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Ataxin-2 as potential disease modifier in *C9ORF72* expansion carriers

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Abstract

Repeat expansions in chromosome 9 open reading frame 72 (*C9ORF72*) are an important cause of both motor neuron disease (MND) and frontotemporal dementia (FTD). Currently, little is known about factors that could account for the phenotypic heterogeneity detected in *C9ORF72* expansion carriers. In this study, we investigated four genes that could represent genetic modifiers: ataxin-2 (*ATXN2*), non-imprinted in Prader-Willi/Angelman syndrome 1 (*NIPA1*), survival motor neuron 1 (*SMN1*) and survival motor neuron 2 (*SMN2*). Assessment of these genes, in a unique cohort of 331 *C9ORF72* expansion carriers and 376 controls, revealed that intermediate repeat lengths in *ATXN2* possibly act as disease modifier in *C9ORF72* expansion carriers; no evidence was provided for a potential role of *NIPA1*, *SMN1* or *SMN2*. The effects of intermediate *ATXN2* repeats were most profound in probands with MND or FTD/MND (2.1% versus 0% in controls, P=0.013), whereas the frequency in probands with FTD was identical to controls. Though intermediate *ATXN2* repeats were already known to be associated with MND risk, previous reports did not focus on individuals with clear pathogenic mutations, such as repeat expansions in *C9ORF72*. Based on our present findings, we postulate that intermediate *ATXN2* repeat lengths may render *C9ORF72* expansion carriers more susceptible to the development of MND; further studies are needed, however, to validate our findings.

Keywords

C9ORF72; ataxin-2; ATXN2; motor neuron disease; amyotrophic lateral sclerosis; frontotemporal dementia; disease modifier

1. Introduction

To date, hexanucleotide repeat expansions in chromosome 9 open reading frame 72 (*C9ORF72*) are the most frequent genetic cause of two fatal neurodegenerative diseases: motor neuron disease (MND) and frontotemporal dementia (FTD) (DeJesus-Hernandez et al., 2011; Renton et al., 2011). It is largely unknown, however, why some of those expansion carriers develop MND, whereas others develop FTD or a combination of both diseases. We have already shown that GGGGCC expansion size and the presence of

additional mutations in FTD-associated genes could act as disease modifiers in expansion carriers (van Blitterswijk et al., 2013a; van Blitterswijk et al., 2013b). Moreover, we recently reported that variants in transmembrane protein 106 B (*TMEM106B*) protect against developing FTD in subjects harboring *C9ORF72* repeat expansions (van Blitterswijk et al., 2014).

In general, an intermediate CAG repeat length in ataxin-2 (*ATXN2*) (Elden et al., 2010; Lee et al., 2011; Ross et al., 2011; Van Damme et al., 2011), an increased GCG repeat length in non-imprinted in Prader-Willi/Angelman syndrome 1 (*NIPA1*) (Blauw et al., 2012b), and abnormal copy numbers of survival motor neuron 1 (*SMN1*) and/or survival motor neuron 2 (*SMN2*) (Blauw et al., 2012a; Corcia et al., 2006; Corcia et al., 2002; Veldink et al., 2005; Veldink et al., 2001) seem to be associated with MND risk. In our present study, we investigated whether variants in these four genes may act as disease modifiers in the presence of a *C9ORF72* repeat expansion.

2. Methods

2.1. Study population

Our study cohort comprised 331 carriers of *C9ORF72* repeat expansions (Table 1), provided by the Mayo Clinic (n=121), Coriell Research Institute (n=71), University of British Columbia, Canada (n=58), University of California, San Francisco (n=38), Robarts Research Institute (n=11), Northwestern University Feinberg School of Medicine (n=9), Drexel University College of Medicine (n=7), University of Western Ontario, Canada (n=7), Banner Sun Health Research Institute (n=5), and University of Tübingen (n=4). Based on clinical and/or pathological data available these subjects were diagnosed with MND (n=127), FTD/MND (n=78) or FTD (n=92), with another diagnosis (n=7; e.g. Alzheimer's disease, alcohol abuse or behavioral impairment), or they were asymptomatic at time of last evaluation (n=27; age at evaluation: 43.6±12.7).

We focused our primary analysis on the 266 unrelated probands with MND (n=120), FTD/MND (n=71) or FTD (n=75) in order to fulfill the statistical assumption of independent measurements, and on a group of neurologically normal controls of similar age and gender obtained through the Mayo Clinic (n=376; Table 1). The 65 remaining expansion carriers who were family members or who had received another diagnosis were included in secondary analyses to examine the sensitivity of our results.

