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Hypermethylation of the CpG-island near the *C9orf72* G_4C_2-repeat expansion in FTLD patients

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The G_4C_2-repeat expansion in *C9orf72* is a common cause of frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS). *C9orf72* transcription is reduced in expansion carriers implicating haploinsufficiency as one of the disease mechanisms. Indeed, our recent ALS study revealed that the expansion was associated with hypermethylation of the CpG-island (5′of the repeat) in DNA samples obtained from different tissues (blood, brain and spinal cord). However, the link between FTLD and methylation of the CpG-island is unknown. Hence, we investigated the methylation profile of the same CpG-island by bisulfite sequencing of DNA obtained from blood of 34 FTLD expansion carriers, 166 FTLD non-carriers and 103 controls. Methylation level was significantly higher in FTLD expansion carriers than non-carriers ($P = 7.8E-13$). Our results were confirmed by two methods (HhaI-assay and sequencing of cloned bisulfite PCR products). Hypermethylation occurred only in carriers of an allele with >50 repeats, and was not detected in non-carriers or individuals with an intermediate allele (22–43 repeats). As expected, the position/number of methylated CpGs was concordant between the sense and anti-sense DNA strand, suggesting that it is a stable epigenetic modification. Analysis of the combined ALS and FTLD datasets (82 expansion carriers) revealed that the degree of methylation of the entire CpG-island or contribution of specific CpGs ($n = 26$) is similar in both syndromes, with a trend towards a higher proportion of ALS patients with a high methylation level ($P = 0.09$). In conclusion, we demonstrated that hypermethylation of the CpG-island 5′of the G_4C_2-repeat is expansion-specific, but not syndrome-specific (ALS versus FTLD).

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INTRODUCTION

Amyotrophic lateral sclerosis [ALS (MIM 612069)] and frontotemporal lobar degeneration [FTLD (MIM 600274)] are two ends of a clinico-pathological spectrum of overlapping neurodegenerative syndromes (1–4).

FTLD is caused by degeneration of the frontal/temporal lobes of the cerebral cortex leading to behavioral and/or language disruptions (5); while ALS is caused by degeneration of upper motor neurons in the cerebral cortex as well as lower motor neurons of the brainstem and anterior horn of the spinal cord resulting in paralysis (6). The co-occurrence of both syndromes can be observed within the same family and individual patients (4). Yet, there are many patients presenting with either pure FTLD or pure ALS even among individuals with identical pathological mutations (7). Such a phenotypic split is unexplained and could be the result of environmental, genetic or epigenetic modifications. The latter is investigated in the current study.

ALS and FTLD are genetically complex and might be explained by mutations in several often overlapping genes (7). Discovery of the hexanucleotide G_4C_2-repeat expansion (up to several thousand repeats) within the non-coding region of *C9orf72* (MIM 614260) as the most common known cause of both ALS and FTLD provides additional evidence of shared pathological mechanisms (8–10). It is critical to understand whether expansion with different sizes have the same pathological consequence, however even the lower limit of repeat number for pathological expansions has not been determined. Most *C9orf72* studies are using a 30-repeat cutoff, which likely will be corrected based on the cumulative evidence of numerous reports including the current study. It is important to uncover functionally relevant markers (e.g. epigenetic modifications investigated here) that can be exploited to circumvent the technical difficulty in measuring the length of the expansion by Southern blot.

Proposed *C9orf72*-related mechanisms include the formation of toxic RNA foci consisting of transcribed repeat sequence (8); the generation of aggregating dipeptide-repeat proteins due to non-ATG translation of the repeat (11,12); and haploinsufficiency of *C9orf72* (8), which might affect the Rab-dependent vesicular trafficking process (13,14). It is also likely that all three mechanisms contribute to the diverse phenotypes to a variable extent.

Importantly, the G_4C_2-repeat is mapped directly or proximal to the *C9orf72* promoter (depending on the transcript); adjacent to two cytosine-phosphate-guanine (CpG) islands and enriched in CpGs itself (15). Hypermethylation of CpG-islands at the promoter region could lead to gene expression silencing reported for other repeat expansion diseases such as Friedreich's ataxia (MIM 229300) (16–18), Fragile X syndrome (MIM 300624) (19–21) and myotonic dystrophy (MIM 160900) (22–24). Indeed, the G_4C_2-repeat expansion lead to an ~50% reduction of *C9orf72* transcripts in brain or blood of mutation carriers (8,10,15). In agreement with the pathological impact of reduced *C9orf72* expression, down-regulation of the zebrafish *C9orf72* orthologue led to altered motor neuron axon morphology and locomotor deficits rescued upon overexpression of human *C9orf72* (25). Similarly, a null mutation of the *Caenorhabditis elegans C9orf72* orthologue caused age-dependent motility defects leading to paralysis and the specific degeneration of GABAergic motor neurons (26).

Our recent ALS study further supports the loss-of-function model to be one of the disease mechanisms. At the CpG-island 5′ (but not 3′) of the G_4C_2-repeat, methylation level was significantly higher in ALS expansion carriers than in non-carriers (ALS and controls) (15). In addition, qRT-PCR results supported that hypermethylation may underlie the reduced *C9orf72* expression, since carriers of larger expansions (>50 repeats) showed a high methylation level together with a reduction in *C9orf72* expression, while individuals carrying unmethylated wild-type or intermediate alleles had a normal level of expression (15).

