Modulation of methylation in the FMR1 promoter region after long term treatment with L-carnitine and acetyl-L-carnitine

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Fragile X syndrome (FXS) is a triplet repeat disorder caused by a large expansion of the CGG repeat in the 5'-untranslated region (UTR) of the fragile X mental retardation (FMR1) gene.1,2 Full mutation alleles are almost always associated with extensive hypermethylation of the repeat and of the upstream CpG island, which correlates with gene silencing and absence of the FMR1 protein. Cognitive function ranging from severe mental retardation to learning disabilities are found in affected people of both sexes. Many mildly affected people show “mosaic” methylation at the FMR1 promoter.3,4 Unusual alleles carrying a completely or partially unmethylated full mutation have been described.1,5 It was shown that in male patients with FXS with unmethylated alleles in the full mutation range, the FMR1 mRNA level is higher than in normal controls. This finding shows that upregulation of the FMR1 gene occurs in cells with unmethylated full mutation alleles and that the CGG triplet expansion does not suppress transcription directly.6,7 Thus, abnormal hypermethylation of the FMR1 promoter suppresses gene transcription. This hypothesis is also supported by the ability of 5-azadeoxycytidine (5-azadC) to restore the FMR1 gene expression in lymphoblastoid cell lines from patients with non-mosaic full mutation FXS by inducing DNA demethylation.8,9

The silencing of the hypermethylated FMR1 gene is consistent with a model in which methylation is coupled with the histone acetylation state. It has been found that the 5′ end of the FMR1 gene of patients with FXS is associated with deacetylated histones H3 and H410 and that the treatment of fragile X cells with 5-azadC results in the reassociation of acetylated histones with the FMR1 promoter and transcriptional reactivation. This finding suggests that both methylation and histone deacetylation are linked to transcriptional inactivity.11,12 In fact, it has been shown that fragile X cell lines treated with histone hyperacetylating drugs can markedly potentiate the effect of 5-azadC on FMR1 gene expression.13 However, when used alone, such drugs induce only a modest reactivation of the FMR1 gene.14 The same pattern of dominance of DNA methylation over histone acetylation has also been reported for other genes, the promoter of which resides in a CpG island.15

Changes of the methylation patterns over a five year period of alleles from five brothers variably affected by FXS indicate that methylation of individual CpG cytosines is strikingly variable in hypermethylated genotypes obtained from an individual patient.16 A reduced frequency of hypermethylated alleles occurred in the leucocytes of the two mildly affected brothers. These findings suggest that maintenance of cytosine methylation is a dynamic process that favours unmethylated alleles. It is conceivable that some compounds can be identified that may modulate this process and achieve gene reactivation.

Carnitine is a well known naturally occurring compound with an essential role in intermediary metabolism, mainly at the mitochondrial level. Acetyl-L-carnitine (γ-trimethyl-β-acetyl-butyrobetaine) is the carnitine ester naturally present in the central nervous system, differentially distributed in the various areas.17 The enzyme carnitine acetyltransferase catalyses both the formation of acetyl-L-carnitine from carnitine and acetyl-coenzyme A (acytetyl-CoA) and the reversible reaction. The modulation of the intracellular concentration of free CoA and acetyl-CoA is recognised to be a common mechanism for the various physiological activities of acetyl-L-carnitine,16,17 such as the acetylation of H4 histones.18 The chemical structure of acetyl-L-carnitine is similar to that of the acetylating agent butyrate. It has been shown that acetyl-L-carnitine, as well as butyrate, inhibits cytogenetic expression of the fragile X site in cultured lymphocytes of patients,19 suggesting that the interaction of these substances with the chromatin structure at the fragile site was present. Carnitine was also shown to suppress position effect variegation in

**Key points**

- We report the effect of acetyl-L-carnitine and L-carnitine on the methylation of the FMR1 promoter in long term cultures. The methylation status of the FMR1 promoter region containing 52 CpG sites was analysed in lymphoblastoid cell lines derived from healthy subjects and patients with FXS by a sensitive bisulphite based technique. We also analysed the 23 CpG sites in exon 1 of the SNRPN gene.
- CpG sites in control cultures from healthy subjects remained unmethylated in all the experimental conditions described. No changes were seen in the SNRPN gene. The promoter region of the untreated fragile X cell lines remained generally hypermethylated although the methylation level of individual CpGs was variable. Both acetyl-L-carnitine and L-carnitine induced a modest though evident decrease of the FMR1 promoter hypermethylation in two of the three fragile X cell lines.
- Our data suggest that long term treatment with the two carnitines has a mild but detectable effect against methylation of the FMR1 alleles.

