Yes

Are the conclusions drawn adequately supported by the results? Yes

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.