The huntingtin protein in Huntington disease

Schut, M.H.

Chapter 5

Huntingtin aggregation in adult onset and juvenile HD.

M.H. Schut[1], I. Hegeman[2], M. Eszes[3], R.L.M. Faull[3], G.J.B. van Ommen[1], and W.M.C. van Roon-Mom[1]

1 Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands

2 Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands

3 Centre for Brain Research and Department of Anatomy with Radiology, University of Auckland, Auckland, New Zealand

Partly published in GLIA (2017)

Abstract

The presence of protein aggregates within the brain is a key hallmark of Huntington disease. To better understand regional differences in HD pathology and the link with htt aggregation, it is important to quantify htt aggregates in different brain regions. In this small-scale pilot study we have assessed the correlation between the presence of insoluble htt protein as determined by the filter-trap assay, and counted aggregates in brain tissue sections. Our results indicate that the filter-trap assay is a promising technique to quantify protein aggregates in *post-mortem* human brain tissue.

Introduction

Huntington disease (HD) is an autosomal dominant neurodegenerative disease [1] caused by elongation of an exonic CAG-repeat within the *HTT* gene [2]. This mutation results in a mutant huntingtin protein (mutHtt) with an expanded polyglutamine repeat (polyQ) [3] that is prone to aggregation [4]. The presence of mutHtt protein aggregates is a key hallmark of HD [2, 5]. However, the link between mutHtt aggregates and HD pathology is elusive. A pathological role of mutHtt aggregates in HD has been suggested in some studies [6, 7]. However other studies suggests that mutHtt aggregate formation is beneficial for cells [8, 9], which indicates that soluble mutHtt is more toxic. The quantification of mutHtt aggregates in HD brain tissue should aid to further elucidate their role in HD pathogenesis. Quantification of protein aggregates can be performed by counting aggregates in tissue sections using a microscope, which is a cumbersome procedure. The filter-trap assay is a simple assay used to objectively quantify mutHtt aggregates [10] and has been used with PC12 cell lysates [11], and brain homogenates of R6/2 mice [12]. In this pilot study, we have used the filter-trap assay to quantify mutHtt aggregation in *post-mortem* human brain tissue. Our results indicate that there could be a correlation between the filter-trap assay and the number of aggregates as counted in tissue-sections. Furthermore, we found more mutHtt aggregates in cortical tissue compared with striatal tissue in one juvenile Huntington disease case which is in concordance with the immunohistochemistry results from [8]. Our results suggest that the filter-trap assay can be used to quantify mutHtt aggregates in *post-mortem* human brain tissue.

Materials and Methods

Post-mortem human brain tissue lysates.

Post-mortem human cortical and striatal brain tissue from control and HD subjects was obtained from the Neurological Foundation of New Zealand Human Brain Bank, Centre for Brain Research, University of Auckland and the department of pathology, Leiden University Medical Centre. Tissue was obtained with the approval of the institutes ethics committee and informed consent. Clinical information in **table 1**.

Table 1. Clinical information of subjects

Type	Subject	Grade	Gender	PMD	CAG	Age	A.o.O.
Control	H121	n.a.	F	6	18 : 23	64	n.a.
HD	HC105	1	F	9	15 : 42	67	47
HD	HD166	2	M	32	17 : 42	80	>70
HD	HD193	3	M	18	9 : 44	68	44
HD	HC90	3	M	NA	23 : 41	61	51
HD	HC140	3	M	22	17 : 40	62	44
HD	HC125	3	F	13	17 : 43	67	45
HD	HC139	3	F	5	14 : 41	67	15
jHD	HC104	3	M	15	18 : 53	40	15
jHD	HD29	NA	F	11	20 : 84	11	8
jHD	HD86	3	F	20	17 : 84	20	16
jHD	HD192	NA	M	NA	17 : 51	37	NA
jHD	HD208	NA	M	NA	15 : 68	37	~ 23

HD = Huntington disease, jHD = Juvenile Huntington disease, F=Female, M=Male, PMD = Post-mortem delay, A.o.O. = Age of onset, n.a. = not applicable. NA = not assessed.HD = subject with a mutant CAG repeat between 35 and 50. jHD = subject with a mutant CAG repeat of 50 or more.