2.2. Genetic analysis

The presence of a GGGGCC repeat in *C9ORF7*2 was determined using a 2-step protocol (DeJesus-Hernandez et al., 2011). Briefly, genomic DNA was PCR-amplified with genotyping primers and one fluorescently labeled primer, followed by fragment length analysis. Repeat-primed PCR was performed for those individuals who were shown to be homozygous for *C9ORF72* repeats. A characteristic stutter pattern was considered evidence of a *C9ORF72* repeat expansion.

ATXN2 repeat length was assessed in cases and controls using fragment analysis with fluorescently labeled primers on an ABI 3730 Genome Analyzer (Applied Biosystems) and

GeneMapper software (primer sequences are available upon request). The repeat length of *NIPA1* was also determined in cases and controls with fragment analysis, as described elsewhere (Blauw et al., 2012b). *SMN1* and *SMN2* copy numbers were investigated in our cases with multiplex ligation-dependent probe amplification (MLPA) assays (MRC Holland, the Netherlands), using the manufacturer's instructions.

2.3. Statistical analysis

We compared the distribution of repeat lengths and copy numbers between *C9ORF72* expansion carriers and controls, utilizing Fisher's exact test. The following categorization was used: normal (≤ 27 repeat units) and intermediate (>27 repeat units) for *ATXN2*, short (≤ 6 repeat units), normal (7–8 repeat units) and long (>8 repeat units) for *NIPA1*, and homozygous deletion (0 copies), heterozygous deletion (1 copy), normal (2 copies), duplication (3 copies) and triplication (4 copies) for both *SMN1* and *SMN2*. The distribution was compared to controls for our entire cohort, and also separately for our disease subgroups. For *ATXN2* and *NIPA1* we used control data generated as part of this study, whereas a previously published meta-analysis was used for *SMN1* and *SMN2* (Blauw et al., 2012a). We also assessed associations of repeat lengths and copy numbers with age at onset using a Wilcoxon rank sum test or a Kruskal-Wallis rank sum test.

To allow further investigations of repeat lengths in *ATXN2*, we also used an alternative categorization of ≤23 repeat units versus >23 repeat units. This alternative categorization facilitated comparisons of both age at onset and survival after onset in our cases, because of the larger number of subjects in each category. For this extra analysis we used a Wilcoxon rank sum test (age at onset) and a log-rank test (survival after onset). Additionally, in making these comparisons, we utilized linear regression models adjusted for gender and disease subgroup (age at onset comparisons) and Cox proportional hazards regression models adjusted for age at onset, gender, and disease subgroup (survival after onset comparisons) to address the potential confounding influences of these variables. P-values ≤ 0.05 were considered as statistically significant. All statistical analyses were performed using R Statistical Software (version 2.14.0; R Foundation for Statistical Computing).

3. Results

The *ATXN2* repeat length ranged from 14 to 31 repeat units in *C9ORF72* expansion carriers, and from 17 to 27 repeat units in controls, with 22 and 23 repeats being most common (allele frequency of 96%). Intermediate *ATXN2* repeat lengths were identified in 1.5% of our 266 MND, FTD/MND and FTD probands as compared to 0% of our 376 controls (P=0.029; Table 2). When focusing on disease subgroups, intermediate repeat lengths were detected in 2.1% of probands with either MND or FTD/MND (P=0.013; versus controls), in 1.7% of probands with MND (P=0.058; versus controls), in 2.8% of probands with FTD/MND (P=0.025; versus controls), and in 0% of probands with FTD (P=1.00; versus controls). These findings were comparable when including the 65 remaining expansion carriers who were family members or who had received another diagnosis (e.g. 2.1% of all expansion carriers [P=0.005; versus controls], and 2.0% of MND or FTD/MND patients [P=0.015; versus controls]; Supplementary Table 1).

The distribution of *NIPA1* repeat lengths did not differ significantly between all probands and controls (P=0.93), or between any of the disease subgroups and controls (P≥0.90; Table 2). Eight repeat units (allele frequency of 79%) and 7 repeat units (allele frequency of 19%) were most prevalent, followed by 10 repeat units (allele frequency of <2%). For *SMN1* and *SMN2* we did not detect significant differences in copy number between all probands and controls (P=0.98 and P=0.11), or when comparing disease subgroups and controls (P≥0.23 and P≥0.21; Table 2). All findings were similar when including the remaining expansion carriers (Supplementary Table 1).