It is critical to know whether DNA hypermethylation also occurs in FTLD expansion carriers and whether it is essential in modifying the disease phenotype (ALS versus FTLD). Hence, we conducted a methylation analysis of the 5′ CpG-island in an FTLD dataset, and compared the methylation profile between FTLD and ALS expansion carriers. We report that hypermethylation of the CpG-island occurs exclusively in expansion carriers at a rate similar for both syndromes.

RESULTS

Analysis of FTLD dataset

FTLD patients ($n = 200$) and origin-matched controls ($n = 103$) were genotyped for the *C9orf72* repeat region (Table 1). None of the controls had the expansion (≤20 repeats). Based on the electropherogram with saw-tooth peaks (Supplementary Material, Fig. S1), 34 FTLD expansion carriers were detected (including a case with a 31-repeat allele). In addition, we identified four carriers of an intermediate allele (22, 23, 28 and 30 repeats). Compared with FTLD non-carriers, FTLD expansion carriers have a significantly younger age of onset ($P = 0.00005$), a higher percentage of cases with familial history ($P = 0.004$) and an FTLD/ALS diagnosis ($P = 0.008$), all of which are known associations (10,27).

All samples were studied by direct bisulfite sequencing (a representative chromatogram is shown in Fig. 1). As in the previous ALS study (15), the total number of methylated CpGs obtained for each sample was used to categorize samples to three methylation levels: 0 (no methylation); 1–3 (low methylation); and 4–26 (high methylation). Among the 34 expansion carriers, 56% ($n = 19$) were in the low or no methylation category and 21% ($n = 7$) were in the high methylation category. In contrast, no highly methylated samples were found in the 269 non-carriers and only 6% of them ($n = 16$) were methylated at a low level (Table 2). The methylation level was significantly higher in FTLD expansion carriers versus FTLD non-carriers ($P = 7.8E-13$); and in FTLD expansion carriers versus controls ($P = 8.3E-9$), while no difference was found between the two non-expansion groups (FTLD versus controls, $P = 0.128$). Similar results were obtained when comparing the high methylation group to the combined no/low methylation group (FTLD expansion carriers versus FTLD non-carriers, $P = 2.4E-6$; FTLD expansion carriers versus controls $P = 3.5E-5$).

The defined methylation categories were confirmed by sequencing of cloned bisulfite PCR (BSP) products from six randomly selected expansion carriers (two samples per methylation category). A representative bisulfite sequence chromatogram is shown in Supplementary Material, Fig. S2A. Of note, all

Table 1. Clinical characteristics and *C9orf72* repeat genotypes for the investigated dataset

Characteristic of the dataset		FTLD patients Expansion carriers	Non-expansion carriers	Controls Non-expansion carriers
Total number		34	166	103
Age of sample collection (± SD)		63.0 ± 8.3	70.5 ± 8.1	73.2 ± 6.3
Age at onset (± SD)		58.6 ± 8.7	66.2 ± 8.8	–
P-value[a] (FTLD carrier versus non-carrier)		0.00005*	–	–
Female (no/frequency)		18 (0.53)	84 (0.51)	57 (0.55)
P-value[b] (FTLD carrier versus non-carrier)		0.804	–	–
Family history (no/frequency)		22 (0.65)	63 (0.38)	–
P-value[b] (FTLD carrier versus non-carrier)		0.004*	–	–
Diagnosis (no/frequency)	FTLD	29 (0.85)	162 (0.98)	–
	FTLD/ALS	5 (0.15)	4 (0.02)	–
P-value[b] (FTLD carrier versus non-carrier)		0.008*	–	–
C9orf72 repeat number (range)	Small allele	2–11	2–12	2–9
	Big allele	31, >50 repeat expansion	2–30	2–20

[a]The independent samples *t*-test was used.
[b]Two-sided Pearson χ^2 test or Fisher's exact test was used (when expected value is <5).
*$P < 0.05$.

