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The C9orf72 repeat expansion itself is methylated in ALS and FTLD patients

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Abstract

The most common cause of both amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) is a G4C2-repeat expansion in C9orf72. However, the lower limit for pathological repeats has not been established and expansions with different sizes could have different pathological consequences. One of the implicated disease mechanisms is haploinsufficiency. Previously, we identified expansion-specific hypermethylation at the 5' CpG-island near the G4C2-repeat, but only in a fraction of carriers (up to 36 %). Here, we tested the hypothesis that the G4C2-repeat itself could be the main site of methylation. To evaluate (G4C2)n-methylation, we developed a novel assay, which was validated by an independent methylation-sensitive restriction enzyme assay. Notably, both assays are qualitative but not quantitative. Blood DNA was available for 270 unrelated individuals, including 71 expansion carriers. In addition, we investigated blood DNA from family members of 16 probands, and 38 DNA samples from multiple tissues of 10 expansion carriers. Finally, we tested DNA from different tissues of an ALS patient carrying a somatically unstable 90-repeat. We demonstrated that the G4C2-expansion is generally methylated in unrelated carriers of alleles >50 repeats (97 %), while small (<22 repeats) or intermediate (22–90 repeats)alleles were completely unmethylated. The presence of (G4C2)n-methylation does not separate the C9orf72-phenotypes (ALS vs. ALS/FTLD vs. FTLD), but has the potential to predict large vs. intermediate repeat length. Our results suggest that (G4C2)n-methylation might sometimes spread to the 5'-upstream region, but not vice versa. It is stable over time, since (G4C2)n-methylation was detected in carriers with a wide range of ages (24–74 years). It was identified in both blood and brain tissues for the same individual, implying its potential use as a biomarker. Furthermore, our findings may open up new perspectives for studying disease mechanisms, such as determining whether methylated and unmethylated repeats have the same ability to form a G-quadruplex configuration.

Keywords ALS · FTLD · C9orf72 · G4C2-repeat · Methylation

Introduction

It has become recognized that amyotrophic lateral sclerosis (ALS [MIM 612069]) and frontotemporal lobar degeneration syndromes (FTLD [MIM 600274]) are the extremes of a disease spectrum, sharing clinical, genetic and neuropathological overlaps [22]. This has been firmly established by the discovery of G4C2-repeat expansions in C9orf72 [MIM 614260] as the most common known genetic cause of ALS and FTLD [11, 41], accounting for up to 37 % familial and 7 % sporadic cases in whites [39]. In these instances, ALS and FTLD frequently co-occur within the same family, and often the same individual [46]. Why members of the same family can exhibit disparate clinical presentations is unclear, but may be related to genetic/environmental modifiers and/or expansion size. Notably, the lower limit for pathological repeat number has not been established and it is possible that expansion alleles with different sizes (typically ranging from hundreds to thousands repeats) could have different pathological consequences.

The mechanism of disease caused by C9orf72 is complex, possibly involving a multitude of molecular pathways. Three major hypotheses, each with varying degrees of evidence, have been promulgated. Two of them suggest toxic gain of function through either formation of RNA foci [11, 12, 17, 28, 33, 54] or the accumulation of dipeptide repeat (DPR) proteins through repeat-associated non-ATG (RAN) translation [2, 9, 32, 34, 35, 38]. The third possibility is loss of function through haploinsufficiency [11]. Notably, all three mechanisms could be linked to a secondary structure (G-quadruplex) formed by DNA and RNA containing the G4C2-repeat [14, 20, 40].

The focus of the current study is a loss-of-function mechanism that is supported by evidence gathered at multiple molecular levels and from different disease models. Firstly, C9orf72 transcription can be decreased by up to 50 % in expansion carriers [11, 18]. Such a reduction has been observed in several independent studies [6, 8, 12, 16, 52]. Although the translational consequence of the expansion is elusive due to the lack of reliable antibodies, a recent report indicated that C9orf72 protein levels are decreased in the frontal cortex of expansion carriers, most likely as a consequence of down-regulation of transcription [47]. Secondly, neurons differentiated from induced pluripotent stem cells obtained from C9orf72 ALS patients also exhibit reduced C9orf72 RNA levels [12]. Thirdly, the down-regulation of C9orf72 orthologues caused axonal degeneration of motor neurons in zebrafish [6] and agedependent motility defects in Caenorhabditis elegans [44]. Moreover, the G-quadruplex formed by the G4C2-repeat was shown to cause repeat-length-dependent accumulation of aborted transcripts of C9orf72, which in turn could impede full-length RNA transcription [20].

Abnormal epigenetic changes that lead to gene silencing is a hallmark of many repeat expansion diseases, such as Friedreich's ataxia [MIM 229300] [1, 13, 19], Fragile X syndrome [MIM 300624] [3, 37, 43] and Myotonic dystrophy [MIM 160900] [26, 27, 45]. In C9orf72-associated disease, epigenetic alterations may also explain the observed reduction in gene product. We previously studied DNA methylation of the CpG-island located in the promoter region of C9orf72 (~200 bp upstream of the 5' end of the G4C2-repeat). In both ALS and FTLD, hypermethylation of this CpG-island only occurred in expansion carriers [49, 52]. A higher degree of methylation was significantly associated with familial ALS and segregated with the expansion in family members [52]. Our findings were validated by independent studies [4, 30]; and all the reports revealed that the hypermethylated samples accounted only for a portion of expansion carriers (e.g., 36 % in ALS and 17 % in FTLD cohorts [49]). Since the transcriptional reduction of C9orf72 is a general phenomenon in carriers, it is possible that other regulatory regions could be a target of DNA methylation. Notably, the G4C2-repeat is predicted to form an extra CpG-island that only occurs in expansion carriers. Therefore, we hypothesized that the G4C2-repeat itself could be the main target of methylation.

