Studies of the epigenetic disease mechanism in FSHD

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No effect of folic acid and methionine supplementation on D4Z4 methylation in patients with facioscapulohumeral muscular dystrophy

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Abstract

Facioscapulohumeral muscular dystrophy (FSHD) is associated with a contraction of the D4Z4 allele on chromosome 4qter. There is also marked DNA hypomethylation of the D4Z4 allele. The DNA hypomethylation may have a central role in the pathogenesis of FSHD. Supplemental folic acid can boost DNA methylation. We evaluated the effect of oral folic acid and methionine supplementation on the methylation level of 4qter D4Z4 alleles in peripheral blood lymphocytes of nine patients affected with FSHD and six healthy controls. Methylation levels did not change, while recommended serum folate concentrations were reached.

Introduction

Autosomal dominant facioscapulohumeral muscular dystrophy (FSHD) is associated with contraction of the polymorphic D4Z4 repeat array on chromosome 4qter. In healthy individuals, D4Z4 consists of 11–100 units on both chromosomes, whereas individuals with FSHD carry one 4q array of 1–10 units (4q-linked FSHD). This contraction is associated with marked hypomethylation of the shortened D4Z4 allele. About 5% of individuals with phenotypic FSHD do not have a contraction of D4Z4 on chromosome 4q. However, both their D4Z4 alleles show hypomethylation. These findings suggest a central role of D4Z4 hypomethylation in the pathogenesis of FSHD [1].

Chromatin of eukaryotic organisms can roughly be divided into relatively hypomethylated euchromatin and highly methylated heterochromatin. In general, euchromatin has an open chromatin structure and is associated with active DNA transcription. In contrast, heterochromatin tends to be tightly packed and is associated with transcriptional inactivity or repression. DNA methylation comprises the addition of a methyl group to the carbon 5 position of cytosine within the so-called CpG dinucleotides. Approximately 70–80% of all CpG dinucleotides are methylated, except for dense CpG clusters, termed CpG islands, which are often located in or near promoter or coding sequences. The chromatin structure of these hypomethylated CpG islands is open, which makes the DNA sequence accessible for transcription. The CpG islands not associated with promoting or coding sequences, i.e., transcriptional silent sequences, are often methylated. Therefore, the level of DNA methylation has an important function in these interrelated processes of chromatin structure modulation, transcriptional regulation and gene silencing. The three best-characterized genetic diseases caused by impaired DNA

methylation-dependent gene control pathways are ICF, Rett and fragile X syndromes [2]. Changes in levels and patterns of DNA methylation also have an important role in oncogenesis [3].

In healthy individuals, the chromatin structure of the D4Z4 region on chromosome 4q35 is highly methylated, and resembles that of heterochromatin or transcriptional inactive euchromatin [4-6]. A currently favored hypothesis is that the marked hypomethylation of the D4Z4 region in FSHD might cause a change in the chromatin structure, and consequently a transcriptional deregulation of one or more genes in the vicinity, or at a distance of the D4Z4 repeat array [1, 6].

As DNA methylation and demethylation are reversible processes, DNA methylation levels and patterns can potentially be influenced [2]. Folic acid and vitamin B12 are essential for the synthesis of methionine and S-adenosyl methionine (SAM), the common methyl donor required for the maintenance of DNA methylation. When the concentration of folic acid, vitamin B12 and methionine is low, SAM synthesis is reduced, leading to a reduced methylation of DNA [7]. Optimal micronutrient levels and dietary requirements for DNA functioning and maintenance are not yet known. Intervention studies in humans taking folic acid and/or vitamin B12 supplements show that DNA hypomethylation, chromosome breaks and uracil misincorporation are minimized when serum concentration of folate is higher than 34 nmol/l, and serum vitamin B12 concentration is higher than 300 pmol/l. These concentrations can only be achieved at intake levels of more than 400 µg folic acid and more than 2 µg vitamin B12 per day [3, 8].

We performed a pilot study to evaluate the effect of supplemental folic acid and methionine on the methylation level of D4Z4 alleles on chromosome 4qter in peripheral blood lymphocytes (PBLs) of patients with FSHD (both 4q-linked FSHD and phenotypic FSHD) and in healthy controls in order to decide whether a larger clinical trial might be warranted.

Material and methods

Subjects

We recruited a convenient sample of six patients clinically affected with 4q-linked FSHD and one young, asymptomatic patient, all with proven contraction and

hypomethylation of D4Z4 at 4q35 (in PBLs). We also included two clinically affected sibs from a known phenotypic FSHD family without a contraction of D4Z4, but with proven hypomethylation of the repeat array. A convenient sample of five non-affected, first-degree relatives and one spouse served as controls. Patients and controls were between 18 and 60 years. Exclusion criteria were: serum vitamin B12 < 160 pmol/l; use of folic acid, vitamin B12 or multivitamin supplements during the last 6 months; cardiovascular disease or more than two cardiovascular risk factors (e.g., hypertension, diabetes mellitus, smoking); pregnancy or breastfeeding. The local ethics committee approved the study. Informed consent was obtained from all subjects involved in the study.