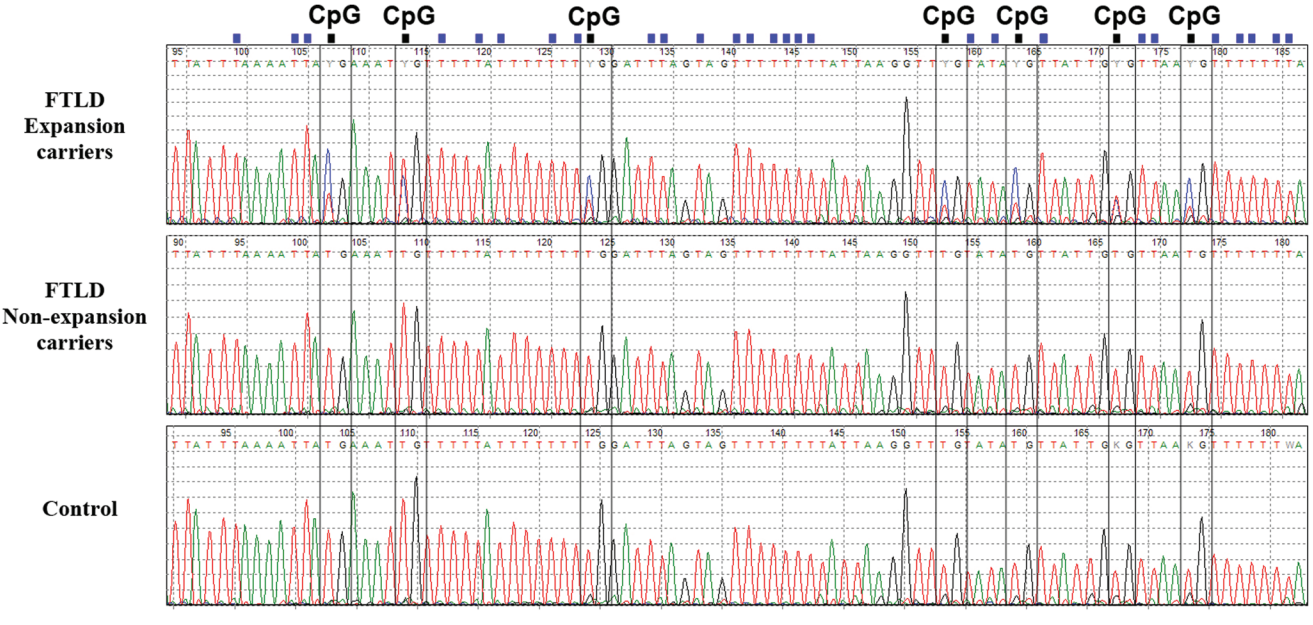

Figure 1. Bisulfite sequencing result. Representative chromatograms of a sequence containing seven CpG-sites (indicated by black squares at the top of the sequence diagram) are shown for an FTLD expansion carrier, an FTLD non-expansion carrier and a control. All non-CpG cytosine (indicated by blue squares) were successfully converted to thymine.

investigated carriers are heterozygous for the expansion; therefore, the highest methylation percentage should be up to ~50%, if only the expanded allele is methylated. Indeed, the percentage of methylation was highest in the high methylation category (18–51%) versus the low (0.4–4%) or no (0.4–0.8%) methylation categories (Supplementary Material, Fig. S2B).

The direct bisulfite sequencing results were further confirmed by the methylation sensitive HhaI-assay developed previously (15). The observed methylation (OM) ratio was significantly higher in the group of 34 FTLD expansion carriers (mean ± SD = 0.46 ± 0.40) versus 166 FTLD non-carriers (mean ± SD = 0.26 ± 0.21, Mann–Whitney *U* test $P = 0.005$) or versus 103 controls (mean ± SD = 0.15 ± 0.13, Mann–Whitney *U* test $P = 9.7E-8$). A representative gel image of 8 samples from each group is shown in Supplementary Material, Fig. S3. A scatter plot of the OM ratio against the number of methylated CpGs revealed a significant correlation between the two assays ($P < 0.0001$) (Supplementary Material, Fig. S3).

Regarding the individual CpG-sites, we observed that any of the 26 evaluated CpGs could be methylated in expansion carriers (Fig. 2). No correlation was found in the expansion carriers between the number of methylated CpGs and wild-type allele (2–11 repeats in our dataset): Spearman's correlation coefficient = −0.068, $P = 0.708$. Of note, samples from all 5 FTLD patients carrying an intermediate allele (22–31 repeats)

Table 2. Methylation level of blood DNA from FTLD expansion carriers, FTLD non-carriers and controls

Methylation level (number of methylated CpG)	FTLD expansion		FTLD non-expansion		Control	
	N	Frequency	*N*	Frequency	*N*	Frequency
No methylation (0)	15	0.44	159	0.96	94	0.91
Low methylation (1–3)	12	0.35	7	0.04	9	0.09
High methylation (4–26)	7	0.21	0	0	0	0
Total	**34**		**166**		**103**	
P^a: compared with FTLD carriers			**7.8E − 13***		**8.3E-9***	
P^a: compared with FTLD non-carriers					0.128	

[a]Two-sided Pearson χ^2 test or Fisher's exact test was used (when expected value <5).
* $P < 0.05$.

were completely unmethylated. In expansion carriers, methylation level did not correlate with age of onset of FTLD (44–74 years old; Spearman's correlation coefficient = −0.006, $P = 0.975$), age at time of examination (46–76 years old; Spearman's correlation coefficient = 0.002, $P = 0.991$), or gender (Fisher exact test $P = 0.132$). Among the seven expansion carriers who had high methylation levels, five were pure FTLD patients and two were ALS/FTLD patients. Of note, all five FTLD patients had positive family history; however the association between degree of methylation and FTLD family history did not reach statistical significance likely due to the small sample size (Supplementary Material, Table S1).

Combined analysis of FTLD and ALS datasets

To address the question whether DNA methylation plays a role in modifying disease phenotypes (ALS versus FTLD), we conducted a combined analysis of the FTLD dataset, our published ALS dataset (15) and 11 recently identified ALS expansion carriers. In total we analyzed 82 expansion carriers (42 ALS, 29 FTLD and 11 ALS/FTLD patients). A comparison of the three disease categories revealed a similar distribution of methylation level ($P > 0.05$), suggesting that it is not a major modifying factor for disease phenotype (Supplementary Material, Table S2).