The methylation study of the G4C2-repeats is challenging, since the expansion cannot be amplified/sequenced by standard methods. Here, we have developed a novel assay that combines repeat-primed PCR (rp-PCR) and methylation-specific PCR (MSP) to directly evaluate methylation of the G4C2-repeat itself, referred to as (G4C2)n-methylation.

Table 1 Characteristics of samples obtained from blood

	Expansion carriers ($n = 71$)			Non-expansion carriers ($n = 199$)				Intermediate allele carriers ($n = 9$)
	ALS	FTLD	ALS/FTLD	ALS	FTLD	ALS/FTLD	Control	FTLD, PD, ALS, unaffected
Sample number	35	25	11	54	59	10	76	9
Age of sample collection (average ± SD)	60.7 ± 8.5	62.6 ± 8.8	61.3 ± 6.9	65.0 ± 8.2	74.3 ± 7.0	74.7 ± 10.3	73.0 ± 7.6	71.2 ± 8.5
Age at onset (average ± SD)	57.9 ± 9.8	58.4 ± 8.6	57.5 ± 7.7	61.4 ± 10.6	68.2 ± 8.7	72.3 ± 10.4	–	–[a]
Female (no/freq)	16 (46 %)	12 (48 %)	1 (9 %)	19 (35 %)	33 (56 %)	6 (60 %)	48 (63 %)	6 (67 %)
Family history (no/freq)	20 (57 %)	17 (68 %)	6 (0.55)	5 (9 %)	23 (33 %)	0 (0 %)	–	–[a]
C9orf72 small allele (range)	2–11	2–16	2–10	2–8	2–8	2–5	2–8	2–6
C9orf72 large allele (range)	exp (>50)	exp (>50)	exp (>50)	2–21	2–15	2–15	2–19	22–43

[a] As intermediate allele carriers include both affected and unaffected individuals, data of average age at onset and frequency of familial samples cannot be calculated

Table 2 List of studied tissue samples (brain and spinal cord) and results of $(G_4C_2)_n$-methylation assay

	ALS expansion carriers		ALS/FTLD expansion carriers		Healthy controls	
	Number of samples	Frequency of methylated samples (%)	Number of samples	Frequency of methylated samples (%)	Number of samples	Frequency of methylated samples (%)
Cervical spinal cord	7	100	3	100	–	–
Frontal cortex	7	100	3	100	13	0
Temporal cortex	6	100	3	100	–	–
Motor cortex	6	100	3	100	–	–
Total	26	100	12	100	13	0

For the first time, we demonstrate that the G4C2-repeat expansion is generally methylated in unrelated carriers of alleles with >90 repeats (100 %), while the small (<22 repeats) or intermediate (22–90 repeats) alleles are completely unmethylated. Our results contribute to understanding the consequences of the expansion and might help to establish a more accurate cut-off for pathogenic repeat.

Materials and methods

Human samples

Informed consent was obtained from all participants in accordance with the respective ethics review boards. The ALS participants were recruited at the ALS Clinic, Sunnybrook Health Sciences Centre, Toronto, Canada and the FTLD participants were recruited from multiple hospitals specializing in neurodegenerative disorders. All affected individuals were diagnosed using established clinical criteria [5, 7, 24].

Sample characteristics are presented in Table 1. Blood DNA was available for 270 unrelated individuals, including 71 expansion carriers diagnosed with ALS (n = 35), FTLD (n = 25), and ALS/FTLD (n = 11); as well as 199 non-carriers with ALS (n = 54), FTLD (n = 59), ALS/ FTLD (n = 10), and normal controls (n = 76). In addition, we investigated the blood DNA from family members of 16 probands with an expansion (11 with ALS and 5 with FTLD). We also studied blood DNA from nine carriers of intermediate alleles (22–43 repeats), including cases with FTLD (n = 4), ALS/FTLD (n = 1), Parkinson disease (n = 3), and one asymptomatic member of an ALS family. Finally, we evaluated 38 DNA samples obtained from multiple

brain regions and cervical spinal cord of 10 autopsyconfirmed expansion carriers with ALS or ALS/FTLD; DNA from frontal cortex of 13 normal controls was used as a comparison group (Table 2). In addition, DNA from seven different tissues of an ALS patient carrying a somatically unstable 90-repeat was studied [15].

Genotyping of C9orf72 and principle of the (G4C2)n-methylation assay

The C9orf72 genotypes were mainly obtained from our previous study [51] using a two-step strategy (expansion carriers were detected when the rp-PCR showed sawtooth peaks beyond 50 repeats). Since controls generally carry 22 repeats that was measurable by the two-step genotyping (up to 50 repeats) was defined as an intermediate allele carrier. To visualize the small expansion carried by case #1740, undiluted PCR products obtained in the first step were resolved on a 3100 DNA analyzer (amplicon length analysis). Figure 1 presents the experimental principle of the (G4C2)n-methylation assay, which was designed based on the combination of two well-established techniques: MSP [23] and rp-PCR [48]. A similar approach was successfully applied in Fragile X syndrome to detect methylation of the CGG-expansion [53]. In MSP, DNA was initially modified by sodium bisulfite to convert all unmethylated, but not methylated, cytosines to uracils. After PCR, uracils were replaced by thymines. Hence, the (GGGGCC)n repeat was converted either to (GGGGTC)n when methylated, or (GGGGTT)n when unmethylated. Subsequent PCR using primers specific for methylated vs. unmethylated DNA discriminates between the two states. To distinguish the PCR products on an ABI DNA analyzer, the primers were labeled differently according to their targets: FAM for methylated DNA amplification (blue channel) and HEX for unmethylated DNA amplification (green channel). If the G4C2-repeat is methylated in all DNA copies, only the blue channel is expected to have products; and if the G4C2-repeat is unmethylated in all DNA copies, only the green channel is expected to have products. However, if the G4C2-repeat is methylated in some copies and unmethylated in others, both channels will have products.

