C9orf72 expansions are the most common genetic cause of Huntington's disease phenocopies

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Abstract

Objective: In many cases where Huntington's disease (HD) is suspected, the genetic test for HD is negative: these are known as HD phenocopies. A repeat expansion in the *C9orf72* gene has recently been identified as a major cause of familial and sporadic frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS). Our objective was to determine whether this mutation causes HD phenocopies.

Methods: A cohort of 514 HD phenocopy patients were analysed for the *C9orf72* expansion using repeat-primed PCR. In cases where the expansion was found, Southern hybridisation was performed to determine expansion size. Clinical case notes were reviewed to determine the phenotype of expansion-positive cases.

Results: 10 subjects (1.95%) had the expansion, making it the commonest identified genetic cause of HD-phenocopy presentations. The size of expansion was not significantly different from that associated with other clinical presentations of *C9orf72* expanded cases. The *C9orf72* expansion-positive subjects were characterised by the presence of movement disorders including dystonia, chorea, myoclonus, tremor and rigidity. Furthermore the age of onset in this cohort was lower than previously reported for subjects with the *C9orf72* expansion, and included one case with paediatric onset.

Discussion: This study extends the known phenotype of the *C9orf72* expansion, both in age of onset and movement disorder symptoms. We propose a revised clinico-genetic algorithm for the investigation of HD-phenocopy patients based on these data.

Introduction

Huntington's disease (HD) is an autosomal dominantly inherited neurodegenerative condition typically characterised by a triad of psychiatric, movement and cognitive impairment. In many cases where HD is suspected clinically, patients lack the CAG repeat expansion that causes HD[1-4]. Such individuals are said to have HD phenocopy syndromes or HD-like disorders[5]. Wild & Tabrizi[3] reviewed genes identified in different HD phenocopy cohorts to determine that Spinocerebellar ataxia 17 (*TBP*) accounts for 1.1%, Huntington's Disease-Like 2 (*HDL2*) for 0.7%, Friedreich's ataxia (*JPH3*) for 0.35% and inherited prion disease (*PRNP*) for 0.24% of HD phenocopies. Testing for these mutations is now routinely performed; however the majority of HD phenocopy patients still do not attain a formal genetic diagnosis.

In 2011 an expanded hexanucleotide repeat in the *C9orf72* gene was identified in large kindreds with FTLD and ALS[6, 7]. This expansion is recognised as the commonest genetic cause of ALS and FTLD in many but not all populations[6-9]. The mutation is intronic, in a highly conserved gene[6, 10] which has homology with the DENN-like superfamily suggesting a role as regulator of membrane traffic[10-12], and which may be involved in other neurological conditions[13]. Several hundred-thousands of repeats have been documented in pathogenic expansions[14]. Elucidating the pathogenic mechanism of this expansion has generated much interest; several non-mutually exclusive possibilities exist[6, 15-19].

In this study we undertook to examine whether the *C9orf72* expansion causes HD phenocopy clinical presentations, and hence whether testing for it should be considered in the routine genetic work-up of this patient group.

Subjects and methods

Case ascertainment

As previously described[20], subjects were classified as having HD phenocopy syndromes on the basis of a clinical presentation consistent with HD when assessed by an experienced neurologist or neurogeneticist, and a negative test for the expanded CAG repeat in the *HTT* gene which causes HD (<36 repeats). At the Neurogenetics Unit of the National Hospital for Neurology and Neurosurgery (NHNN), London, UK, 63.5% of diagnostic HD tests (those done on symptomatic patients) are negative for HD. A cohort of 514 HD phenocopy cases who underwent negative diagnostic genetic testing for HD at NHNN were identified. The average age at onset in this cohort was 48.8 years in those with precise onset data (SD 19.3, N=176). 300 subjects were seen at NHNN, 214 at other hospitals. Of those seen at NHNN, 45.3% were seen by a Movement Disorders Consultant, 15.3% by a Cognitive Disorders Consultant, 14.3% by a Neurogenetics Consultant and 25% by other Consultant Neurologists.

Clinical summaries were reviewed for all cases, and all available clinical case notes reviewed for cases positive for the *C9orf72* expansion mutation. Demographic data, family history, examination findings, first symptoms and age of onset were recorded. Where available, neuropsychometry reports were reviewed, and additional investigations were documented including electrophysiological assessments, MRI, CSF and tissue biopsies. HTT CAG repeat length was recorded. Fisher's exact test (Stata software) was used to examine the relationship between the presence of particular clinical signs and gene test outcome.