Immunohistochemistry

Slide preparation: Tissue sections (thickness 8µm) were formalin fixed and embedded in paraffin. After paraffin removal, tissue sections were rehydrated and washed with PBS + Triton X-100. Sections were incubated in 50% methanol. Primary antibody (rabbit anti huntingtin): 3702-1 (Epitomics, Burlinggame CA, USA), or mouse anti htt EM48 (Millipore, Billerica MA, USA). Primary antibodies were diluted 1:500. Negative control: sections not incubated with a primary antibody. Secondary antibody: goat anti-rabbit or goat anti-mouse (Santa Cruz, Dallas TX, USA) diluted 1:1000. Streptavidin/DAB: Tissue sections were incubated with Streptavidin-HRP (diluted 1:2000 in 1% normal goat serum), followed by 10 minutes of incubation in DAB solution. Sections were Nissl-counterstained and examined for aggregates by microscope. Qualitative determination of aggregate abundance: Total number of aggregates for any combination of HD (adult-onset/juvenile), antibody (EM48/3702-1) and brain region (cortex/striatum) was subtracted by the number of aggregates counted in the corresponding negative control. The resulting number determines the qualitative abundance of aggregates where "+" = 1-5, "++" = 6-10, "+++" = 11-15, "++++" = > 15.

Preparation of insoluble protein fractions

Homogenization of human brain tissue sections: bullet blender (Next Advance, str 8, 3 min). Homogenization buffer: 150mM Sucrose, 15mM HEPES pH7.9, 60mM KCl, 0.5mM EDTA pH8 and 0.1mM EGTA pH8 (W/V ratio: 1:5). Added Triton X-100 (final concentration 1%). Stored on ice (1 hour). Preparation of pellet fraction: Homogenized tissue was centrifuged (14000rpm/10min) followed by 3x wash in wash-buffer (60mM Tris) subsequently followed by pellet-resuspension in 15% SDS and an o.n. incubation at 95ºC. The bicinchoninic (BCA) assay kit (Thermo Fisher Scientific,Waltham, USA) was used to determine protein concentration.

Filter-trap assay

Dot-blot: 100µg of pellet fraction in 300 µl of 15% SDS was applied onto a cellulose acetate membrane (Schleicher & Schuell,St Louis, USA, 0.2µm pore-size) with the Bio-Rad Bio-Dot apparatus (Bio-Rad, #1706545). Wash: 2x in 0.2% SDS. Fixation: 0.5% glutaraldehyde (20 min). Blocking of blot membranes was performed with 4% non-fat milk (Nutricia, Schiphol, The Netherlands) in TBST. Primary antibody (rabbit anti huntingtin): 3702-1, diluted 1:5000 (Epitomics). Secondary antibody: HRP conjugated goat anti rabbit, diluted 1:10000 (Santa Cruz). Visualization of results: ECL (#32106, ThermoFisher) and Hyperfilm ECL (#28906837, GE healthcare, Little Chalfond, United Kingdom). Quantification of signal intensity was performed with ImageJ-software. Results represent the average of three independent filter-trap assays.

Results

Immunohistochemistry reveals more huntingtin aggregates in juvenile HD compared with adult-onset HD tissue sections.

Immunohistochemistry was performed on four adult-onset HD subjects (HC90, HC125, HC139 and HC140, average age = 64.3y ±3.2y, average mutant CAG = 41.3 ± 1.3), and four juvenile HD subjects (HD86, HC104, HC192 and HD208, average age = 33.5y ±9.1y, average mutant CAG = 64.0 ± 15.3). In **Table 1**, a qualitative overview of the amount of aggregates in adult-onset HD or juvenile HD cases is shown. On average, htt aggregates were more abundant in the juvenile HD tissue sections.