In addition, no significant difference between ALS and FTLD was found in methylation frequency of individual CpGs, although the methylation frequency for many CpGs was marginally higher in ALS versus FTLD (Supplementary Material, Fig. S4). A tendency for more highly methylated samples was observed in ALS (36% cases) versus FTLD (17% cases) ($P = 0.09$; Supplementary Material, Table S2). A similar result was obtained when comparing the FTLD expansion carriers to the ALS and ALS/FTLD carriers combined ($P = 0.08$).

Methylation analysis of the CpG-island on anti-sense DNA strand

All of the methylation data above was obtained from the sense strand encoding *C9orf72*. This is of note since the bisulfite sequencing assay can only obtain information from a single strand (after bisulfite conversion the DNA double strands are no longer complementary to each other). To get further insight into the methylation patterns, we studied the same 26 CpGs on the anti-sense strand in 14 FTLD expansion carriers randomly selected based on different methylation degree of the sense strand: high methylated ($n = 4$), low methylated ($n = 4$) and unmethylated samples ($n = 6$). The results revealed that the position and number of methylated CpGs was mostly concordant between the strands (Supplementary Material, Fig. S5A).

DISCUSSION

We report the first investigation of DNA methylation at the *C9orf72* locus in an FTLD cohort. Methylation level of the 5′ CpG-island was significantly higher in FTLD expansion carriers than non-carriers. Methylation equally affected both the sense and anti-sense DNA strands, suggesting that it is likely to be a stable epigenetic modification maintained by DNA (cytosine-5)-methyltransferase 1 rather than a transitional phenomenon of active or passive demethylation (28–30). Importantly, the investigated CpG-island is mapped to the promoter of *C9orf72* and not any other neighboring gene on either strand (Supplementary Material, Fig. S5B). Therefore, hypermethylation would likely only affect *C9orf72*.

The current study was limited to DNA isolated from blood, since brain tissue was not available for our clinical cohort of FTLD patients. However, the methylation data obtained from blood DNA likely well-reflects the degree of methylation in brain tissue, since in a prior ALS study we observed high concordance of methylation level across different tissues (blood, frontal cortex and cervical spinal cord) using two independent assays (15). Moreover, while our manuscript was in preparation, Belzil *et al*. (31) detected DNA hypermethylation in cerebellum of an FTLD expansion carrier using our bisulfite sequencing protocol.

Combined analysis of the FTLD and ALS datasets revealed that the hypermethylation is expansion-specific, since a high level of methylation was not observed in any of the 415 non-carriers, irrespective of their disease status (FTLD, ALS or controls) and ethnic origin (Italian or Canadian). Of note, DNA samples from individuals with intermediate alleles (up to 43 repeats) were unmethylated, challenging the current 30-repeat cutoff for pathological alleles.

Analysis of all 82 expansion carriers suggested that methylation level is not a major modifying factor for disease phenotype (ALS versus FTLD). However, a tendency for more highly methylated samples was detected in ALS ($P = 0.09$). This observation could be important if proved to be significant in a larger dataset. Methylation degree was known to increase with the number of GAA-repeats in Friedreich's ataxia (32). The trend