All *C9orf72*-positive cases were given a modified Goldman score[21, 22], which was used to quantify the strength of the autosomal dominant family history.

Standard Protocol Approvals, Registrations, and Patient Consents

Ethical approval to undertake these analyses was given by the local NHNN/ION ethics committee. Informed consent for genetic studies was obtained from all participants.

Repeat primed PCR (rpPCR)

To test for the presence of an expansion at *C9orf72*, rpPCR was carried out as previously described[7]. Fragment length analysis was undertaken on an ABI 3730xl automated sequencer. Analysis of repeat primed PCR electropherograms was performed using Peak Scanner v1.0 (ABI). Expansions with a characteristic 'saw-tooth' pattern were identified and put forward for Southern blotting.

Rs3849942 genotyping

The surrogate marker rs3849942, reported to be associated with an increased risk of mutation[6, 14], was genotyped by allelic discrimination using the 5' nuclease assay in conjunction with Minor Groove Binding (MGB) probes. The assay was performed on the SDS7500 Fast Real Time PCR system (ABI) and genotyping calls were made using software v2.0.6.

Southern Hybridisation

A recently described Southern hybridisation protocol was used[14]. This combined the use of an oligonucleotide $(GGGGCC)_5$ probe which targets multiple sites within the expansion and genomic DNA (gDNA) digested with two frequently cutting restriction endonucleases whose

sites closely flanked the repeat region. Hexanucleotide repeat number was estimated by interpolation of autoradiographs using a plot of $\log_{10}$ base pair number against migration distance which was created in Microsoft Excel.

Results

Genetic analyses

Of the 514 HD phenocopy cases screened, 10 probands (1.95%, 95% CI 1-4) were positive for the *C9orf72* expansion, making this mutation the commonest identified cause of HD phenocopy syndromes in a UK cohort[20].

Genotyping of the *C9orf72*-positive cases was consistent with all previous reports in that these individuals were either heterozygous or homozygous for the rs3849942 A allele[6] (Table 1). No *C9orf72*-positive cases had intermediate sized HD CAG repeats in the Huntingtin gene, and there was no correlation between the larger HD normal allele and age of onset.

Table 1.

Southern hybridisation (Table 1 and Figure 1) of 8/10 subjects for whom there was sufficient DNA demonstrated that the size of expansion in this HD Phenocopy case series was not significantly different from that found in series with other clinical presentations of the *C9orf72* expansion[14]. There was no significant difference in expansion size between those with and without chorea/dystonia.

Figure 1: 'Southern Blot of eight HD phenocopy patient DNAs'.

Of the entire cohort, 19.5% had a family history of similar neurodegenerative disease whereas 70% of *C9orf72*-positive cases had a positive family history (see Goldman scores, table 1). These results suggest that there is a predominance of those with family history, but sporadic *C9orf72*-positive cases may be possible.

Clinical features of *C9orf72* expansion gene carriers (table 2)

The mean age of onset was 42.7 years, range 8-60. Early psychiatric and behavioural problems were common; they were the first recorded symptoms in six of the cohort. Depression occurred in four, obsessions in two, apathy in two and psychosis in two cases.

Movement disorders were a prominent feature in this cohort - three exhibited chorea, four dystonia, four myoclonus and three tremor. Six of the ten subjects had rigidity and five bradykinesia. Chorea was observed periorally in one, was generalised with predominant head and arm involvement in one, and in the left arm and leg in another. Of the four subjects with dystonia, three were observed to have torticollis. In four of the ten subjects upper motor neuron signs were noted; lower motor neuron signs were not observed in any.

Cognitively, executive dysfunction was noted in six subjects, and memory impairment was present in six; in subject 6 for whom limited history was available, 'cognitive impairment' was noted.

Of eight cases with available MRI reports four had generalised atrophy.

Case 4 was found to be homozygous for the *C9orf72* expansion mutation and has been described in detail in Fratta *et al*[23].

Comparisons between *C9orf72* positive cases and the rest of the HD phenocopy cohort

To examine whether there are particular HD phenocopy cases in whom *C9orf72* testing should be prioritized, we compared the frequencies of symptoms and signs between the whole cohort and those with the expansion (table 3). Fisher's exact test was performed to investigate association between each clinical feature and the outcome of the *C9orf72* genetic test. The presence of cognitive and psychiatric features, and some movement disorder features (dystonia, bradykinesia/rigidity, tremor, myoclonus and upper motor neuron features), were significantly associated with a positive *C9orf72* test (table 3). Though there may be some degree of ascertainment bias as more clinical detail was recorded for positive cases, it remains clear that many symptoms characteristic of HD phenocopies are associated with a *C9orf72* gene expansion.