We also investigated associations of repeat lengths and copy numbers with age at onset in all probands and in the subgroup of probands with either MND or FTD/MND; however, there was no evidence of a difference in age at onset for any of the genes investigated in this study (Table 3), and these findings did not change when including the remaining expansion carriers (Supplementary Table 2).

To further investigate *ATXN2* repeat length, we also used an alternative categorization (≤23 versus >23). Due to the larger number of samples in each category this alternative categorization has more power to detect associations, and allows adjustment of potential confounding variables in age at onset and survival after onset analyses. We did not detect a significant difference in age at onset (P≥0.81; Supplementary Table 3) or survival after onset (P≥0.12; Supplementary Table 4) with this alternative categorization in all probands nor in probands with either MND or FTD/MND. These findings were consistent when including additional expansion carriers (Supplementary Table 3 and Supplementary Table 4), and when performing a multivariable analysis adjusted for age at onset, gender and disease subgroup (data not shown).

4. Discussion

We demonstrate that intermediate repeat lengths in *ATXN2* might modify the disease phenotype of *C9ORF72* expansion carriers. These intermediate repeats were more frequently encountered in our expansion carriers than in controls. Interestingly, they were present in 2.1% of our probands with MND or FTD/MND (P=0.013; versus controls), but in none of our probands with FTD (P=1.00; versus controls). We did not find associations between *ATXN2* repeat length and age at onset or survival after onset. Furthermore, no significant differences were detected in repeat length or copy number of other genes investigated in this study (*NIPA1*, *SMN1*, and *SMN2*). Based on our findings, we speculate that intermediate *ATXN2* repeats, previously shown to increase MND risk, may also predispose to the development of MND in carriers of *C9ORF72* expansions, influencing their phenotype.

Ataxin-2 plays a vital role in RNA metabolism, associates with RNA-binding proteins, and affects many cellular processes, including calcium signaling, glutamate toxicity and mitochondrial stress (van den Heuvel et al., 2014). Importantly, a yeast screen also identified ataxin-2 as a potent enhancer of transactive response DNA-binding protein 43 (TDP-43) toxicity (Elden et al., 2010). Moreover, it has been shown that intermediate *ATXN2* repeats are associated with MND (Elden et al., 2010). In *Drosophila*, these intermediate repeats

result in even more pronounced TDP-43 toxicity than repeats in the wild-type range (Kim et al., 2013). The interaction between ataxin-2 and TDP-43 is probably mediated by poly(A)-binding protein (PABP) (Kim et al., 2013), and is thought to promote the recruitment of TDP-43 to stress granules, to affect the ability of stress granules to dissolve and/or to impair the return of TDP-43 to the nucleus, especially upon exposure to stress (Li et al., 2013).

Since the initial report in 2010, many studies have confirmed the association between intermediate *ATXN2* repeat lengths and MND risk (Chen et al., 2011; Conforti et al., 2012; Corrado et al., 2011; Daoud et al., 2011; Gellera et al., 2012; Gispert et al., 2012; Laffita-Mesa et al., 2013; Lahut et al., 2012; Lee et al., 2011; Liu et al., 2013; Ross et al., 2011; Soraru et al., 2011; Van Damme et al., 2011; Van Langenhove et al., 2012). Based on these studies and on our present findings, we reanalyzed MND cases included in our original *ATXN2* report (Ross et al., 2011) supplemented with new MND cases. When excluding carriers of *C9ORF72* repeat expansions, and when using our cut-off of 28 *ATXN2* repeats, approximately 3% of our 525 MND patients harbored intermediate *ATXN2* repeats. It should be emphasized, therefore, that the frequency of intermediate *ATXN2* repeats in studies investigating MND risk seems similar to that detected in our current study focusing on those patients with *C9ORF72* repeat expansions. The effects of *ATXN2* on MND risk, thus, are not specific to *C9ORF72* expansion carriers, but it is interesting that even on the background of a strong pathogenic mutation such as a repeat expansion in *C9ORF72*, *ATXN2* is still able to confer MND risk, thereby modulating the disease phenotype.