Information on the repeat expansion was obtained by rpPCR (a method commonly used to detect the expansion in C9orf72 [51]). In brief, a locus-specific primer flanking the repeat (the fluorescently labeled forward primer) and a primer amplifying from multiple priming sites within the repeat (the reverse primer targeting the converted G4C2-sequence) were used. An anchor primer was also added to enhance PCR efficiency. On the fluorescence trace, repeat-primed PCR showed the G4C2-repeat as a peak ladder of 6 bp increments in amplicon length. For large repeat expansions, the peak ladder gradually decayed without a clear stop. For small repeat alleles, however, the peak ladder was relatively short and ended with a sudden stop. To better distinguish between repeat expansions and non-expansions, the reverse primer was designed to cover the G4C2-repeat as well as its flanking sequence. Such a design would stop the peak ladder for small repeat alleles with a spike that represents the allele size, but does not influence the peak ladder for repeat expansions (Fig. 1).

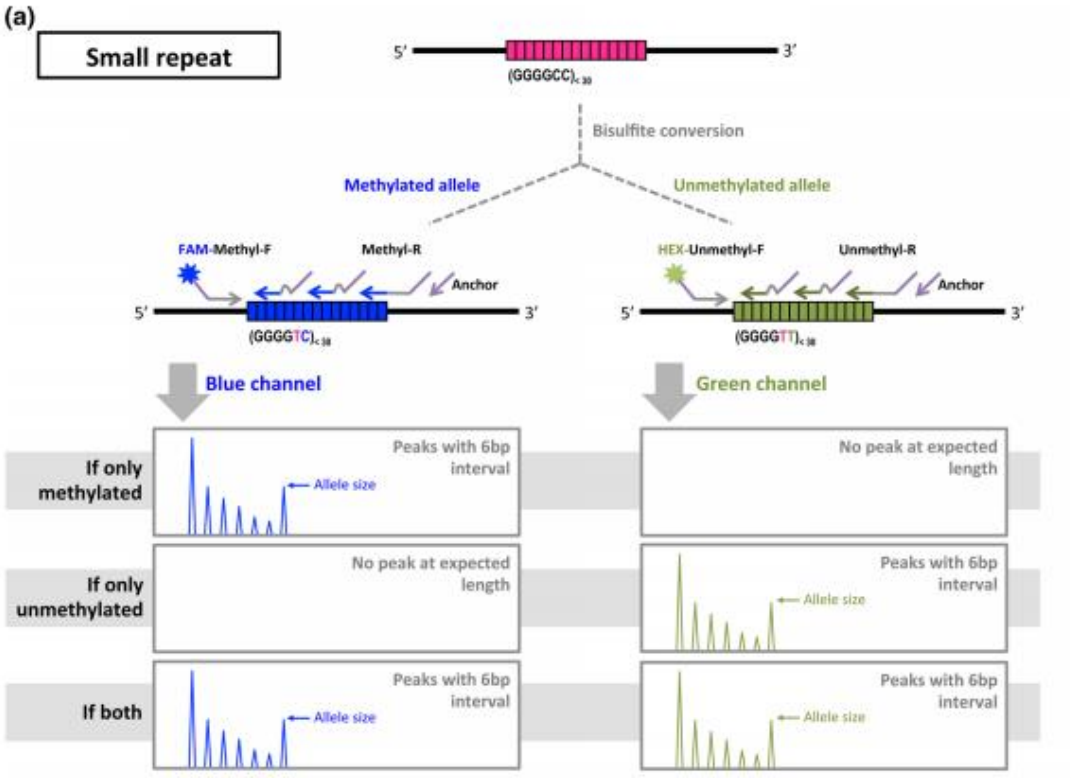
Experimental conditions of the (G4C2)n-methylation assay

Bisulfite treatment was performed with 0.5–1.0 μg of DNA using the EZ DNA Methylation-Lightning™ Kit (Zymo) according to the manufacturer's protocol. The treated DNA was eluted in 10 μl elution buffer and equally split to be used as template for the following methyl-specific (M) and unmethyl-specific (U) PCR. The M-primers and U-primers were designed partially by MethPrimer [29] software and partially by modifying the rp-PCR primers used for C9orf72 genotyping [51]. The locus covered by the MSP primers is shown in supplementary Figure 1 and primers are listed in supplementary Table 1. MSP reactions were performed in 50 μl using FastStart PCR Master Mix (Roche) with 800 nM of 7-Deaza-dGTP (Roche). A slow-down PCR program was used to amplify the MSP product. Specifically, the PCR was initiated with a denaturation step at 96.5 °C for 13 min and followed by two stages of amplification. In stage one, the annealing temperature was decreased from 70 to 63 °C at a rate of −1 °C/2 cycles and then from 61 to 57 °C

at a rate of −2 °C/2 cycles. In stage two, PCR was continued with an annealing temperature at 55 °C for 35 cycles. For each cycle, 1 min was used for the denaturation and annealing steps, and 3 min was used for the extension step. The ramp rate was adjusted to 3.2 °C/s for the denaturation and extension steps and 1.3 °C/s for the annealing step. The program was finalized by a 10-min extension at 72 °C. After amplification, 2.5 µl of PCR product was mixed with HiDi Formamide (Applied Biosystems) and GeneScan 400HD Rox size standard (Applied Biosystems) and resolved on an ABI 3100 DNA Analyzer. Data were visualized by Genotyper software (version 3.6, Applied Biosystem).