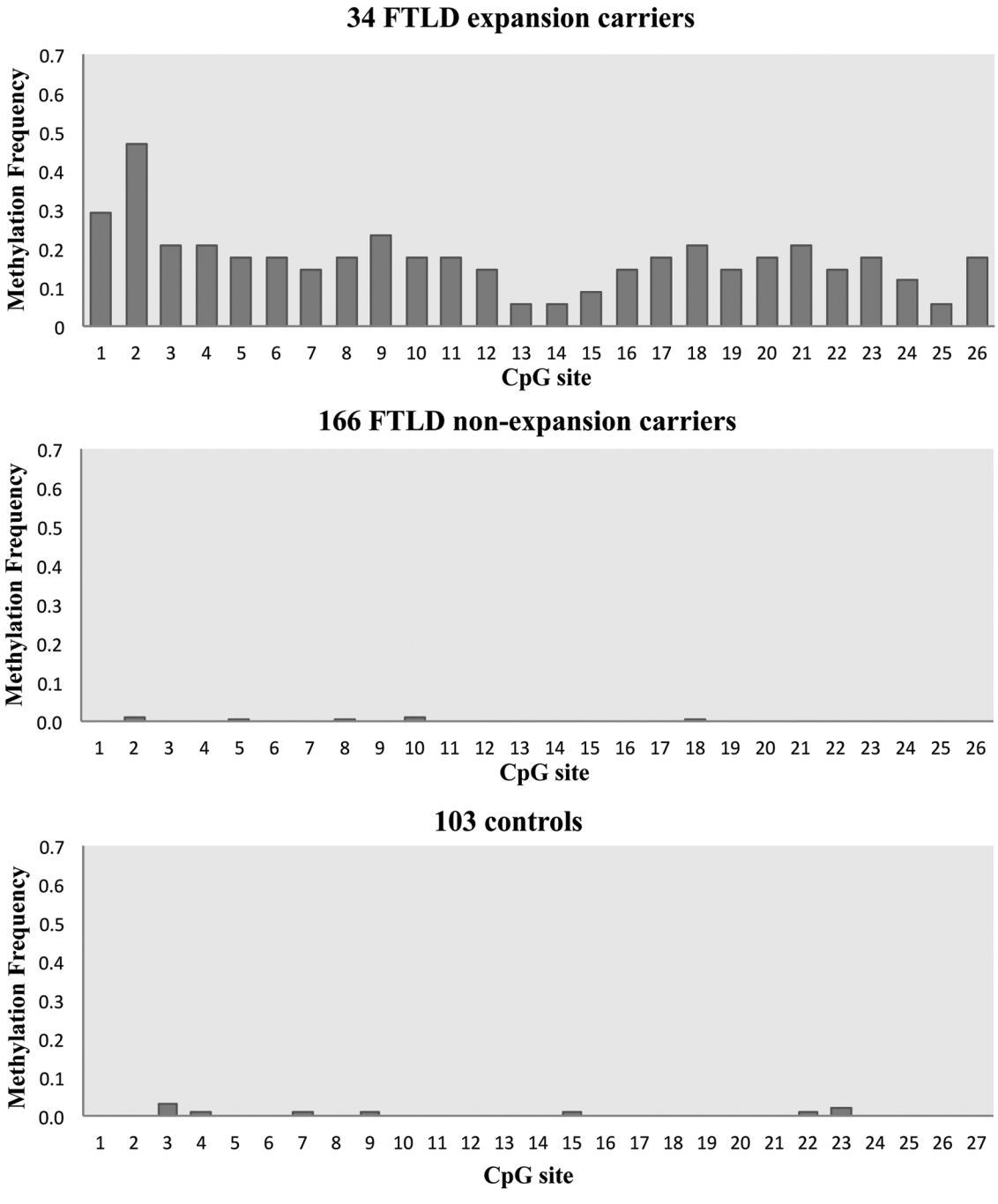

Figure 2. Methylation frequency of each CpG-site for the three investigated groups: FTLD expansion carriers, non-carriers and controls. The proportion of methylated samples to all samples in each subgroup is presented.

observed in our study may suggest that ALS is associated with the higher repeat number than FTLD. Such a possibility is in agreement with a Southern blot study of 60 expansion carriers, which showed that despite a substantial overlap in repeat length in peripheral blood, ALS patients had a higher repeat number than FTLD patients (33). However, this finding requires further validation given that an independent study did not detect any statistical difference in repeat length between motor neuron disease (MND; the most common form of which is ALS), FTLD and FTLD/MND in frontal cortex ($n = 41$), cerebellum ($n = 40$) or blood ($n = 47$) (34); although, the median repeat length appears to be higher in the blood of MND than FTLD patients.

Intriguingly, we detected a high methylation level of the CpG-island only in 36% of expansion carriers, yet down-regulation of *C9orf72* expression seems to be more prevalent, since it was previously reported in all evaluated expansion carriers (8,10,25,35–37). Reduced RNA levels (both mRNA and pre-mRNA) were also detected in neurons differentiated from induced pluripotent stem cells obtained from *C9orf72* ALS patients (37). It is possible that hypermethylation of another

CpG-island located physically distant from but conformationally close to the repeat is also involved. Moreover, it is possible that we studied only the tail end of the methylated region, since the G_4C_2-repeat forms an extra CpG-island exclusively in expansion carriers and thus could also be a target for DNA methylation. However, studying the methylation of the G_4C_2-repeat itself is challenging and requires the development of a novel approach, since the expansion cannot be amplified/sequenced by standard methods.

In addition, other epigenetic modifications such as histone methylation could contribute to down-regulation of *C9orf72*. Indeed, a recent small-scale study reported that trimethylation of histones H3 and H4 at several lysine residues was related to the reduced expression of *C9orf72* (38). Treating fibroblasts derived from expansion carriers with 5′-aza-2′-deoxycytydine (a demethylating agent of both DNA and histones) increased *C9orf72* expression. Such findings indicate that both DNA and histone methylation could be important in the regulation of *C9orf72*. In epigenetic gene regulation, histone methylation provides labile transcriptional repression, whereas DNA methylation is a stable long-term silencing marker (39). Both could trigger the other and restrict transcriptional factors from accessing DNA as a result of heterochromatin formation (39). In expansion carriers, reduced transcription of mRNA and increased binding of mutant *C9orf72* to trimethylated histones was found in both patients and asymptomatic carriers (15,38), thus the repression of *C9orf72* seems to be a long-term effect that likely involves DNA methylation. Moreover, the global methylation pattern of DNA and histone modifications are known to change with aging (40) and thus could be linked to the mid-adulthood onset of disease. Further studies are needed to determine whether the DNA and histone methylation are coordinated with each other and together contribute to the disease mechanism. However, in the current study we had access only to DNA samples that cannot be used in a histone analysis, which requires either cell lines or whole blood.

In conclusion, we have shown that hypermethylation of the CpG-island 5′ of the repeat is expansion-specific in FTLD patients, and occurred at a rate comparable to that seen in ALS study (15). A trend towards a higher proportion of ALS patients with a high methylation level versus FTLD may suggest that more ALS samples are in the high range of repeat number. Further validation of such observations may help improve future diagnosis of *C9orf72*-related diseases. Also, future studies have to include the investigation of DNA methylation along with other epigenetic mechanisms (e.g. histone modifications); as well as large consortium studies assessing the link between epigenetic markers and clinical parameters (e.g. disease duration).