An illustrative case:

Case 5, a right-handed Caucasian woman, had a normal birth and development and was university educated. She worked in a professional job and was well until a sudden bereavement when she was fifty after which she became depressed.

At around 55y increasing fatigue was noted and she had her first falls, initially backwards. She stopped working, and developed a change in personality with decreased interest in her environment and child-like behaviour. She developed hypophonia and slurred speech.

By 58y she was having difficulty mobilizing and within 12 months went from independent-living to being mute, profoundly bradykinetic and requiring a hoist to transfer. She developed dystonic posturing of her feet and hands, and involuntary movements and a tremor in her lower limbs.

In her family history, her father died of dementia without motor problems aged 69y.

She was admitted to hospital for investigation aged 60y. On examination there was akinetic mutism with marked axial rigidity. There was left laterocollis, minor right torticollis, perioral movements and occasional right cheek movements. There was broken pursuit and slow broken saccades. There was moderate rigidity with spasticity in the upper limbs and severe rigidity in the lower limbs. Plantars were extensor. Palmomental and pout reflexes were present. There was perseveration and frontal features. MMSE (mini mental state examination) was 16/25. (See supplementary information, case 1, for more detail of clinical investigations undertaken.)

An unusual case:

Case 7, a right-handed Caucasian man, had a normal birth and early development. Aged three at nursery school, it was noted that he did not mix well with the other children. At primary school aged five he was found to have slight difficulties with writing; aged six he was unable to follow basic lessons. Soon thereafter he was seen by an educational psychologist and was diagnosed as having moderate learning difficulties and was transferred to special needs school.

By age 8y, he had abnormal movements under stress, particularly affecting his hands and head. These became a lot more prominent from 21y when they affected his walking. Occasionally his right leg was noted to jerk uncontrollably from under him, and he had some falls. The ‘fidgeting’ and jerking movements of hands and neck deteriorated. From 21y he had increased frustration and aggression.

His parents are non-consanguineous. His maternal grandmother died of motor neuron disease; both parents were well.

Aged 23y he was admitted to hospital for investigation. Gait was slightly broad based, with both arms tending to hold slightly dystonic postures, particularly on the right. There was decreased arm swing, nuchal more than axial rigidity, unsteadiness on heel-toe walking, and Romberg's test was negative. Eye movements were abnormal, with poor gaze initiation, impaired pursuit, saccadic hypometria with head thrusts, and reduced vertical up-gaze. There was generalised chorea with mainly head and arm involvement, oro-buccal chorea, myoclonic movements of the head and neck, and some additional dystonic elements with mild bradykinesia. In the limbs there were prominent irregular myoclonic jerks, exacerbated by movement and stimuli. Reflexes and sensation were normal.

MMSE was 20/28. On Neuropsychological examination, the Wechsler Adult Intelligence Scale-Revised was within the defective range consistent with learning difficulties. There was evidence of memory impairment for visual and verbal memory.

MRI scan showed one small lacune. Nerve conduction studies and electromyography were normal. Electroencephalography revealed a diffuse and non-specific excess of theta activity with only a trace of alpha like activity. Although the bursts of high voltage slow activity had a bursting paroxysmal quality no definite epileptiform activity was seen**.** (See supplementary information, case 2, for more detail of clinical investigations undertaken.)

Discussion

Huntington's disease is the most common genetically determined neurodegenerative disease with a prevalence of at least 12.4 per 100,000 people[24], but in those in whom HD is suspected but patients do not have a CAG repeat expansion in HTT, attaining genetic diagnosis has been rare (2.8%[20]). Here we present data demonstrating that the *C9orf72*

expansion is the commonest-identified genetic cause of HD phenocopy presentations in a UK cohort, with a prevalence of 1.95% (95% CI 1-4).

