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ORIGINAL ARTICLE

Analysis of the GCG repeat length in NIPA1 gene in *C9orf72*-mediated ALS in a large Italian ALS cohort

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Abstract
Amyotrophic lateral sclerosis (ALS) is characterized by degeneration of upper and lower motor neurons. The hexanucleotide repeat expansion in *C9orf72* gene (*C9orf72*-HRE) is the most frequent genetic cause of ALS. Since many ALS pedigrees showed incomplete penetrance, several genes have been analyzed as possible modifiers. Length of the GCG repeat tract in *NIPA1* (non-imprinted in Prader-Willi/Angelman syndrome 1) gene has been recently investigated as a possible modifier factor for *C9orf72*-HRE patients with contrasting findings. To disclose the possible role of *NIPA1* GCG repeat length as modifier of the disease risk in *C9orf72*-HRE carriers, we analyzed a large cohort of 532 Italian ALS cases enriched in *C9orf72*-HRE carriers (172 cases) and 483 Italian controls. This sample size is powered (92% power, $p = 0.05$) to replicate the modifier effect observed in literature. We did not observe higher frequency of *NIPA1* long alleles (> 8 GCG) in *C9orf72*-HRE carriers (3.5%) compared with *C9orf72*-HRE negative patients (4.1%) and healthy controls (5%). For the latter comparison, we meta-analyzed our data with currently available literature data, and no statistically significant effect was observed ($p = 0.118$). In conclusion, we did not confirm a role of *NIPA1* repeat length as a modifier of the *C9orf72* ALS disease risk.

Keywords ALS · *NIPA1* · *C9orf72*-HRE carriers

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal motor neuron disease characterized by motor neuron degeneration in the primary motor cortex, brainstem, and spinal cord. In about 10% of patients, the disease is familial, while the majority of patients are sporadic cases [1]. The hexanucleotide repeat expansion in *C9orf72* gene (*C9orf72*-HRE) is the most frequent genetic cause of ALS [2]. Since many familial cases carrying *C9orf72*-HRE showed incomplete penetrance, and the same expansion is present in apparently sporadic cases (7%) [2], several genetic factors have been analyzed as possible modifier in *C9orf72*-HRE-mediated ALS. Among them, the length of the GCG repeat tract in *NIPA1* gene (non-imprinted gene in Prader-Willi/Angelman syndrome 1), previously identified as a risk factor for ALS susceptibility [3, 4], has been recently investigated as a possible modifier factor for ALS patients carrying *C9orf72-HRE* (HRE carriers), with contrasting findings [4–6]. Our aim is to further disclose the possible role of *NIPA1* GCG repeat length as modifier of the disease risk in *C9orf72*-HRE carriers analyzing a large cohort of Italian ALS cases enriched in *C9orf72* HRE carriers.

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Table 1 *NIPA1* alleles in sporadic (SALS) and familial (FALS) *C9orf72* HRE carriers, *C9orf72* HRE non-carriers, and healthy controls

Number of GCG repeats	ALS HRE carriers			ALS HRE non-carriers			Controls
	Total	FALS	SALS	Total	FALS	SALS	Total
≤ 8	166	22	144	345	21	324	459
> 8	6 (0.035)	0	6 (0.04)	15 (0.041)	0	15 (0.044)	24 (0.05)
Total	172	22	150	360	21	339	483

For each group, a number of tested individuals (and percentage of *NIPA1* long allele carries) are displayed

Methods

Patients and controls

The study cohort included 532 unrelated ALS cases from an Italian cohort recruited by Novara and Torino ALS centers. Patients were recruited following standard diagnostic protocols and previously analyzed for the presence of *C9orf72* expansion with a HRE threshold > 30 repeats (172 HRE carriers and 360 HRE non-carriers) and for mutations in at least other 3 major genes: *SOD1*, *TARDBP* e, and *FUS*. Moreover, we genotyped all the patients for *ATXN2* repeated tract length. Among the HRE carriers, 2 patients resulted to carry a second variant in two different ALS genes (*FUS* p.S135N and *PRPH* p. A133P). The Italian control cohorts consisted of 483 unrelated individuals with no reported family history for neurodegenerative diseases. All patients and controls provided written informed consent for participation in genetic studies for research purposes. The informed consent was approved by the ethical committees of the local hospitals.

Genetic analysis

Genomic DNA was isolated from peripheral blood according to standard protocol. Through a two-step protocol described by DeJesus-Hernandez et al. [7], the hexanucleotide repeat (GGGGCC) in *C9orf72* was analyzed. Furthermore, fragment-length analysis of (GCG) repeats present in the first exon in *NIPA1* was performed. The *NIPA1* repeat-region was amplified using a fluorescent forward primer 5′-CGGA ATGGGGACTGCAGCT-3′ and the reverse primer 5′-ACGATGCCCTTCTTCTGTAGCA-3′. The product fragments were sized by capillary electrophoresis on an ABI Prism 3100XL Genetic Analyzer (Applied Biosystems). The data analysis was performed by GeneMapper program version 4.1. The repeat size has been confirmed by Sanger sequence analysis of a representative product fragment for each of the identified fragment sizes.

Statistical analysis

The difference of *NIPA1* long allele frequencies in the different subgroups was assessed with Fisher's exact test. Student's *t* test was used to compare differences in age of onset in the two categories. Random model meta-analysis between our sample set and literature data [5, 6] was performed by comprehensive meta-analysis software (CMA).