MATERIALS AND METHODS

Human samples

Informed consent was obtained from all participants in accordance with the ethics review boards. DNA extracted from blood was available for 200 unrelated FTLD patients and 103 neurologically normal controls of Italian origin (>62 years old) (Table 1). The FTLD participants, mainly of Italian origin (except 6 from Argentina and 4 from Canada), were recruited from hospitals specializing in neurodegenerative disorders and diagnosed using established clinical criteria (41). To increase the sample size and compare the methylation level between ALS versus FTLD, we also studied blood DNA from 11 new Canadian ALS expansion carriers (including one ALS/FTLD patient) in addition to the reported cohort of 37 ALS expansion carriers (15).

C9orf72 genotyping

The *C9orf72* G_4C_2-repeat was genotyped by a two-step strategy as previously described (27). Briefly, the first step was the fluorescent fragment length genotyping to obtain the number of repeats of the small alleles (<50 repeats). The second step was the repeat-primed PCR to determine the presence of the expansion (>50 repeats). Pathological expansions were defined using the previously suggested 30-repeat cutoff (8,9).

Bisulfite sequencing

As described previously (15), each DNA sample was sequenced following bisulfite conversion, after which unmethylated C are read as T, while methylated C remain unchanged. The methylation status of each CpG was classified as unmethylated (T peak) or methylated (T/C double peaks). Only samples with >95% conversion rates of non-CpG C were included in the analyses. For each sample, we obtained the total number of methylated CpGs. All primers and experimental conditions for genotyping or methylation analyses are available in previous reports (15,27). For bisulfite sequencing of the anti-sense DNA strand (non-coding for *C9orf72*), the same CpG-island was amplified by a semi-nested PCR (in two tandem amplicons) and sequenced (Supplementary Material, Table S3). To validate the results of direct bisulfite sequencing, PCR products were cloned (TOPO TA cloning kit, Invitrogen) and sequenced in both directions using commercial vector primers (M13 Forward and M13 Reverse). For each sample, 10 clones were sequenced and the methylation percentage was calculated as follows: (the overall methylated CpG sites/the total studied CpG sites) × 100%.

Methylation sensitive restriction enzyme assay

As described previously (15), each DNA sample was amplified after incubation with or without HhaI. The PCR product from the digested and undigested DNA was resolved on a 1.5% agarose gel and quantified using black/white inverted gel images to obtain the OM ratio.

Statistical analyses

A linear regression analysis was performed between the OM ratio (HhaI-assay) and number of methylated CpG sites (bisulfite sequencing assay) to assess the correlation between the two assays. Spearman's correlation coefficients were used to measure the correlation between independent variables: repeat size (<50 repeats), age, disease duration and methylation level. The independent samples *t*-test or the non-parametric Mann–Whitney *U* test was used to compare continuous variables between two groups as appropriate. The two-sided Pearson χ^2 test or Fisher's exact test (when expected value <5) was

used to compare categorical variables. All analyses were performed using SPSS (version 20).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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REFERENCES