Methylation-sensitive restriction enzyme (MSRE) assay

Blood DNA (1 µg) was digested overnight at 37 °C with 10 U HpaII or MspJI (New England Biolabs), followed by a 10-min inactivation at 97 °C. HpaII digests only unmethylated DNA, hence the following PCR produces an amplification product only for methylated DNA. MspJI digests only methylated DNA and the following PCR produces a product only for unmethylated DNA. Duplicate reactions were prepared without HpaII or MspJI but with their respective digestion buffers to serve as internal controls. The digested and undigested DNA (100 ng) was amplified in parallel by the same primer sets used in rp-PCR of C9orf72 genotyping [51]. PCR conditions were described previously [51]. Fragment length analysis was conducted using an ABI 3100 DNA Analyzer and data were visualized by Genotyper software (version 3.6, Applied



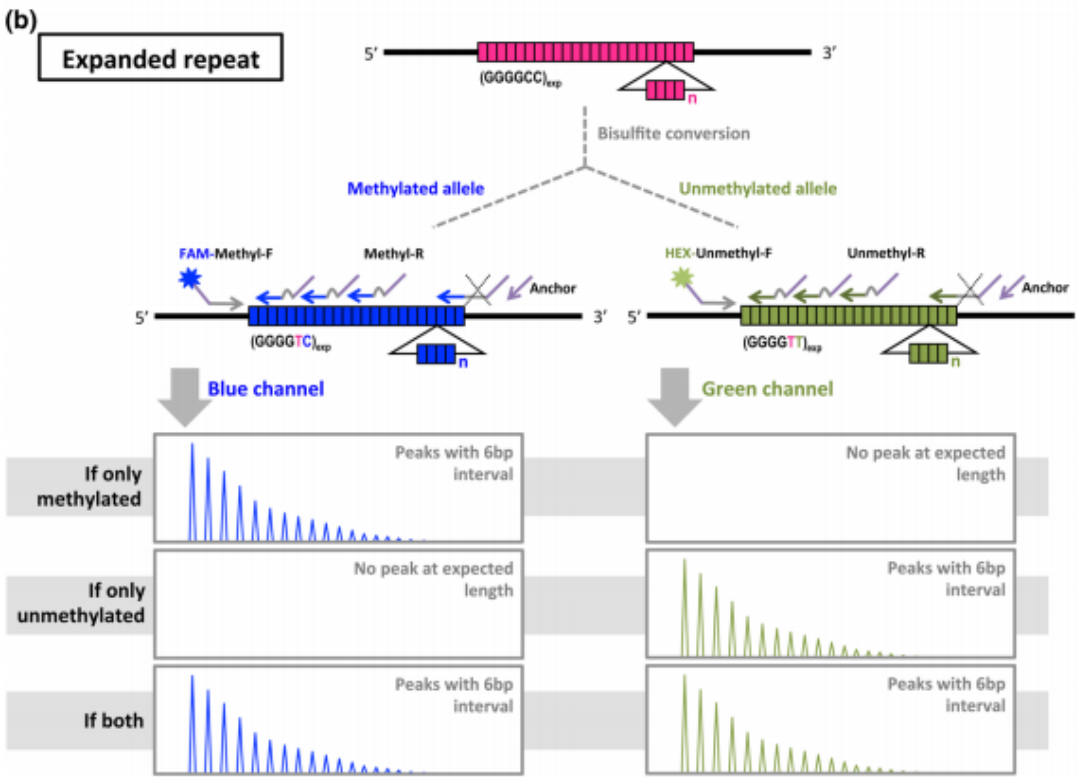


Fig. 1 Schematic showing the experimental principle of the (G4C2)nmethylation assay. a The expected result for small repeat alleles. Peak spikes represent the allele size. b The expected result for expanded repeat alleles. “X” on the Methyl-R primer represents the inability to amplify the full-length G4C2-repeat expansion

