ELECTRONIC LETTER

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

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J Med Genet 2003;40:e76(<http://www.jmedgenet.com/cgi/content/full/40/6/e76>)

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.^{5,6} 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.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.^{10,11} 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.¹² However, when used alone, such drugs induce only a modest reactivation of the *FMR1* gene.¹² 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.¹³

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.¹⁴ 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, differently distributed in the various areas.¹⁵ The enzyme carnitine acetyltransferase catalyses both the formation of acetyl-L-carnitine from carnitine and acetyl-coenzyme A (acetyl-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

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.

Drosophila,²⁰ another indication of a direct effect on chromatin. Finally, recent evidence suggests that acetyl-L-carnitine, the physiological form of carnitine, acts as a histone hyper-acetylating agent at the *FMR1* locus in fragile X cells.²¹

Because transcription of the *FMR1* gene in fully mutated patients was obtained by treatment with butyrate,¹² we decided to evaluate the effect of acetyl-L-carnitine and L-carnitine in lymphoblastoid cultures from patients with FXS. In the present study we investigated the effect on the CpG island methylation present in the *FMR1* promoter after treatment with L-carnitine and acetyl-L-carnitine. We assessed the methylation status at 52 CpG sites of the *FMR1* promoter using bisulphite treated, polymerase chain reaction (PCR) amplified genomic DNA obtained from lymphoblastoid cell cultures from healthy subjects and patients with FXS with CGG repeat expansions of different lengths. Furthermore, we controlled the effect of the two compounds on the methylation status of the putative promoter and exon 1 region of the gene called small nuclear ribonucleoprotein polypeptide N (*SNRPN*). This gene is reported to be involved in Prader-Willi syndrome and Angelman syndrome.^{22–25} It was chosen because in normal subjects only one allele is methylated, so each of the 23 CpGs present in this region will result in 50% methylated. In this way we could evaluate the effects of L-carnitine and acetyl-L-carnitine on another DNA region and our experimental approach at the same time.

MATERIALS AND METHODS

Three Epstein-Barr virus transformed lymphoblastoid cell lines were established from peripheral blood samples of healthy subjects and three from patients with FXS (M, L, and F) harbouring different degrees of triplet expansion. Cell line M had mosaicism in the methylation pattern and a full mutation between 1.3 and 3.7 kb. In cell line L, the expansion was less than 1 kb, and in cell line F it was more than 2.5 kb. From each cell line three subcultures were derived: one was left untreated, another was treated with 1 mmol/l L-carnitine, and the third one was treated with 1 mmol/l acetyl-L-carnitine. The two compounds were constantly present in the medium. Fresh L-carnitine and acetyl-L-carnitine were added every three to four days at each medium change. Cells were grown in RPMI 1640 medium supplemented with 20% fetal calf serum and penicillin/streptomycin at 37°C, in a 5% CO₂ atmosphere. They were cultured for 90–100 replicative cycles (for about three months) and their concentration was maintained between 3×10⁵/ml and 1.5×10⁶/ml.

DNA was extracted from cell cultures by the salting out method and modified by the sodium bisulphite technique as

reported previously,²⁴ 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 desulphonation, 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: GTTATTGAGTGATTGTTGTAGAAATGGG (positions 2297–2325 of GenBank sequence X61378) and 3R: CCTCTCTCTCAAATAACCTAAAAAC (positions 2492–2466) under the following conditions: 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 20 seconds for 35 cycles. For the amplification of the 239 bp fragment the following primers were used: 7F GGTATTGAGAGAGAGGG (positions 2473–2492 of GenBank sequence X61378) and 6R: CAC(A/G)CCCCCTAACAAAC (positions 2696–2711, fig 1); the conditions were as follows: 94°C for 30 seconds, 47°C for 30 seconds, and 72°C for 20 seconds for 35 cycles. 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 an automatic sequencer using both forward and reverse primers using independent pools. Amplification by PCR and sequencing of the *SNRPN* promoter and exon 1 region was performed on the same sodium bisulphite converted DNA used for the *FMR1* promoter analysis. Amplification was carried out by a nested PCR protocol previously described.²³

FMR1 gene expression was analysed by reverse transcriptase-PCR (RT-PCR). Total RNA was extracted from the cells with Trizol (Gibco-BRL) following the manufacturer's instructions. A total of 5 µg RNA was preincubated at 65°C for 10 minutes. Synthesis of cDNA was carried out at 40°C for 40