HD is an autosomal dominant condition, classically presenting with a triad of movement, cognitive and psychiatric symptoms. However there is clinical heterogeneity, particularly early in disease, and not all characteristic features may be apparent: 90% of adults with HD develop chorea, but the clinical spectrum is broad, including Parkinsonian akinetic-rigid syndromes and relatively pure dystonic, ataxic and psychiatric presentations[25]. Around 8% of patients with HD present without an apparent family history of HD[26]. Because of this clinical diversity, it is accepted[3, 20] that any definition of Huntington's disease phenocopy syndromes need encompass not only the classical triad of HD but also syndromes having a major degree of overlap with HD, and those without a known autosomal dominant family history. Those patients with a clear family history of HD and with classical manifest HD are more likely to have HD, however many patients seen by Neurologists do not present in such a clear cut manner. Our cohort is composed of patients seen by experienced neurologists in whom the diagnosis of HD was considered thus it reflects clinical reality. It is UK-based, and given that UK-based cohorts have similar ethnic descent to other European, Australian and North American cohorts, our findings are likely to be representative of cohorts from these areas. In patients of African origin (particularly Southern Africans), *JPH3* expansion remains the commonest cause of HD-like presentations[27]. Identifying the causes of HD phenocopy syndromes is of importance to the diagnosis and management of patients with these presentations, as well as the counselling of such individuals and their relatives in matters of genetic testing, life choices and reproduction[3].

Diagnostic tests for this novel mutation have recently become available. Many symptoms characteristic of HD were associated with the subject being *C9orf72* positive; given this, and the high frequency of *C9orf72* expansion among HD phenocopies mean that we believe that it should be tested for in all HD phenocopy cases. In the future it is likely that multi-gene 'disease panels' will supersede the need for sequential genetic testing, however since *C9orf72,* like many other causes of HD phenocopies is an expansion mutation, it will remain important for the clinician to be aware of which tests are most appropriate for different patients and request them accordingly. We propose a revised clinico-genetic algorithm for the investigation of HD phenocopy cases in Figure 2.

Figure 2: 'Algorithm for the investigation of HD phenocopy cases'.

The effects of the *C9orf72* expansion are known to be both clinically and pathologically varied[28] and it is the major cause of both familial and sporadic ALS and FTLD, which are themselves phenotypically heterogeneous conditions. Parkinsonism, particularly rigidity and bradykinesia, has been previously noted in *C9orf72*-positive individuals[29-31]; the *C9orf72* mutation has been found in some cohorts of patients with Parkinson's disease[32] and not others[30, 33, 34]. In this study we have demonstrated that the clinical phenotypes caused by *C9orf72* expansion mutations are broader than previously noted to date. It can present with a movement disorder including chorea, dystonia, myoclonus and tremor. The combination of movement disorder, cognitive decline and psychiatric and behavioural problems, often with a family history of similar problems, explains why *C9orf72*-positive cases can have a presentation very similar to HD. It is notable that ALS-type symptoms were relatively infrequent in the HD phenocopy *C9orf72* cases: none had lower motor neuron signs, while 40% had upper motor neuron signs. By contrast, symptoms more

characteristic of FTLD such as cognitive impairment were much more prevalent, suggesting that there is more overlap between the HD-like and FTLD-like cases.

The average age of onset for *C9orf72* in published reports is around 57 years[7, 9, 31, 35], in this study it is lower at 42.7 years, with range 8 – 60, suggesting that the condition should be considered in the differential diagnosis not only in a wider range of clinical presentations, but in a wider demographic group than previously identified.

We examined whether the difference in phenotype could be accounted for by a different size of expansion by Southern hybridisation: the size of expansion in our HD phenocopy cohort was not significantly different from that of other cohorts[14]. Furthermore, among the 8 *C9orf72*-positive subjects examined here, there is no statistically significant association between expansion size and age of onset. Case 7, who had motor onset at 8y, underwent whole-exome sequencing; no large-scale structural abnormalities were detected. An important caveat is that there is evidence of reduced penetrance of the *C9orf72* expansion given that the population frequency of *C9orf72* expansion is 1 in 691[14] in the UK population, so there is a small possibility of false positives accounting for one or more of these unusual presentations of *C9orf72* mutations.

Among the ten HD phenocopy *C9orf72* cases, there was a tendency for those with chorea and dystonia to have younger ages of onset than those without them: the average age of onset of subjects with chorea/ dystonia in this cohort is 28.3, whereas the average age of onset of those without them is 54.8 (P=0.019, Independent samples Mann-Whitney U-test). This may reflect our ascertainment criteria, since HD-phenocopy cases are more likely to

be young and have movement disorders than FTLD or ALS cases. However, it is possible that the *C9orf72* expansion with these motor symptoms manifests with earlier onset.