Results

The *NIPA1* GCG repeat was genotyped in 532 Italian ALS patients (150 sporadic and 22 familial *C9orf72*-HRE carriers, 339 sporadic and 21 familial HRE non-carriers) and 483 Italian healthy controls. The *NIPA1* repeat length ranged from 6 to 10 repeats (GCG_6–GCG_{10} alleles) in ALS patients and from 7 to 10 repeats (GCG_7–GCG_{10} alleles) in controls. The most frequent alleles are GCG_7 (15%) and GCG_8 (80%). To evaluate the effect of short or long repeat lengths, as in the literature papers, we dichotomized *NIPA1* alleles as "normal" ($(GCG)_7$ or $(GCG)_8$) or "long" (> 8 GCG repeats) and we compared the allele frequencies in *C9orf72*-HRE carriers versus *C9orf72*-HRE non-carriers and controls (Table 1). *NIPA1* long alleles were detected in 3.5% of the *C9orf72*-HRE

Table 2 Meta-analysis of the association of *NIPA1* long alleles with ***C9orf72***HRE ALS

Dataset	HRE carriers*	Healthy controls*	Odds ratio (95% CI)**	*p* value**
Dekker (2016)	46	956	4.458 (1.869–10.631)	0.001
Van Blitterswijk (2014)	331	376	1.050 (0.472–2.335)	0.904
Tazelaar (2018)	322	5051	1.434 (0.861–2.387)	0.166
Present study	172	483	0.691 (0.278–1.721)	0.428
Meta-analysis (random effects)	871	6866	1.468 (0.748–2.280)	0.265

* Number of tested individuals

** Statistics refers to the comparison of *NIPA1* long allele frequencies in ALS HRE carriers vs healthy controls

Table 3 Association of *NIPA1* allele length with age and site of onset in the C9orf72 HRE carriers cohort

Number of GCG repeats	Number	Age at onset mean	Site of onset bulbar
> 8	6	54.6 (12.8)	1 (17%)
≤ 8	166	57.9 (9.9)	64 (38%)
		P value = 0.43	*P* value = 0.412

* Student's *t* test

** Two-tailed Fisher test

Mean (± standard deviation) values are displayed

carriers, in 4.1% of HRE non-carriers, and in 5% of controls. Hence, *NIPA1* long alleles do not appear to be more frequent in *C9orf72*-HRE carriers compared with HRE non-carriers ($p = 0.81$) or controls ($p = 0.53$).

To further test the co-occurrence of *NIPA1* long repeat tract and *C9orf72* repeat expansion in ALS, we performed a random-effects meta-analysis between our study and available literature data, which allowed to compare the frequency of long *NIPA1* alleles in HRE carriers versus controls from Dekker et al. (15.2% vs 3.9%, OR = 4.458), Blitterswijk et al. (3.6% vs 3.5%, OR = 1.050), and Tazelaar et al. (5.3% vs 3.74%, OR = 1.434). We did not observe any statistically significant association ($p = 0.265$, OR meta-analysis = 1.468, Table 2).

To verify a possible role of *NIPA1* long alleles as disease phenotype modifier, we investigated associations of *NIPA1* repeat length with age and site of onset in the *C9orf72* HRE carriers (Table 3). Age or site of onset between *NIPA1* long and normal allele carriers was not significantly different (Table 3).

Discussion

In our cohort of Italian ALS patients, we did not observe a higher frequency of *NIPA1* long alleles in HRE carriers compared with HRE non-carriers and to healthy controls (Table 1). Thus, we did not confirm the role of *NIPA1* as a disease risk modifier in *C9orf72*-HRE carriers recently proposed by [5] reporting a higher frequency of *NIPA1* long alleles in sporadic *C9orf72*-HRE carriers (15.2%) compared with HRE non-carriers (4.9%) and controls (3.9%), although the sample size of our dataset allowed to replicate the modifier effect observed in the literature study (92% power, at alpha 0.05). In addition, our data are in keeping with other two papers. The first one reported *NIPA1* long alleles in 3.6% of ALS/frontotemporal dementia-HRE carriers compared with 3.5% of controls [6]. In a second recent paper, a higher than expected number of ALS cases carrying *NIPA1* and *C9orf72* repeat expansion were reported, but these data did not reach a statistical significance ($p = 0.06$) [4]. The lack of replication of findings from Dekker et al. could be due to a population-specific effect due to the presence of additional mutations in ALS genes, or to the small sample size of *C9orf72*-HRE carriers in Dekker's cohort. To overcome these limitations, we have enriched our ALS cohort in HRE carriers, and performed a meta-analysis with currently available literature data. From random-effects meta-analysis performed between our data and currently available literature data, no statistically significant effect was observed among ALS *C9orf72*-HRE vs healthy controls ($p = 0.265$). *NIPA1* allele length did not appear to influence clinical features of HRE carriers, although our study has some limitation due to the low number of *NIPA1* long alleles carriers and we could not analyze survival time and cognitive impairment, because these data were not available for all the patients of the *C9orf72*-HRE-carriers cohort. Although we observed a reduced age of onset and a predominately spinal onset in the subgroup of *NIPA1* long allele carriers, as previously reported by Dekker et al., our sample set (6 patients) is not powered to confirm these data. To verify whether the different mean age of onset between our and Dekker 's cohorts could be the main factor explaining the lack of data replication, we compared the mean age of onset between the two cohorts. The mean age of onset of the two *C9orf72* cohorts was similar (57.9 versus 58.7, respectively) suggesting that age at onset seems not to be a main confounder factor.

In conclusion, despite our sample size allowed to replicate the modifier effect observed in the literature study, we did not confirm a possible role of *NIPA1* repeat length as disease risk modifier in *C9orf72* ALS. This is also supported by our meta-analysis with all literature data. Further studies are needed to find genetic modifier factors explaining the incomplete penetrance observed in *C9orf72* carriers.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical standards Informed consent was obtained from all individual participants involved in the study. The informed consent was approved by the ethical committees of the local hospitals.

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