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 RNAGuard (Amersham Pharmacia Biotech), and 100 U M-MLV-RT (Gibco-BRL). The PCR conditions for both *FMRI* 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 *FMRI* cDNA amplification were CACTTTCGGAGTCTGCGCAC and TAGCTCCAATCTGTGCGCAACTGC. Primers for *HPRT* were CGTGGGGTCCTTTTCACCAGCAAG and AATTATGGACAGG ACTGAACGTC.¹⁰ 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 *FMRI* gene by the bisulphite sequencing technique and on the transcription of the fully mutated *FMRI* 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.²⁵

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 *FMRI* 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 M cell line the CpG sites that are partially unmethylated increased from six and eight in the starting and untreated cultures, to 10 and 14 in cells treated with L-carnitine and acetyl-L-carnitine, respectively. 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 13 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 hypermethylated 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

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 *FMRI* 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.

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 *FMRI* gene is a very unlikely event.

Table 1 Overall methylation status (%) after treatment with L-carnitine or acetyl-L-carnitine

	Starting cultures	After long term culturing		
		Untreated	L-carnitine	ALC
F	99.7	97.2*	99.2	98.6
M	99.0	98.7	96.2	95.6
L	98.1	98.5	95.4	95.7

*Lowest methylation is the result of high demethylation percentage of sites 28 and 29.

To control for the effect of our treatments on a non-pathologically methylated sequence, the human *SNRPN* sequence in the putative promoter, exon 1, and 5' region of intron 1 was also analysed. The methylation pattern of all individual 23 CpG sites in the DNA fragment analysed was evaluated. As expected, a 50% methylation was found in both control and fragile X derived starting cultures. The quite striking similarity between related electropherograms enables us to infer that not even a minimal change occurred after long term culturing either with or without the two carnitines (data not shown).

The RT-PCR designed for detecting transcriptional reactivation of the *FMR1* gene in the three tested fragile X cell lines was negative before treatment, as expected, but remained negative even after long term culturing with both acetyl-L-carnitine and L-carnitine; meanwhile *FMR1* gene expression was detected in all cultures from normal subjects.

DISCUSSION

The clinical features of fragile X syndrome are the result of the hampered transcriptional activity of the *FMR1* promoter secondary to its hypermethylated status. Attempts to reactivate gene expression in cells with a CGG expansion in the full mutation range have been successful. This was obtained by reducing DNA methylation with 5-azadC,^{8,9} and, although to a lesser extent, by inducing histone hyperacetylation with drugs such as trichostatin A, 4-phenylbutyrate, and sodium butyrate.¹² A general view recently emerged that histones regulate the access of proteins to the DNA and post-transcriptional histone modifications such as lysine acetylation or methylation mediate the epigenetic effects of DNA methylation patterns.²⁶ It has become apparent that histone deacetylation follows DNA hypermethylation in a cascade process leading to chromatin inactivation¹¹ and it was shown by chromatin immunoprecipitation that the inactive fully mutated *FMR1* gene is associated with relatively hypoacetylated histones.¹⁰

It has been shown that USF1, USF2, and α -Pal/Nrf-1 are the major transcription factors that bind the *FMR1* promoter at the E box and α -Pal/Nrf-1 DNA binding sites, respectively,²⁵ as indicated in fig 1. The binding of α -Pal/Nrf-1 is abolished by methylation, whereas the binding of USF1 and USF2 is only reduced.²⁵ Thus, DNA methylation can have a direct, as well as an indirect, effect on the transcription of the *FMR1* gene and seems to be dominant over histone acetylation, that is, *FMR1* reactivation cannot take place efficiently only with histone hyperacetylating drugs, if DNA is not previously demethylated, as suggested by Chiurazzi *et al.*¹² However, data obtained from studies on *Neurospora crassa*²⁷ suggested that, at least in some instances, histone hyperacetylation may eventually cause DNA demethylation,¹¹ just as DNA demethylation would induce histone reacylation (and gene reactivation). We hypothesised that a long term treatment with histone hyperacetylating drugs may therefore be effective in reactivating *FMR1* expression in fragile X cell cultures. 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.¹² Previous results from our groups have shown that acetyl-L-carnitine increases levels of H4 acetylation,¹⁷ also in the *FMR1* gene itself.²¹ 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 after the three month 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,¹³ 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 cytosine 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, favour 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.

ACKNOWLEDGMENTS

We thank FARMM onlus for financial support.

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