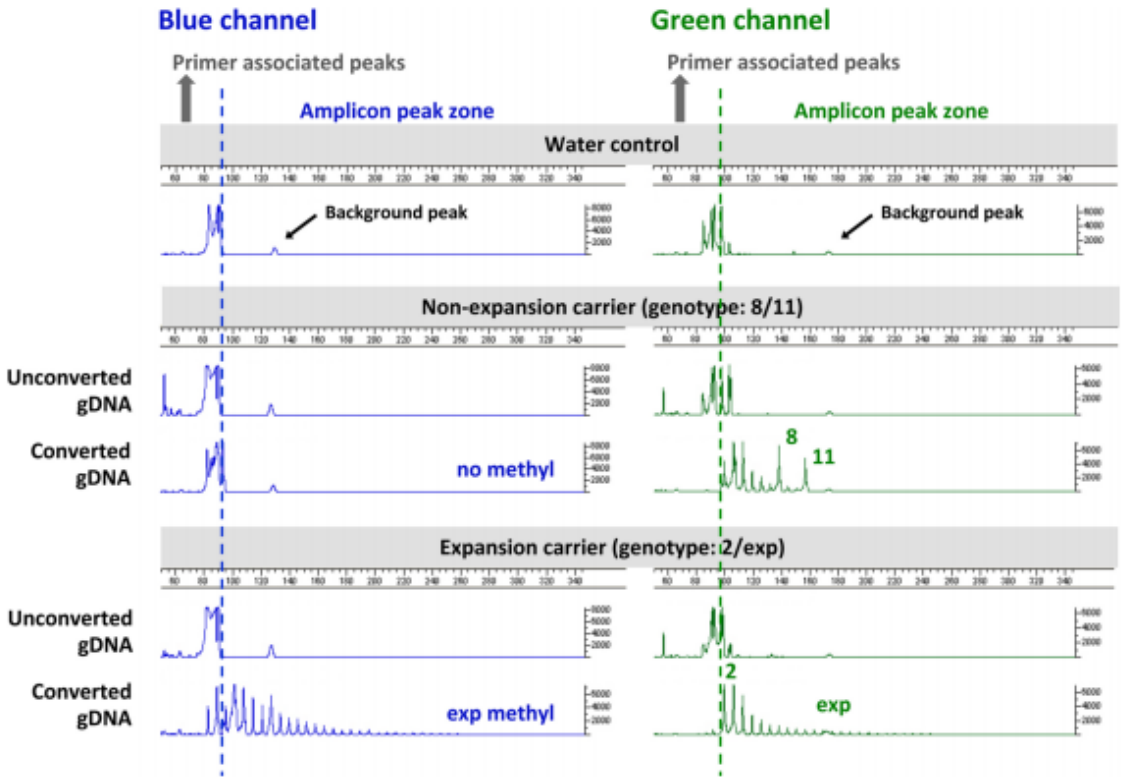


Fig. 2 Representative chromatograms of the test experiment. Results are shown for water, bisulfite-treated/-untreated expansion carriers and non-carrier. Detectable repeat size was marked along with the corresponding peak

Biosystems). Of note, both the (G4C2)n-methylation and MSRE assays cannot be used to quantify the degree of methylation of the G4C2-repeat (it is a qualitative "yes/no" test).

Southern blotting

Southern blotting was performed as previously described [15], using a 1-Kb single copy probe and a double digest with EcoRI/BamHI to generate a 'normal' band of 2.4 Kb. Statistical analysis Two-sided Fisher's exact test was performed using SPSS (version 20) to compare the percentage of methylated cases among the three disease subgroups (ALS, ALS/FTLD, and FTLD). Statistical significance was taken to be $p < 0.05$.

Results

Development of a novel assay for direct detection of methylation of the G4C2-repeat

To test the (G4C2)n-methylation assay, we first examined a small set of samples with known C9orf72 genotypes (3 expansion carriers and 3 non-carriers). Both converted and unconverted DNA were amplified for each sample. A negative control reaction was performed using molecular grade water. As shown in the representative results (Fig. 2), the fluorescence traces revealed a set of continuous peaks in the range of 80–350 bp. The result of the control reaction indicated that the peaks before the dotted line were primer associated, in addition to a small background peak across all the samples. For the converted DNA, amplifications were all successful and amplicon peaks started from approximately 100 with 6 bp increments as expected. The peaks for non-expanded alleles could be visibly distinguished from the gradually decaying peaks for expanded alleles (e.g., 8- and 11-repeat alleles were clearly displayed by two spikes in the unmethylation channel). Importantly, amplification of M-PCR only occurred for the expanded alleles, indicating that the G4C2-repeat is methylated when expanded (at least 30 repeats at the 5' end of the expansion are all methylated, based on visible sawtooth peaks). For unconverted DNA, no amplicon was generated at the expected length, meaning that the MSP primers are specific to the bisulfite-converted DNA and our findings were not due to false-positive results. For clear visualization, all the following results are presented with the amplicon peak zone (95–350 bp) indicated in Fig. 2. Each peak represents the specific amplification from the corresponding repeat (methylated repeat in the blue channel or unmethylated repeat in the green channel).

To further validate the (G4C2)n-methylation assay, we generated methylation control samples using the custom service provided by EpigenDx Inc. One control sample with C9orf72 genotype of 5/5 was chemically and enzymatically treated to generate high-methylated (>85 %) and low-methylated (<5 %) DNA. Control experiments were carried out using a series of samples mixed with both DNA at different percentages. No methylation was detected in the low-methylated DNA (supplementary Figure 2). Only a low level of the unmethylated signal was detected in the high-methylated DNA, probably due to the fact that artificially modified DNA cannot reach a methylation level of 100 %. Importantly, the (G4C2)n-methylation assay was sensitive enough to detect repeat methylation in the mixture containing only 5 % high-methylated DNA. However, it is a qualitative but not quantitative assay, since samples with different methylation percentages showed similar signal intensity.

Before analyzing the entire dataset, we studied DNA input amount in the bisulfite treatment for its influence on the (G4C2)n-methylation assay. The signal for unmethylated DNA could be detected with input

as low as 50 ng, while the methylation signal could only be confidently detected when the DNA input was above 200 ng (supplementary Figure 3). To ensure reliable detection of methylation, we used at least 500 ng of DNA to perform bisulfite treatment for every studied sample.

(G4C2)n-methylation study of carriers of expansions with >50 repeats

A total of 71 unrelated expansion carriers were evaluated using blood DNA (Table 1) and 97 % of them revealed methylated expanded alleles. No difference in the percentage of methylated samples was found among the three disease subgroups (ALS vs. FTLD, p = 0.17; ALS/FTLD vs. FTLD, p = 1.0). All of the 46 ALS or ALS/FTLD carriers demonstrated methylation of the repeat expansion (Figs. 3a, 4). Among the samples from the 25 FTLD carriers, 23 (92 %) were methylated (Fig. 4). The chromatograms for the two FTLD expansion carriers without (G4C2)n-methylation (sporadic patients #9983 and #1740) are shown in supplementary Figure 4. To investigate whether the lack of (G4C2)n-methylation could reflect a relatively small repeat expansion, we performed a Southern blot for sample #9983. Indeed, case #9983 is a carrier of a small expansion with ~60 repeats (range 50–110 repeats, supplementary Figure 4). We did not have enough DNA for Southern blot of sample #1740; however, amplicon length analysis of undiluted PCR product revealed that sample #1740 is also a carrier of a small expansion with ~70 repeats (supplementary Figure 4).

Next, we evaluated blood DNA from family members of 16 probands that included an additional 24 expansion carriers. Again, all these carriers showed methylation of the G4C2-expansion regardless of their age or disease status (supplementary Table 2). Notably, the G4C2-expansion was incompletely methylated in each carrier; since an unmethylated signal was observed for all studied expanded alleles (Figs. 2, 3a, supplementary Figures 3, 5).