Design

After the baseline visit subjects started using folic acid (5 mg, orally, once daily) and methionine (1 g, orally, three times a day) until after the final visit at 12 weeks. The baseline visit consisted of a general medical history and physical examination to verify eligibility, obtain anthropometrical measures, including blood pressure and pulse rate and a clinical severity score. Venous blood samples were taken to measure methylation levels of D4Z4 alleles and total DNA in PBLs, and concentrations of serum folate, serum vitamin B12 and plasma homocysteine. The final visit consisted of an event history (including side effects), anthropometrics and drawing of blood samples.

Outcome measures; clinical severity score

As an indication of disease severity, all subjects were scored according to the 10-grade Clinical Severity Scale (CSS) formulated by Ricci *et al* [9]. The original score ranged from 1 indicating only facial weakness to 10 for wheelchair bound patients. We added score 0 for clinically unaffected patients and healthy controls.

Outcome measures; DNA methylation at D4Z4

Our primary outcome measure was the DNA methylation level of the D4Z4 alleles on 4qter in PBLs. The methylation level of two CpG methylation-sensitive restriction sites (*Bsa*AI and *Fse*I) in the first (proximal) unit of the D4Z4 repeat array on chromosome 4q35 was determined, as described previously [1]. As shown earlier, these results are representative of the entire array [1].

Outcome measures; total DNA methylation

A nonisotopic cytosine extension assay was used to estimate CpG island methylation of the whole genome [10].

Outcome measures; serum folate level

Serum folate concentration was measured using a competitive immunoassay (DPC Immulite 2000 system).

Outcome measures; serum vitamin B12 level

Serum vitamin B12 concentration was measured using a solid-phase, competitive chemiluminescent enzyme immunoassay involving an automated alkaline denaturation procedure (DPC Immulite 2000 system).

Outcome measures; plasma homocysteine level

To check for unintended rises of plasma homocysteine due to methionine loading total, non-fasting homocysteine concentration was measured in EDTA plasma by automated high-performance liquid chromatography with reverse-phase separation and fluorescent detection (Gilson 232-401 sample processor, Spectra-Physics 8800 solvent-delivery system, Spectra-Physics LC 304 fluorometer). We used the method described by Fiskerstrand *et al.* with some modifications [11].

Outcome measures; statistical analysis

Independent-sample *t* tests or Mann-Whitney U tests were used to test for equality between the patient and control group at baseline (P=0.05). To test for statistical differences in outcome measures before and after treatment, paired-sample t tests or Wilcoxon signed-rank tests were performed (P= 0.05).

Results

Subjects

Baseline characteristics are presented in Table 1. CSS scores of the 4q-linked FSHD patients were 0, 4, 5, 8, 10, 10 and 10. The CSS scores of the two phenotypic FSHD sibs were 6 and 7. All controls scored zero. Residual D4Z4 fragment sizes of the 4q-linked FSHD patients were 2, 3, 3, 5, 6, 6 and 7 units. All subjects completed the study.

	FSHD (n=9)	Control (n=6)
Female/male (n)	4/5	2/4
Age (years)	43 ± 16	50 ± 18
Length (cm)	172 ± 16	172 ± 10
Weight (kg)	76 ± 20	89 ± 12

Table 1

Baseline characteristics of patients and controls involved in the study.

Values are mean ± 1 SD.

Baseline values

Baseline values of outcome measures are presented in Table 2. As expected, the methylation level of the two CpG sites was significantly lower in patients as compared to controls (*BsaA*I, *P*=0.003; *Fse*I, *P*=0.001). Serum folate levels of all subjects were below the recommended 34 nmol/l needed to minimize DNA hypomethylation [3, 8]. Four patients and five controls had vitamin B12 values below the recommended 300 pmol/l [3, 8]. Mean vitamin B12 concentration was significantly higher for patients than for controls (*P*=0.036). Homocysteine levels were within normal range for all subjects.

Outcomes

Study medication was well tolerated; no side effects were reported. There were no significant changes in the methylation levels of *BsaA*I and FseI in both patients and controls between the baseline and final visit (Table 2). Total DNA methylation level increased in five patients and four controls, did not change in two patients and one control, and decreased in one patient. Total DNA methylation assay failed in

	FSHD (n=9)	FSHD (n=9)	Control (n=6)	Control (n=6)
	Week 0	**Week 12**	**Week 0**	**Week 12**
*BsaA*I methylation level (%)	35.4 ± 6.2[a]	35.1 ± 4.3	48.9 ± 8.5[a]	50.4 ± 9.4
*Fse*I methylation level (%)	25.8 ± 8.2[a]	26.7 ± 8.1	55.0 ± 18.3[a]	55.3 ± 16.4
Serum folate level (nmol/l)	18.0 ± 7.3[b]	210.9 ±77.8[b]	16.4 ± 7.1[b]	127.7 ± 83.6[b]
Serum vitamin B12 level (pmol/l)	345 ± 109[a]	337 ± 129	248 ± 92[a]	279 ± 124
Plasma homocysteine level (µmol/l)	10.8 ± 2.2	12.1 ± 3.4	11.0 ± 2.0	11.0 ± 1.9

Table 2

D4Z4 DNA methylation, serum folate, serum vitamin B12 and plasma homocysteine levels before and after supplementation.