**Abbreviations:** acetyl-CoA, acetyl-coenzyme A; 5-azadC; 5-azadeoxycytidine; FMR1, fragile X mental retardation gene; FXS, fragile X syndrome; RT-PCR, reverse transcriptase-polymerase chain reaction; SNRPN, small nuclear ribonucleoprotein polypeptide N gene; 5′-UTR, 5′-untranslated region
method and modified by the sodium bisulphite technique as described previously, with some modifications. Briefly, 3 μg of genomic DNA was denatured by a freshly prepared NaOH solution at 0.37 mol/l final concentration for 15 minutes at 75°C. DNA was then treated with sodium bisulphite at 3.6 mol/l final concentration adjusted to pH 5.0 and 1 mmol/l hydroquinone, overlaid with mineral oil, and incubated at 55°C for six hours in the dark. Samples of DNA were purified by the Wizard DNA Clean-Up System according to the manufacturer (Promega). Modification was completed by desulphation, incubating samples with 0.3 mol/l NaOH for 15 minutes at 37°C, and by neutralisation with 3 mol/l ammonium acetate, pH 5.2 overnight.

After ethanol precipitation, DNA samples were dissolved in 20 μl distilled water and the 414 bp region containing 52 CpG sites was analysed by PCR amplification of two DNA amplicons of 196 and 239 bp. Primers were designed to amplify bisulphite converted DNA of the FMR1 promoter. Both amplifications were performed in 100 μl reaction mixture containing 4 μl bisulphite treated genomic DNA, 200 μmol/l dNTPs, 0.4 μmol/l primers, 50 mmol/l KCl, 1.5 mmol/l MgCl2, 10 mmol/l Tris-HCl pH 9, 5% DMSO, and 0.2 μl Taq DNA polymerase (Promega). The first DNA fragment was amplified using primer 2F: GTTATTGAGTTTATTGTAGAAATGGG (positions 2297–2325 of GenBank sequence X61378) and 3R: CGTCTTCACTTGTTAGAAGCG (positions 2492–2466 of GenBank sequence X61378). For each DNA sample a pool of PCR reactions was prepared for both amplicons to evaluate on average the level of induced demethylation. The PCR pools were purified by recovering the bands from a 6% acrylamide gel and nucleotide sequencing was performed by the manufacturer (Promega).

Figure 1  CpG island of the 5’ end of the FMR1 promoter. The sequence analysed corresponds to bases 2297–2711 of the published genomic DNA. The locations of primers used for PCR amplification are indicated. All CpG dinucleotides are in bold print and numbered 1–52 and the conserved protein binding sites are boxed.
minutes in a total volume of 40 µl buffer containing 50 mmol/l Tris HCl pH 8, 3 mmol/l MgCl₂, 75 mmol/l KCl, 10 mmol/l DTT, 0.5 mmol/l dNTPs, 10 µmol/l random hexamers, 12 U RiboGuard (Amersham Pharmacia Biotech), and 100 U M-MLV-RT (Gibco-BRL). The PCR conditions for both FMR1 and HPRT were as follows: 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 40 seconds for 30 cycles. Primers for FMR1 cDNA amplification were CACTTTGGAGTCTGGCACC and TAGCTCCAATCTGCGCAACTGC. Primers for HPRT were CGTGGGTTGCTTTCACGCAGAA and AATTATGACACGG ACTGAAAGTC. Amplifications were performed in 50 µl reaction mixture containing 5 µl of cDNA, 200 mmol/l dNTPs, 1.5 mmol/l MgCl₂, 10 µmol/l primers, 50 mmol/l KCl, 10 mmol/l Tris-HCl, and 0.2 µl Taq DNA polymerase (Promega). The PCR products were electrophoresed on a 1% agarose gel.

RESULTS

In this study we investigated the effect of long term treatment with L-carnitine and acetyl-L-carnitine on the methylation status of the CpG island in the promoter region of the FMR1 gene by the bisulphite sequencing technique and on the transcription of the fully mutated FMR1 gene. Lymphoblastoid cell lines from patients with FXS and from healthy subjects were analysed during cell culture progression in the absence or presence either of L-carnitine or acetyl-L-carnitine. The fragile X cell cultures analysed (L, M, and F) were characterised by different CGG repeat expansions, which remained unchanged throughout the long term culturing as detected by Southern blot analysis. Fig 1 shows the 414 bp sequence analysed corresponding to bases 2297–2711 of the published genomic DNA sequence (GenBank X61378). CpG sites are in bold and numbered, primers used for PCR amplification are underlined, and the protein binding sites described by Kumari and Usdin are boxed.

The methylation status of the 52 tested CpG sites in cell cultures from patients with FXS is depicted in fig 2. The bisulphite sequencing of the FMR1 promoter was performed for each culture at the start of the cell culturing, after long term culturing, and after long term culturing in the presence of L-carnitine or acetyl-L-carnitine. Each column has been calculated as the ratio between the height of the thymine electropherogram peak and the sum of heights of cytosine and thymine peaks at the individual CpG site. Therefore, the black part of each column reflects the percentage of demethylation seen for each CpG site. A few individual CpG dinucleotides were partially unmethylated in all the starting cultures. In the F cell line the number of the sites that showed a partial demethylation increased from two to four in the control culture and to five in cells treated with L-carnitine and acetyl-L-carnitine. In this culture we found a striking spontaneous demethylation of sites 28 and 29. In the L cell line the number of unmethylated CpG sites increased from four in the starting culture to six in the untreated culture, and to 10 in cells treated with L-carnitine and to 11 in cells treated with acetyl-L-carnitine. Only sites 28 and 42 were partially unmethylated in all experimental conditions. In particular, position 28 was unmethylated in up to 70% of the cells in the starting culture and in cultures treated with the two compounds.