Ten of the expansion carriers were autopsy-confirmed ALS or ALS/FTLD cases with DNA available from temporal cortex, frontal cortex, motor cortex and cervical spinal cord. Overall, we evaluated 38 tissue samples. The expansion alleles were methylated in all examined tissues. In contrast, DNA samples from frontal cortex of 13 normal controls (Table 2) were free of methylation. Representative results of an expansion carrier with all studied tissues are presented in supplementary Figure 5.

(G4C2)n-methylation study of carriers of short or intermediate repeat alleles

We evaluated blood DNA of 221 non-carriers, including 199 unrelated individuals and 22 family members from 16 pedigrees (Table 1, supplementary Table 2; Figs. 3b, 4). None of them had (G4C2)n-methylation irrespective of their age and disease status (ALS, FTLD, ALS/FTLD or controls). The (G4C2)n-methylation was also absent in all nine carriers of intermediate alleles (22–43 repeats) (Figs. 3c, 4). In total, 555 chromosomes with less than 43 repeats showed no (G4C2)n-methylation. Importantly, it includes the 95 small alleles inherited with the expansion alleles (all carriers were heterozygous for the expansion), since spikes associated with small alleles were never seen in the methylation channel for expansion carriers (Fig. 3a).

Recently, an ALS patient from the United Kingdom was identified to carry an atypical small expansion that was somatically unstable [15]. In blood, cauda equina, dura mater, and skin fibroblasts, the expansion was ~90-repeat; while in central nervous system tissues (the frontal cortex, cerebellum and spinal cord) the G4C2-repeat expanded to approximately 950–3000 repeats [15]. We studied these seven tissues from the patient and found that (G4C2)nmethylation was dependent on repeat size even within the same individual. No (G4C2)n-methylation was detected if the G4C2-repeat was ~90-repeat; but (G4C2)n-methylation was observed when the G4C2-repeat expanded to 950–3000 repeats (Fig. 5).

Validation by MSRE-based assay

To further validate our findings, we used a second MSREbased method. A similar approach was recently reported by Liu et al. [30] to amplify the 3' end of G4C2-repeat, which

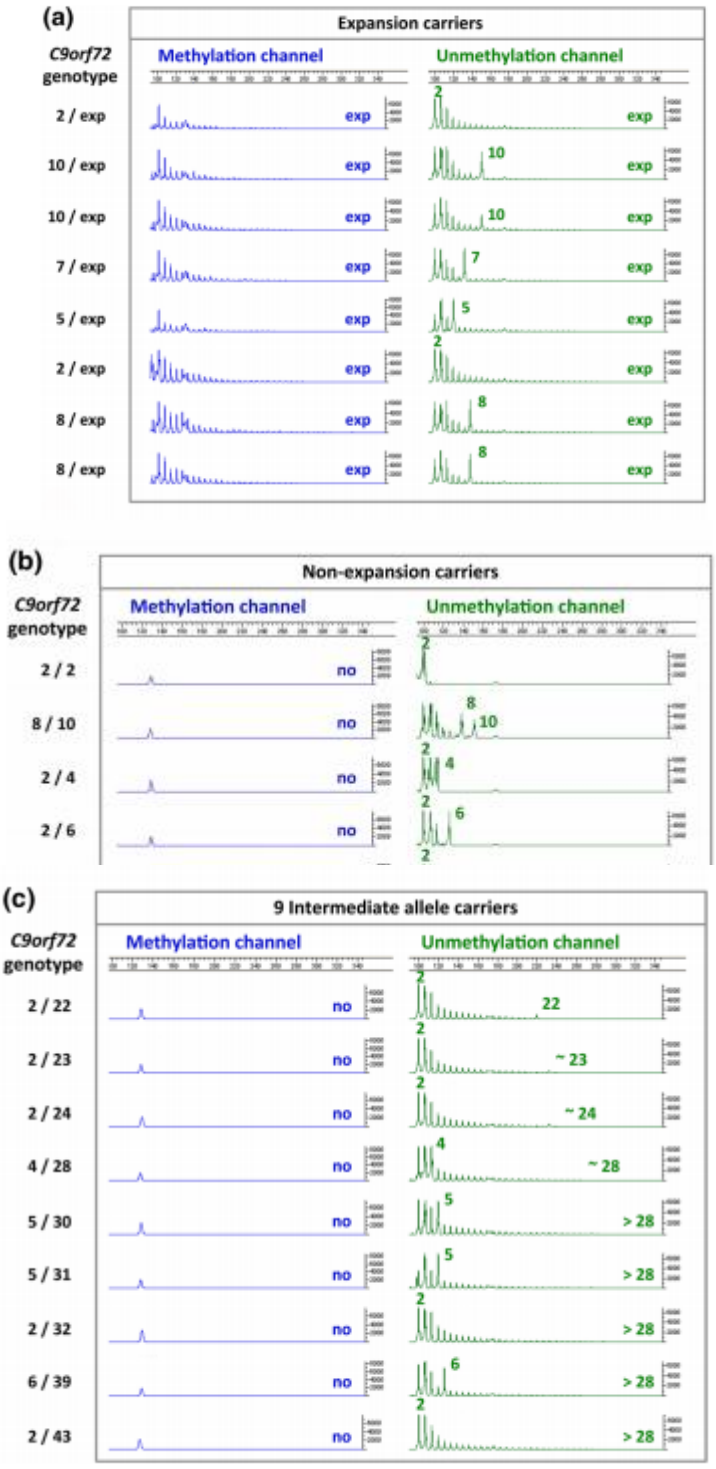


Fig. 3 Representative chromatograms of the $(G_4C_2)_n$-methylation assay for the main cohort (blood DNA). Results are shown for **a** eight expansion carriers, **b** eight non-expansion carriers, and **c** all nine intermediate allele carriers