Incomplete penetrance has been previously suggested in *C9orf72* expanded individuals[36, 13, 31] which has important implications for genetic testing. In this case series there was no reported family history in three cases, and case 7's family history is compatible with incomplete penetrance – the subject's maternal grandmother had MND, but the mother was well.

We have presented a large case series which not only demonstrates that the *C9orf72* expansion is the most frequent cause of HD phenocopy presentations in this UK-based population, but also that the phenotype of the *C9orf72* encompasses a diversity of movement disorders, and a younger age of onset than previously recorded.

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Figure Legends

Figure 1: 'Southern Blot of eight HD phenocopy patient DNAs'. Southern Blot of eight HD phenocopy patient DNAs, showing that C9orf72 repeat expansions can be seen in all cases. The asterisk indicates a GGGGCC containing a short-tandem-repeat genome motif unrelated to C9orf72. The samples are ordered from 1 – 8 from left to right; there was insufficient DNA to blot samples 9 and 10. The blot for cases 1 – 6 has been previously published[14]. (Reprinted with permission from Elsevier.)

Figure 2: 'Algorithm for the investigation of HD phenocopy cases'. Proposed clinico-genetic algorithm for the work-up of Huntington's disease phenocopy patients, highlighting key diagnoses to be considered. SCA, Spinocerebellar ataxia; HDL2, Huntington's disease-like 2, DRPLA, dentatorubral-pallidoluysian atrophy; NBIA, Neurodegeneration with Brain Iron Accumulation.

Subject	Age at onset	Rs3849942 genotype	Expansion size estimated by southern hybridisation	Goldman score
1	60	AA	4010	4.5
2	56	GA	3441	1
3	55	AA	3682	1
4	36	AA	3180	1
5	50	GA	2939	3
6	56	GA	2939	0
7	8	GA	3186	3
8	44	GA	3518	3
9	19	AA	insufficient DNA	4.5
10	58	GA	insufficient DNA	3

Table 1: Age at onset and genetic results of C9orf72 expansion positive cases

Clinical feature	1	2	3	4	5	6	7	8	9	10
Chorea					√		√		√	
Myoclonus	√			√	√		√			
Dystonia				√	√		√		√	
Tremor					√			√		√
Rigidity			√	√	√		√	√		√
Bradykinesia				√	√		√	√		√
Torticollis				√	√			√		
UMN signs				√	√			√		√
Depression			√	√	√			√		
Anxiety	√	√								
Apathy				√	√					
Executive dysfunction	√	√	√	√				√		√
Impaired memory		√	√	√	√		√			√
Impaired face recognition		√	√				√			
Impaired verbal	√				√					√

fluency										

Table 2: Summary of the clinical features of ten C9orf72 expansion-positive cases.

UMN = upper motor neuron.

	Number in C9orf72 negative cases (N=504) (Percentage)	Number in C9orf72 positive cases (N=10) (Percentage)	Number in whole HD phenocopy cohort (N=514) (Percentage)	P value (Fisher's exact test)
All movement disorder features	394 (78%)	8 (80%)	402 (78%)	1
Chorea	154 (31%)	3 (30%)	157 (31%)	1
Dystonia	53 (11%)	4 (40%)	57 (11.1%)	0.017
Bradykinesia/ rigidity	78 (15%)	6 (60%)	84 (16%)	0.002
Tremor	39 (8%)	3 (30%)	42 (8%)	0.041
Ataxia	72 (14%)	1 (10%)	73 (14%)	1
Myoclonus	31 (6%)	4 (40%)	35 (7%)	0.003
UMN features	18 (4%)	4 (40%)	24 (5%)	<0.001
LMN features	8 (1.6%)	0 (0%)	8 (2%)	1
Psychiatric problems	53 (11%)	7 (70%)	60 (12%)	<0.001
Depression	17 (3%)	4 (40%)	21 (4%)	0.035

Anxiety	4 (0.8%)	2 (20%)	6 (1%)	0.005
Cognitive impairment	167 (33%)	9 (90%)	176 (34%)	<0.001
Executive dysfunction	19 (4%)	6 (60%)	25 (5%)	<0.001
Memory problems	29 (6%)	9 (90%)	176 (34%)	<0.001
Family history	98 (19%)	7 (70%)	105 (20%)	0.001

Table 3: Phenotypic features of *C9orf72* negative & positive cases within HD phenocopy cohort, and outcome of Fisher's exact test to test for association between clinical feature and genetic test outcome.

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