Several of the aforementioned studies highlighted that *ATXN2* repeat length did not appear to influence clinical characteristics, including age at onset and survival after onset (Chen et al., 2011; Conforti et al., 2012; Corrado et al., 2011; Daoud et al., 2011; Gispert et al., 2012; Lee et al., 2011; Liu et al., 2013; Soraru et al., 2011; Van Damme et al., 2011), which is well in line with our present findings. Furthermore, our results are consistent with reports that did not find associations between *ATXN2* repeat lengths and FTD (Ross et al., 2011; Van Langenhove et al., 2012).

Our study has some limitations. Because of the relatively low number of subjects in several categories, the possibility of type II error (i.e. a false-negative association) should be considered. Additionally, for our case-control analyses, we used 28 repeats as cut-off to define intermediate *ATXN2* repeat lengths, based on the upper limit observed in our control cohort. In literature, however, there is no consensus on the definition of intermediate *ATXN2* repeats and different cut-offs have been used, depending on the population studied (e.g. 24, 27, 28, 29, 30, 31 and 32 repeats) (Chen et al., 2011; Conforti et al., 2012; Corrado et al., 2011; Daoud et al., 2011; Elden et al., 2010; Gellera et al., 2012; Gispert et al., 2012; Laffita-Mesa et al., 2013; Lahut et al., 2012; Lattante et al., 2012; Lee et al., 2011; Liu et al., 2013; Ross et al., 2011; Soraru et al., 2011; Van Damme et al., 2011; Van Langenhove et al., 2012). Lastly, we used control data from a recent meta-analysis for *SMN1* and *SMN2* (Blauw et al., 2012a). The distribution of our *SMN1* and *SMN2* copy numbers in cases, and our *ATXN2* and *NIPA1* repeat lengths in both cases and controls, however, is very comparable to that reported in literature (Blauw et al., 2012a; Blauw et al., 2012b; Laffita-Mesa et al., 2013), and hence, it seems unlikely that usage of this data severely impacted our findings related to *SMN1* and *SMN2*.

Previously, we showed that variants in *TMEM106B* protect *C9ORF72* expansion carriers from developing FTD (van Blitterswijk et al., 2014). Importantly, our present findings reveal that intermediate repeat lengths in *ATXN2* possibly drive *C9ORF72* expansion carriers towards MND, potentially due to effects on TDP-43 toxicity, stress granule formation, RNA metabolism, and/or other cellular processes involved in MND pathogenesis. Thus, both *TMEM106B* and *ATXN2* may contribute to the phenotypic heterogeneity detected in *C9ORF72* expansion carriers. Further studies are needed, however, to confirm these interesting findings and to elucidate the underlying mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Characteristics of *C9ORF72* expansion carriers and controls

Group	N	Female gender	Age	Age at onset	Pathological diagnosis
Controls	376	173 (46.0%)	61.2 ± 10.2 (35 – 90)	N/A	N/A
All expansion carriers	331	150 (45.3%)	59.3 ± 10.0 (35 – 90)	56.7 ± 9.2 (34 – 83)	124 (37.5%)
MND, FTD/MND, and FTD probands	266	116 (43.6%)	59.6 ± 10.0 (35 – 90)	56.8 ± 9.1 (34 – 83)	113 (42.5%)
MND and FTD/MND probands	191	86 (45.0%)	58.2 ± 8.7 (37 – 83)	56.4 ± 8.8 (34 – 83)	67 (35.1%)
MND probands	120	61 (50.8%)	56.9 ± 8.6 (37 – 83)	56.5 ± 8.7 (37 – 83)	16 (13.3%)
FTD/MND probands	71	25 (35.2%)	60.6 ± 8.5 (39 – 80)	56.2 ± 9.0 (34 – 74)	51 (71.8%)
FTD probands	75	30 (40.0%)	62.8 ± 12.1 (35 – 90)	57.6 ± 9.8 (34 – 79)	46 (61.3%)

Continuous variables are summarized with the sample mean ± standard deviation (range). The age provided is age at blood draw in controls, age at onset in clinically diagnosed patients, and age at death in pathologically diagnosed patients. Information was unavailable regarding age (N=41) and age at onset (N=59).