Values are mean ± 1 SD. a Statistical differences (P<0.05) between the patient and control group at baseline. b Statistical differences (P<0.05) before and after treatment.

two subjects. Serum folate levels rose significantly, and all individual levels reached above the recommended 34 nmol/l. For both groups, mean values for vitamin B12 and homocysteine, weight, blood pressure and pulse rate did not show significant changes.

Discussion

Despite the fact that the recommended serum folate level to minimize DNA hypomethylation was reached in all subjects and total DNA methylation levels increased in the majority of subjects, our pilot study did not show effect of supplemental folic acid and methionine on the methylation level of 4qter D4Z4 alleles in PBLs of patients with FSHD and controls. Several in vivo folate depletion and repletion studies with similar dosing regimens had shown positive effects on genomic DNA methylation and the expression of methylation-regulated genes in PBLs and several body tissues [3, 7, 12]. Other studies indicate that the effect of folate status on DNA methylation is more complex, highly dose-dependent, and, of importance, even tissue-specific [13]. The absence of any noticeable effect in both patients and controls might be caused by a relatively low folic acid dose to change D4Z4 methylation. It can also implicate a, maybe even heritable, folate-resistance status of the methylation level of the two studied D4Z4 CpG sites. At

present, there is insufficient ground for a larger clinical study on folic acid supplements in FSHD. We are considering a second pilot study on the effects of supplemental folic acid, combined with supplemental vitamin B12, on DNA hypomethylation and allelic expression in PBLs and in skeletal muscle cells.

References

[1] Van Overveld PG, Lemmers RJ, Sandkuijl LA, Enthoven L, Winokur ST, Bakels F, Padberg GW, van Ommen GJ, Frants RR, van der Maarel SM. Hypomethylation of D4Z4 in 4q-linked and non-4q-linked facioscapulohumeral muscular dystrophy. Nat. Genet. 35 (2003) 315-317.

[2] Robertson KD, Wolffe AP. DNA methylation in health and disease. Nat. Rev. Genet. 1 (2000) 11-19.

[3] Fenech M. The Genome Health Clinic and Genome Health Nutrigenomics concepts: diagnosis and nutritional treatment of genome and epigenome damage on an individual basis. Mutagenesis 20 (2005) 255-269.

[4] Tsien F, Sun B, Hopkins NE, Vedanarayanan V, Figlewicz D, Winokur S, Ehrlich M. Methylation of the FSHD syndrome-linked subtelomeric repeat in normal and FSHD cell cultures and tissues. Mol. Genet. Metab 74 (2001) 322-331.

[5] Jiang G, Yang F, Van Overveld PG, Vedanarayanan V, van der Maarel S, Ehrlich M. Testing the position-effect variegation hypothesis for facioscapulohumeral muscular dystrophy by analysis of histone modification and gene expression in subtelomeric 4q. Hum. Mol. Genet. 12 (2003) 2909-2921.

[6] van der Maarel SM, Frants RR, Padberg GW. Facioscapulohumeral muscular dystrophy. Biochim. Biophys. Acta 1772 (2007) 186-194.

[7] Fenech M. The role of folic acid and Vitamin B12 in genomic stability of human cells. Mutat. Res. 475 (2001) 57-67.

[8] Fenech M. Recommended dietary allowances (RDAs) for genomic stability. Mutat. Res. 480-481 (2001) 51-54.

[9] Ricci E, Galluzzi G, Deidda G, Cacurri S, Colantoni L, Merico B, Piazzo N, Servidei S, Vigneti E, Pasceri V, Silvestri G, Mirabella M, Mangiola F, Tonali P, Felicetti L. Progress in the molecular diagnosis of facioscapulohumeral muscular dystrophy and correlation between the number of KpnI repeats at the 4q35 locus and clinical phenotype. Ann. Neurol. 45 (1999) 751-757.

[10] Fujiwara H, Ito M. Nonisotopic cytosine extension assay: a highly sensitive method to evaluate CpG island methylation in the whole genome. Anal. Biochem. 307 (2002) 386-389.

[11] te Poele-Pothoff MT, van den Berg M, Franken DG, Boers GH, Jakobs C, de Kroon I, Eskes TK, Trijbels JM, Blom HJ. Three different methods for the determination of total homocysteine in plasma. Ann. Clin. Biochem. 32 (Pt 2) (1995) 218-220.

[12] Ingrosso D, Cimmino A, Perna AF, Masella L, De Santo NG, De Bonis ML, Vacca M, D'Esposito M, D'Urso M, Galletti P, Zappia V. Folate treatment and unbalanced methylation and changes of allelic expression induced by hyperhomocysteinaemia in patients with uraemia. Lancet 361 (2003) 1693-1699.

[13] Sohn KJ, Stempak JM, Reid S, Shirwadkar S, Mason JB, Kim YI. The effect of dietary folate on genomic and p53-specific DNA methylation in rat colon. Carcinogenesis 24 (2003) 81-90.