Table 1 – Qualitative determination of aggregates in HD subjects

Adult-Onset HD	Cortex	Striatum	Juvenile HD	Cortex	Striatum
EM48	+	+	EM48	+++	+++
3702-1	+	+	3702-1	++++	+++

Immunohistochemistry was performed with either EM48 or 3702-1 as primary antibody.
Note: juvenile HD is defined here as any case with a CAG repeat of 50 or longer.

Insoluble huntingtin protein levels in Huntington disease subjects

For the filter-trap assay, we first used cortical brain tissue lysates from five HD subjects with increasing polyQ-repeats, and one non-HD subject as a negative control **(Figure 1)**. As expected, the negative control (H121) did not produce a significant signal indicating no insoluble huntingtin protein is present. Filter-trap analysis on the five HD subjects showed that the average signal-intensity per subject, as measured from three independent filter-trap assays, showed increased intensity of the filter trap assay dots with larger polyQ-repeats **(Figure 1)**. This was in agreement with the qualitative determination of aggregates in HD subjects shown in **table 1**.

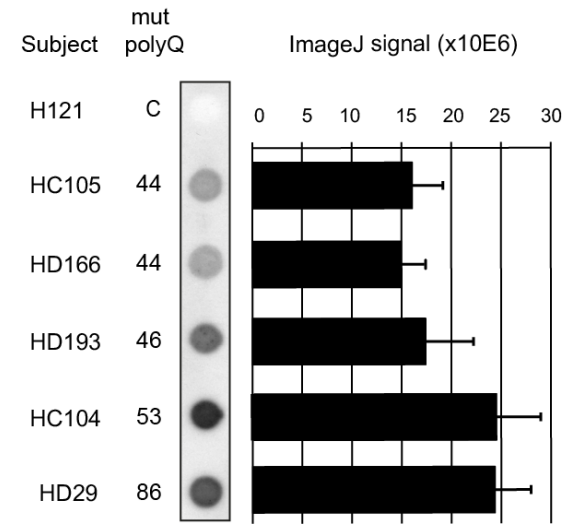

Figure 1 - Insoluble htt proteins in human adult-onset and juvenile HD brain tissue.

Left Filter-trap assay of SDS-insoluble htt protein in cortical human brain tissue of one control subject (H121), three adult-onset HD subjects (HC105, HD166, HD193) and two juvenile HD subjects (HC104, HD29). PolyQ-lengths shown next to dots. 100µg of protein was loaded per dot. Blots were probed with an anti htt antibody. Dot intensity indicates level of insoluble htt protein. *Right* Comparison of dot intensities per subject as determined with imageJ analysis. Black bars represent the average dot intensity from three independent dot blot assays. Standard deviation indicated by error bars.

Insoluble huntingtin protein in subject HD86 Cortex and Striatum

From one juvenile HD case we independently analyzed brain tissue lysates originating from the striatum and cortex using the filter-trap assay **(Figure 2)**. For this subject, our results clearly show a significant difference, with more insoluble huntingtin present in the cortical sample (**Figure 2**).

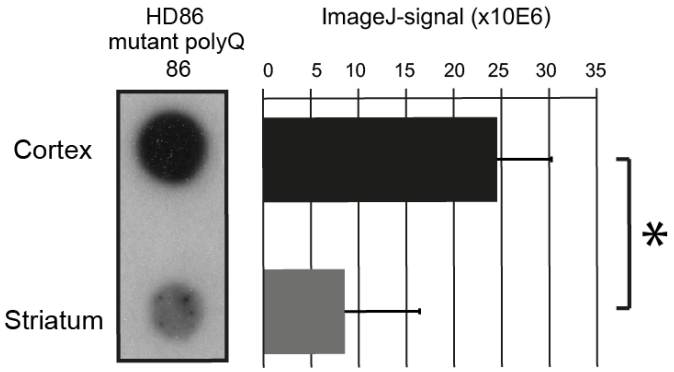

Figure 2 - Insoluble htt protein in cortex and striatum of juvenile HD subject HD86. *Left* Filter-trap assay of SDS-insoluble htt protein in cortical and striatal human brain tissue of one juvenile HD case. 100µg of protein was loaded per dot. Blots were probed with an anti htt antibody. Dot intensity indicates level of insoluble htt protein. *Right* Comparison of dot intensities per area as determined with imageJ analysis. Bars represent average dot intensity from three independent assays. Standard deviation indicated by error bars. * = P<0.05.