The mean methylation value for each culture, averaged on the 52 CpG sites, is summarised in table 1. The overall hypermethylation status of the L and M cell lines did not change after long term culturing with any added compound, but we found a decrease of mean methylation both with L-carnitine and acetyl-L-carnitine. The methylation degree of the F cell line did not change in long term cultures in the presence of the two compounds, although it showed a tendency to decrease methylation as well. The marked reduction of the mean methylation value in the untreated long term culture is the result of the unexpected high demethylation of only two sites (70% for site 28 and 60% for site 29). None of the 52 cytosines analysed in control cultures from healthy subjects was methylated in all the experimental conditions described confirming that methylation over time of the normal FMR1 gene is a very unlikely event.

Figure 2 Effect of L-carnitine and acetyl-L-carnitine (ALC) on the degree of CpG methylation. The column height shows the percentage of unmethylated cytosine for each of the 52 CpGs in the FMR1 promoter for L, M, and F lymphoblastoid cell lines. Each bar represents an individual CpG that is completely methylated. The percentage of unmethylated CpGs is indicated by the black part of the column. The methylation pattern determined at the start of cell culturing, and after long term culturing with and without L-carnitine or acetyl L-carnitine, respectively, are directly compared for each culture derived from fragile X. The 50% methylation is indicated.
acetylation. They hypothesized that a long-term treatment with histone hyperacetylation drugs may therefore be effective in reactivating the FMR1 gene expression in fragile X cell lines. Unfortunately, butyrate cannot be added for too long in culture because it readily causes cell cycle arrest, and previous experiments with sodium butyrate or 4-phenylbutyrate alone had to be limited to short treatments of 24–48 hours. Previous results from our groups have shown that acetyl-L-carnitine increases levels of H4 acetylation, also in the FMR1 gene itself. Therefore we were interested in exploring the DNA methylation status of the FMR1 promoter after long-term treatment with acetyl-L-carnitine and L-carnitine.

In the present study, no change in the CGG repeat expansion was detected by Southern blotting and long-term treatment with the two carnitines that were well tolerated by cells that possibly seemed to benefit in growth and viability. However, no reactivation of FMR1 gene transcription has been shown in treated fragile X cell lines compared with the untreated ones. Experiments with RT-PCR were negative at the start of treatment in cell lines F, M, and L, but remained negative even after the three months treatment with the two compounds. We found that the overall methylation status of the FMR1 promoter in fragile X lymphoblastoid cell lines is quite stable after culturing for about 100 cell duplications without any treatment. However, as previously reported, we found that methylation of individual CpG cytosine is variable in hypermethylated cell lines. On the other hand, we found a modest though measurable reduction of the hypermethylated status of the promoter in two of the three fragile X cell lines (L and M) grown with L-carnitine or acetyl-L-carnitine. It is noteworthy that the two compounds were less efficient in the F-cell line, which harbours an expansion of more than 2.5 kb suggesting that acetyl-L-carnitine and L-carnitine may be somewhat effective in reversing the hypermethylation present in full mutations with smaller CGG expansions.

The SNRPN gene is monoallelically expressed and has been used as an internal control for testing the potential demethylating effect of carnitines on another locus as well as for checking the efficiency of our experimental approach. The SNRPN gene is expressed from the paternal allele and hypermethylation is present only in the maternal allele. We consistently obtained a 1:1 ratio between the cytosome and thymine peaks at each of the 23 CpG sites investigated, before any treatment, confirming that our bisulphite sequencing approach was working well. Treatment with carnitines did not change the hemimethylated status of the SNRPN promoter region.

These data suggest that carnitines do not affect the methylation status of the cell itself but may be effective in the abnormal hypermethylation of the FMR1 gene to move the methylation equilibrium towards the unmethylated status. Overall these data show that methylation of individual CpGs in the FMR1 gene is a dynamic process that seems to favour unmethylated alleles (see sites 28 and 29 of F cell lines). This trend seems to be favoured by the carnitines we used. We suppose that L-carnitine and acetyl-L-carnitine, acting on the histone hyperacetylation process, favours demethylation of the FMR1 gene. It is conceivable to suppose that much longer treatments with acetyl-L-carnitine and L-carnitine might further decrease the methylation status. Any future pharmacological attempt at reactivating the FMR1 gene in vivo should therefore contemplate the use of safe DNA demethylating drugs ideally targeted to the FMR1 promoter region.

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REFERENCES