1. Cruts, M., Gijselinck, I., Van Langenhove, T., van der Zee, J. and Van Broeckhoven, C. (2013) Current insights into the C9orf72 repeat expansion diseases of the FTLD/ALS spectrum. *Trends Neurosci.*, **36**, 450–459.
2. Mackenzie, I.R., Rademakers, R. and Neumann, M. (2010) TDP-43 and FUS in amyotrophic lateral sclerosis and frontotemporal dementia. *Lancet Neurol.*, **9**, 995–1007.
3. Rademakers, R., Neumann, M. and Mackenzie, I.R. (2012) Advances in understanding the molecular basis of frontotemporal dementia. *Nat. Rev. Neurol.*, **8**, 423–434.
4. Van Langenhove, T., van der Zee, J. and Van Broeckhoven, C. (2012) The molecular basis of the frontotemporal lobar degeneration-amyotrophic lateral sclerosis spectrum. *Ann. Med.*, **44**, 817–828.
5. Neary, D., Snowden, J.S., Gustafson, L., Passant, U., Stuss, D., Black, S., Freedman, M., Kertesz, A., Robert, P.H., Albert, M. *et al.* (1998) Frontotemporal lobar degeneration: a consensus on clinical diagnostic criteria. *Neurology*, **51**, 1546–1554.
6. Brooks, B.R., Miller, R.G., Swash, M. and Munsat, T.L. (2000) El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph. Lateral Scler. Other Motor Neuron Disord.*, **1**, 293–299.
7. Hardy, J. and Rogaeva, E. (2013) Motor neuron disease and frontotemporal dementia: sometimes related, sometimes not. *Exp. Neurol.* [Epub ahead of print].
8. DeJesus-Hernandez, M., Mackenzie, I.R., Boeve, B.F., Boxer, A.L., Baker, M., Rutherford, N.J., Nicholson, A.M., Finch, N.A., Flynn, H., Adamson, J. *et al.* (2011) Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron*, **72**, 245–256.
9. Renton, A.E., Majounie, E., Waite, A., Simon-Sanchez, J., Rollinson, S., Gibbs, J.R., Schymick, J.C., Laaksovirta, H., van Swieten, J.C., Myllykangas, L. *et al.* (2011) A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. *Neuron*, **72**, 257–268.
10. Gijselinck, I., Van Langenhove, T., van der Zee, J., Sleegers, K., Philtjens, S., Kleinberger, G., Janssens, J., Bettens, K., Van Cauwenberghe, C., Pereson, S. *et al.* (2012) A C9orf72 promoter repeat expansion in a Flanders-Belgian cohort with disorders of the frontotemporal lobar degeneration-amyotrophic lateral sclerosis spectrum: a gene identification study. *Lancet Neurol.*, **11**, 54–65.
11. Mori, K., Weng, S.M., Arzberger, T., May, S., Rentzsch, K., Kremmer, E., Schmid, B., Kretzschmar, H.A., Cruts, M., Van Broeckhoven, C. *et al.* (2013) The C9orf72 GGGGCC repeat is translated into aggregating dipeptide-repeat proteins in FTLD/ALS. *Science*, **339**, 1335–1338.
12. Ash, P.E., Bieniek, K.F., Gendron, T.F., Caulfield, T., Lin, W.L., Dejesus-Hernandez, M., van Blitterswijk, M.M., Jansen-West, K., Paul, J.W. 3rd, Rademakers, R. *et al.* (2013) Unconventional translation of C9ORF72 GGGGCC expansion generates insoluble polypeptides specific to c9FTD/ALS. *Neuron*, **77**, 639–646.
13. Levine, T.P., Daniels, R.D., Gatta, A.T., Wong, L.H. and Hayes, M.J. (2013) The product of C9orf72, a gene strongly implicated in neurodegeneration, is structurally related to DENN Rab-GEFs. *Bioinformatics*, **29**, 499–503.
14. Zhang, D., Iyer, L.M., He, F. and Aravind, L. (2012) Discovery of novel DENN proteins: implications for the evolution of eukaryotic intracellular membrane structures and human disease. *Front Genet.*, **3**, 283.
15. Xi, Z., Zinman, L., Moreno, D., Schymick, J., Liang, Y., Sato, C., Zheng, Y., Ghani, M., Dib, S., Keith, J. *et al.* (2013) Hypermethylation of the CpG Island Near the GC Repeat in ALS with a C9orf72 Expansion. *Am. J. Hum. Genet.*, **92**, 981–989.
16. Al-Mahdawi, S., Pinto, R.M., Ismail, O., Varshney, D., Lymperi, S., Sandi, C., Trabzuni, D. and Pook, M. (2008) The Friedreich ataxia GAA repeat expansion mutation induces comparable epigenetic changes in human and transgenic mouse brain and heart tissues. *Hum. Mol. Genet.*, **17**, 735–746.
17. Greene, E., Mahishi, L., Entezam, A., Kumari, D. and Usdin, K. (2007) Repeat-induced epigenetic changes in intron 1 of the frataxin gene and its consequences in Friedreich ataxia. *Nucleic Acids Res.*, **35**, 3383–3390.
18. Evans-Galea, M.V., Carrodus, N., Rowley, S.M., Corben, L.A., Tai, G., Saffery, R., Galati, J.C., Wong, N.C., Craig, J.M., Lynch, D.R. *et al.* (2012) FXN methylation predicts expression and clinical outcome in Friedreich ataxia. *Ann. Neurol.*, **71**, 487–497.
19. Sutcliffe, J.S., Nelson, D.L., Zhang, F., Pieretti, M., Caskey, C.T., Saxe, D. and Warren, S.T. (1992) DNA methylation represses FMR-1 transcription in fragile X syndrome. *Hum. Mol. Genet.*, **1**, 397–400.
20. Pieretti, M., Zhang, F.P., Fu, Y.H., Warren, S.T., Oostra, B.A., Caskey, C.T. and Nelson, D.L. (1991) Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell*, **66**, 817–822.
21. Bell, M.V., Hirst, M.C., Nakahori, Y., MacKinnon, R.N., Roche, A., Flint, T.J., Jacobs, P.A., Tommerup, N., Tranebjaerg, L., Froster-Iskenius, U. *et al.* (1991) Physical mapping across the fragile X: hypermethylation and clinical expression of the fragile X syndrome. *Cell*, **64**, 861–866.
22. Klesert, T.R., Otten, A.D., Bird, T.D. and Tapscott, S.J. (1997) Trinucleotide repeat expansion at the myotonic dystrophy locus reduces expression of DMAHP. *Nat. Genet.*, **16**, 402–406.
23. Thornton, C.A., Wymer, J.P., Simmons, Z., McClain, C. and Moxley, R.T. 3rd (1997) Expansion of the myotonic dystrophy CTG repeat reduces expression of the flanking DMAHP gene. *Nat. Genet.*, **16**, 407–409.
24. Korade-Mirnics, Z., Tarleton, J., Servidei, S., Casey, R.R., Gennarelli, M., Pegoraro, E., Angelini, C. and Hoffman, E.P. (1999) Myotonic dystrophy: tissue-specific effect of somatic CTG expansions on allele-specific DMAHP/SIX5 expression. *Hum. Mol. Genet.*, **8**, 1017–1023.
25. Ciura, S., Lattante, S., Le Ber, I., Latouche, M., Tostivint, H., Brice, A. and Kabashi, E. (2013) Loss of function of C9orf72 causes motor deficits in a zebrafish model of Amyotrophic Lateral Sclerosis. *Ann. Neurol.*, **74**, 180–187.
26. Therrien, M., Rouleau, G.A., Dion, P.A. and Parker, J.A. (2013) Deletion of C9ORF72 results in motor neuron degeneration and stress sensitivity in *C. elegans*. *PLoS One*, **8**, e83450.
27. Xi, Z., Zinman, L., Grinberg, Y., Moreno, D., Sato, C., Bilbao, J.M., Ghani, M., Hernandez, I., Ruiz, A., Boada, M. *et al.* (2012) Investigation of C9orf72 in 4 neurodegenerative disorders. *Arch. Neurol.*, **69**, 1583–1590.
28. Arand, J., Spieler, D., Karius, T., Branco, M.R., Meilinger, D., Meissner, A., Jenuwein, T., Xu, G., Leonhardt, H., Wolf, V. *et al.* (2012) *In vivo* control of CpG and non-CpG DNA methylation by DNA methyltransferases. *PLoS Genet.*, **8**, e1002750.
29. Laird, C.D., Pleasant, N.D., Clark, A.D., Sneeden, J.L., Hassan, K.M., Manley, N.C., Vary, J.C. Jr, Morgan, T., Hansen, R.S. and Stoger, R. (2004) Hairpin-bisulfite PCR: assessing epigenetic methylation patterns on complementary strands of individual DNA molecules. *Proc. Natl. Acad. Sci. U.S.A.*, **101**, 204–209.
30. Ehrlich, M. and Lacey, M. (2013) DNA hypomethylation and hemimethylation in cancer. *Adv. Exp. Med. Biol.*, **754**, 31–56.
31. Belzil, V.V., Bauer, P.O., Gendron, T.F., Murray, M.E., Dickson, D. and Petrucelli, L. (2014) Characterization of DNA hypermethylation in the cerebellum of c9FTD/ALS patients. *Brain Res.* [Epub ahead of print].