Discussion

The results of our pilot-study are encouraging for the use of the filter-trap assay in the quantification of protein aggregates in *post-mortem* human HD brain tissue. No discernible signal was obtained with the non-HD control subject which indicates that, despite the use of human brain material and the rigorous procedure to suspend the protein pellet, background is not an issue. We observed that the signal intensity for insoluble htt protein showed an increase with increasing polyQ repeats. One possible explanation is that increased polyQ repeats are more prone to aggregate formation [13]. The current study, does not elucidate whether the increased signal is due to more efficient aggregation of larger polyQ-repeats, or is merely mirroring differences in protein expression levels. In an earlier study, we have looked at soluble wild-type and mutant huntingtin protein levels in *post-mortem* human HD brain tissue [14]. Results from that study indicate that soluble mutant huntingtin protein levels were lower for juvenile HD cases. This suggests an inverse correlation between levels of aggregated and soluble mutant huntingtin, which is in accordance with results obtained by Baldo *et al* [12]. In addition, we demonstrated the filter-trap assay's ability to assess differences in the level of insoluble htt protein within different brain regions. The difference in insoluble huntingtin protein levels we found in our juvenile HD subject HD86 are in concordance with the results by Gutekunst *et al* [8] who found that huntingtin aggregation was predominantly present within the cortex. In conclusion, the data from this pilot-study provide a proof-of-principle of the filter trap assay to be used as a quantitative assay for determining the level of huntingtin protein aggregation in *post-mortem* human brain tissue.

References

1. Roos, R.A., *Huntington's disease: a clinical review.* Orphanet J Rare Dis, 2010. **5**: p. 40.
2. *A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group.* Cell, 1993. **72**(6): p. 971-83.
3. Aronin, N., et al., *CAG expansion affects the expression of mutant Huntingtin in the Huntington's disease brain.* Neuron, 1995. **15**(5): p. 1193-201.
4. Legleiter, J., et al., *Mutant huntingtin fragments form oligomers in a polyglutamine length-dependent manner in vitro and in vivo.* J Biol Chem, 2010. **285**(19): p. 14777-90.
5. DiFiglia, M., et al., *Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain.* Science, 1997. **277**(5334): p. 1990-3.
6. Yang, W., et al., *Aggregated polyglutamine peptides delivered to nuclei are toxic to mammalian cells.* Hum Mol Genet, 2002. **11**(23): p. 2905-17.
7. Costanzo, M., et al., *Transfer of polyglutamine aggregates in neuronal cells occurs in tunneling nanotubes.* J Cell Sci, 2013. **126**(Pt 16): p. 3678-85.
8. Gutekunst, C.A., et al., *Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology.* J Neurosci, 1999. **19**(7): p. 2522-34.

9. Arrasate, M., et al., *Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death.* Nature, 2004. **431**(7010): p. 805-10.
10. Heiser, V., et al., *Identification of benzothiazoles as potential polyglutamine aggregation inhibitors of Huntington's disease by using an automated filter retardation assay.* Proc Natl Acad Sci U S A, 2002. **99 Suppl 4**: p. 16400-6.
11. Scotter, E.L., et al., *High throughput quantification of mutant huntingtin aggregates.* J Neurosci Methods, 2008. **171**(1): p. 174-9.
12. Baldo, B., et al., *TR-FRET-based duplex immunoassay reveals an inverse correlation of soluble and aggregated mutant huntingtin in huntington's disease.* Chem Biol, 2012. **19**(2): p. 264-75.
13. Li, S.H. and X.J. Li, *Aggregation of N-terminal huntingtin is dependent on the length of its glutamine repeats.* Hum Mol Genet, 1998. **7**(5): p. 777-82.
14. Evers, M.M., et al., *Making (anti-) sense out of huntingtin levels in Huntington disease.* Mol Neurodegener, 2015. **10**: p. 21.