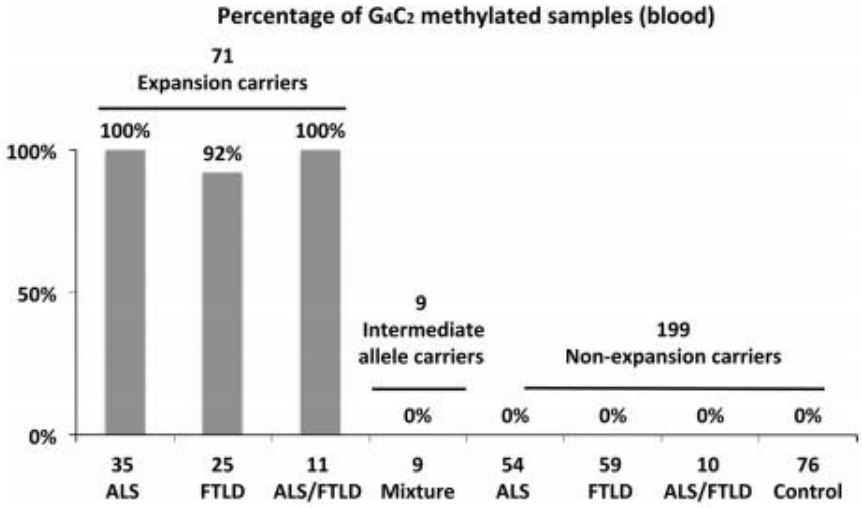


Fig. 4 Percentage of G_4C_2-repeat methylated samples in diagnostic subgroups (clustered based on repeat size)

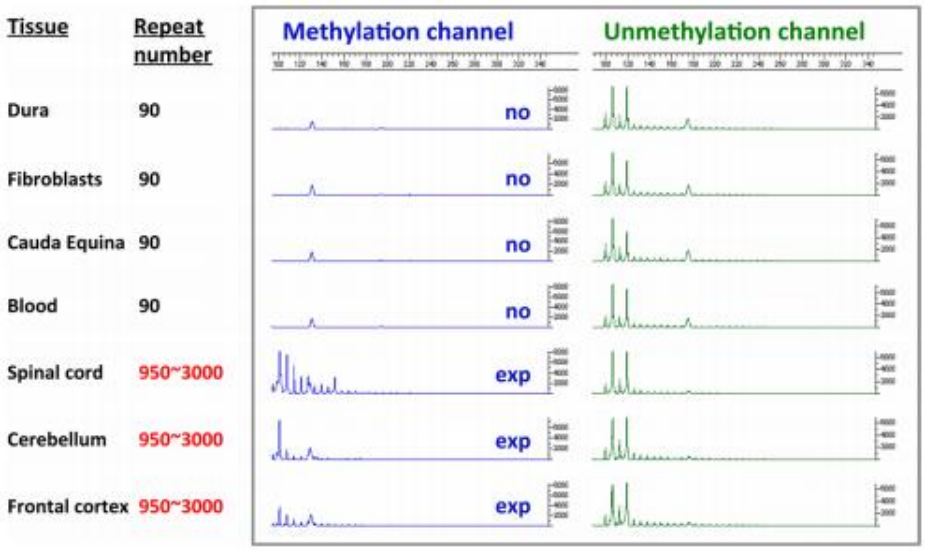


Fig. 5 The $(G_4C_2)_n$-methylation results of all seven tissues from the atypical 90-repeat carrier

did not detect methylation, likely due to the fact that they only studied the 3' end of G4C2-repeat, while in the current study all primers are designed to study the 5' end of the G4C2-repeat.

Of note, it was not possible to evaluate our entire dataset, since the MSRE-based assay requires ~4 µg of DNA for each sample (4 digestion reactions). In total, we investigated 12 samples, including 6 expansion carriers, 3 intermediate allele carriers and 3 non-carriers. Representative chromatograms are shown in supplementary Figure 6. As expected, only the expanded alleles were methylated. For example, in the sample with a C9orf72 genotype of 8/ expansion, only the expansion allele was amplifiable after HpaII digestion, but not the 8-repeat allele (supplementary Figure 6). Alternatively, expanded, intermediate, and nonexpanded alleles can all be amplified after MspJI digestion, meaning that they all have unmethylated

copies. Hence, the results from the MSRE-based assay are in line with the results from our (G4C2)n-methylation assay.

Discussion

We report on a novel assay that we have developed to directly evaluate presence or absence of CpG methylation of the G4C2-repeat, the most disease-relevant sequence for C9orf72 epigenetic studies. By screening a comprehensive cohort for (G4C2)n-methylation, including 95 expansion alleles (>50 repeats), 9 intermediate alleles (22–43 repeats), and 546 small alleles (<22 repeats), we found that (G4C2)n-methylation is expansion specific and detected in the majority of expansion carriers (97 %). The expansion and (G4C2)n-methylation co-occur in both blood and brain samples collected from affected or asymptomatic carriers (family members). The (G4C2)n-methylation is stable over time, since it was detected in carriers with a wide range of ages (24–74 years) (supplementary Table 2).