Table 2

Associations of *ATXN2*, *NIPA1*, *SMN1*, and *SMN2* with disease – analysis of MND, FTD/MND, and FTD probands

Repeat length or copy number	Controls [a]	MND, FTD/MND, and FTD probands (N=266)	MND and FTD/MND probands (N=191)	MND probands (N=120)	FTD/MND probands (N=71)	FTD probands (N=75)
ATXN2 repeat length						
Normal	376 (100.0%)	262 (98.5%)	187 (97.9%)	118 (98.3%)	69 (97.2%)	75 (100.0%)
Intermediate	0 (0.0%)	4 (1.5%)	4 (2.1%)	2 (1.7%)	2 (2.8%)	0 (0.0%)
Comparison with controls	N/A	P=0.029	P=0.013	P=0.058	P=0.025	P=1.00
NIPA1 repeat length						
Short	2 (0.5%)	1 (0.4%)	1 (0.5%)	1 (0.8%)	0 (0.0%)	0 (0.0%)
Normal	361 (96.0%)	257 (96.6%)	184 (96.3%)	115 (95.8%)	69 (97.2%)	73 (97.3%)
Long	13 (3.5%)	8 (3.0%)	6 (3.1%)	4 (3.3%)	2 (2.8%)	2 (2.7%)
Comparison with controls	N/A	P=0.93	P=1.00	P=0.90	P=1.00	P=1.00
SMN1 copy number						
1	39 (2.2%)	5 (2.0%)	5 (2.7%)	3 (2.5%)	2 (3.0%)	0 (0.0%)
2	1673 (94.0%)	239 (94.1%)	172 (93.0%)	112 (94.9%)	60 (89.6%)	67 (97.1%)
3	67 (3.8%)	10 (3.9%)	8 (4.3%)	3 (2.5%)	5 (7.5%)	2 (2.9%)
4	1 (<0.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Comparison with controls	N/A	P=0.98	P=0.76	P=0.82	P=0.23	P=0.66
SMN2 copy number						
0	147 (8.3%)	30 (11.8%)	22 (11.9%)	13 (11.0%)	9 (13.4%)	8 (11.6%)
1	663 (37.2%)	81 (31.9%)	61 (33.0%)	36 (30.5%)	25 (37.3%)	20 (29.0%)
2	888 (49.9%)	134 (52.8%)	95 (51.4%)	63 (53.4%)	32 (47.8%)	39 (56.5%)
3	79 (4.4%)	8 (3.1%)	6 (3.2%)	5 (4.2%)	1 (1.5%)	2 (2.9%)
4	3 (0.2%)	1 (0.4%)	1 (0.5%)	1 (0.8%)	0 (0.0%)	0 (0.0%)
Comparison with controls	N/A	P=0.11	P=0.21	P=0.21	P=0.44	P=0.46

P-values results from Fisher's exact test, comparing the repeat length or copy number distribution in the given group to that of controls.

[a] Data for *SMN1* and *SMN2* in control subjects was not obtained in the current study (Blauw et al., 2012a).

Table 3

Associations of *ATXN2*, *NIPA1*, *SMN1*, and *SMN2* with age at disease onset – analysis of MND, FTD/MND, and FTD probands

	MND, FTD/MND, and FTD probands (N=244)		MND and FTD/MND probands (N=176)	
Repeat length or copy number	**N**	**Mean (range) age at onset**	**N**	**Mean (range) age at onset**
ATXN2 repeat length				
Normal	240	56.8 (34 – 83)	172	56.5 (34 – 83)
Intermediate	4	55.6 (52 – 59)	4	55.6 (52 – 59)
Test of difference		P=0.78		P=0.87
NIPA1 repeat length [a]				
Normal	236	56.7 (34 – 83)	170	56.3 (34 – 83)
Long	8	60.1 (50 – 68)	6	60.2 (50 – 68)
Test of difference		P=0.22		P=0.19
SMN1 copy number				
1	4	50.3 (46 – 60)	4	50.3 (46 – 60)
2	219	56.9 (34 – 83)	159	56.7 (34 – 83)
3	9	54.3 (43 – 65)	7	53.2 (43 – 61)
Test of difference		P=0.22		P=0.21
SMN2 copy number				
0	26	58.1 (41 – 83)	19	56.8 (41 – 83)
1	75	56.1 (34 – 74)	57	55.8 (34 – 74)
2	123	56.8 (34 – 80)	88	56.6 (39 – 80)
3 or 4	8	56.4 (46 – 66)	6	57.6 (46 – 66)
Test of difference		P=0.97		P=0.95

[a] Due to the fact that only 1 patient had a short *NIPA1* repeat length, short and normal *NIPA1* repeat length categories were combined in age at onset association analysis. Tests of difference in age at onset between groups result from a Wilcoxon rank sum test (*ATXN2* repeat length, *NIPA1* repeat length), or a Kruskal-Wallis rank sum test (*SMN1* copy number, *SMN2* copy number).