32. Castaldo, I., Pinelli, M., Monticelli, A., Acquaviva, F., Giacchetti, M., Filla, A., Sacchetti, S., Keller, S., Avvedimento, V.E., Chiariotti, L. *et al.* (2008) DNA methylation in intron 1 of the frataxin gene is related to GAA repeat length and age of onset in Friedreich ataxia patients. *J. Med. Genet.*, **45**, 808–812.
33. Dols-Icardo, O., Garcia-Redondo, A., Rojas-Garcia, R., Sanchez-Valle, R., Noguera, A., Gomez-Tortosa, E., Pastor, P., Hernandez, I., Esteban-Perez, J., Suarez-Calvet, M. *et al.* (2013) Characterization of the repeat expansion size in C9orf72 in amyotrophic lateral sclerosis and frontotemporal dementia. *Hum. Mol. Genet.*, **23**, 749–754.
34. van Blitterswijk, M., Dejesus-Hernandez, M., Niemantsverdriet, E., Murray, M.E., Heckman, M.G., Diehl, N.N., Brown, P.H., Baker, M.C., Finch, N.A., Bauer, P.O. *et al.* (2013) Association between repeat sizes and clinical and pathological characteristics in carriers of C9ORF72 repeat expansions (Xpansize-72): a cross-sectional cohort study. *Lancet Neurol.*, **12**, 978–988.
35. Fratta, P., Poulter, M., Lashley, T., Rohrer, J.D., Polke, J.M., Beck, J., Ryan, N., Hensman, D., Mizielinska, S., Waite, A.J. *et al.* (2013) Homozygosity for the C9orf72 GGGGCC repeat expansion in frontotemporal dementia. *Acta Neuropathol.*, **126**, 401–409.
36. Cooper-Knock, J., Higginbottom, A., Connor-Robson, N., Bayatti, N., Bury, J.J., Kirby, J., Ninkina, N., Buchman, V.L. and Shaw, P.J. (2013) C9ORF72 transcription in a frontotemporal dementia case with two expanded alleles. *Neurology*, **81**, 1719–1721.
37. Donnelly, C.J., Zhang, P.W., Pham, J.T., Heusler, A.R., Mistry, N.A., Vidensky, S., Daley, E.L., Poth, E.M., Hoover, B., Fines, D.M. *et al.* (2013) RNA Toxicity from the ALS/FTD C9ORF72 Expansion Is Mitigated by Antisense Intervention. *Neuron*, **80**, 415–428.
38. Belzil, V.V., Bauer, P.O., Prudencio, M., Gendron, T.F., Stetler, C.T., Yan, I.K., Pregent, L., Daughrity, L., Baker, M.C., Rademakers, R. *et al.* (2013) Reduced C9orf72 gene expression in c9FTD/ALS is caused by histone trimethylation, an epigenetic event detectable in blood. *Acta Neuropathol.*, **126**, 895–905.
39. Cedar, H. and Bergman, Y. (2009) Linking DNA methylation and histone modification: patterns and paradigms. *Nat. Rev. Genet.*, **10**, 295–304.
40. Fraga, M.F. and Esteller, M. (2007) Epigenetics and aging: the targets and the marks. *Trends Genet.*, **23**, 413–418.
41. 1994) Clinical and neuropathological criteria for frontotemporal dementia. The Lund and Manchester Groups. *J. Neurol. Neurosurg. Psychiatry*, **57**, 416–418.