Importantly, (G4C2)n-methylation happens only when the G4C2-repeat gets largely expanded, with all nine intermediate alleles being completely unmethylated. In addition, we studied a carrier of small expansion (~90-repeat in blood DNA) that was reported to be somatically unstable in different tissues [15]. Our results show that there is no (G4C2)nmethylation of the 90-repeat allele in contrast to the hypermethylation detected for its expanded variant (>950 repeats). The fact that such opposite epigenetic signatures arose at the same genomic locus in the same person strongly implicates the tight link between repeat length and repeat methylation. It is possible that the only two FTLD patients from our cohort lacking (G4C2)n-methylation also have a small expansion in blood but large expansions in the brain. Indeed, we proved that blood DNA of both sporadic FTLD patients (#9983 and #1740) have a small expansion (60–70 repeats), indicating the predictive value of our (G4C2)n-methylation assay to separate large expansions from small ones. This result brings the frequency of expansion carriers with (G4C2)n-methylation to 100 % among carriers of large alleles (>90 repeats).

The current study evaluated a cohort of 279 unrelated samples, 271 of which were previously investigated for methylation of the 5′ CpG-island adjacent to G4C2-repeat and hypermethylation was detected in 36 % of the expansion carriers [49, 52]. All carriers of small and intermediate alleles are free from methylation at both the 5′ CpG-island and G4C2-repeat, indicating that the repeat and its adjacent sequences are unmethylated under normal conditions. In contrast, all carriers of large expansions (>90 repeats) revealed (G4C2)n-methylation irrespective of whether they were hypermethylated at the 5′ CpG-island. For instance, a recently reported pair of ALS-discordant monozygotic twins, who are free from 5′-methylation [50], display (G4C2)n-methylation. Cumulatively, our results suggest that (G4C2)n-methylation might sometimes spread to the 5′-upstream region and not the other way around. Methylation spreading is a well-documented phenomenon [25] and has been observed in Fragile X syndrome [36], although in that case methylation spreads from 5′-upstream to the downstream promoter and the CGG-repeat. Notably, Liu et al. [30] did not observe (G4C2)n-methylation at the 3′ end of the G4C2-repeat and in our previous study we did not observe methylation at the 3′ CpG-island downstream of the G4C2-repeat [52]. Collectively, these data may suggest that DNA methylation at the C9orf72 locus concentrates at the 5′ end of the G4C2-repeat, with its 5′ edge sometimes reaching the upstream CpG-island and its 3′ edge located somewhere within the G4C2-repeat.

The mechanism and the pathological significance of methylation spreading at the C9orf72 locus remain unknown. Despite the low occurrence in expansion carriers, 5′-methylation was inversely correlated to disease duration in our ALS cohort [52], highlighting its potential pathological relevance or ability to reflect the (G4C2)n-methylation level. Likewise, a recent study reported an association between 5′-methylation and age at death in FTLD patients [42]. However, these data need to be interpreted with caution, as both were derived from a limited number of samples. Clinical correlation analysis of the (G4C2)nmethylation is

not possible due to the technical difficulty in quantifying (G4C2)n-methylation, which is a limitation of the current study.

Another issue which needs to be considered for the 5'- and (G4C2)n-methylation is that they occur at different positions in relation to the C9orf72 gene structure. The 5' CpG-island is upstream of all three C9orf72 transcripts and expected to mostly contribute to an overall suppression of C9orf72 expression, although such suppression may lead to a reduction of toxic repeat RNA and RAN translation products. However, the G4C2-repeat can be either in the promoter of C9orf72 transcript 2 (NM_018325.3) or intron 1 of transcript 1 and 3 (NM_145005.5 and NM_001256054.1) [52].

In addition to the classic pathway of promoter inactivation, (G4C2)n-methylation is possibly involved in the pathophysiology through multiple mechanisms. A recent discovery has shown that the G4C2-repeat expansion within the sense strand forms a secondary DNA structure, called a G-quadruplex, during gene transcription, leaving the antisense strand free to bind the nascent C9orf72 mRNA and promoting RNA–DNA hybrids that disrupt normal transcription [20]. There was also a suggestion that G4C2-repeat G-quadruplex is a possible cause of downstream molecular cascades, such as the accumulation of toxic RNA foci and the generation of DPR [20]. Indeed, G-quadruplex structure is also formed by G4C2-repeat containing RNA [14, 40] and preferentially binds to an essential nucleolar protein, nucleolin, that was found to co-localize with the G4C2-RNA foci in the motor cortex neurons of C9orf72 ALS patients [20]. Interestingly, G4-motifs (nucleotide sequences with the potential to adopt G-quadruplex structure) are widely prevalent in the human genome with essential biological functions [31]. Genome-wide studies suggest that G4-motifs are generally hypomethylated [10, 21]. Taken together with our current findings, methylation on the G4C2-expansion might hinder G4-quadruplex structure formation; it may reflect a protective response, however, at the cost of interfering with normal C9orf72 expression. In such a scenario, the unmethylated G4C2-expansion copies detected in all studied carriers might be the suitable basis for G-quadruplex formation. Future studies, such as determining whether methylated and unmethylated G4C2-repeats have the same ability to form a G-quadruplex configuration will help to understand the relationship between (G4C2)n-methylation and G-quadruplex structure. If the (G4C2)n-methylation is truly involved in both protective and damaging mechanisms, the extent to which the two mechanisms outweigh each other might be a player in the high clinical heterogeneity in C9orf72 diseases.

In conclusion, we have developed a novel assay for detection of (G4C2)n-methylation and investigated its use in a comprehensive cohort. For the first time, we showed that (G4C2)n-methylation is the direct consequence of the mutation, since large expansions are methylated while small or intermediate alleles are not. The presence of (G4C2)n-methylation does not differ between the C9orf72-related phenotypes (ALS vs. ALS/FTLD vs. FTLD), but has the potential to predict large vs. intermediate repeat length. Except for the atypical 90-repeat carrier, (G4C2)n-methylation was always detected in blood, brain and spinal cord tissues of the same individual, implying its potential use in clinical applications as a biomarker. Our findings may open up new perspectives for studying disease mechanisms such as the relationship between the (G4C2)n-methylation and the G-quadruplex structure.

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