Studies of the epigenetic disease mechanism in FSHD

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Hypomethylation is restricted to the D4Z4 repeat array in phenotypic FSHD

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Abstract

Background: Patients with facioscapulohumeral muscular dystrophy (FSHD) show a contraction of the D4Z4 repeat array in the subtelomere of chromosome 4q. This D4Z4 contraction is associated with significant allele-specific hypomethylation of the repeat. Hypomethylation of D4Z4 is also observed in patients with phenotypic FSHD without contraction of D4Z4 and in patients with the immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome, an unrelated disease that does not present with muscular dystrophy and is in part caused by *DNMT3B* mutations.

Methods: In order to identify the gene defect and to find the pathogenetic epigenetic pathway in phenotypic FSHD, we have aimed to identify the differences and commonalities in phenotypic FSHD and ICF by 1) investigation of DNA methylation of non-D4Z4 repeat arrays, 2) analysis of mitogen-stimulated lymphocytes to detect pericentromeric abnormalities involving chromosomes 1, 9, and 16, 3) determination of IgA, IgG, and IgM levels, and 4) mutational analysis of candidate genes to identify a second disease locus involved in the pathogenesis of phenotypic FSHD.

Results: Our results do not show epigenetic or phenotypic commonalities between phenotypic FSHD and ICF other than the earlier observed D4Z4 hypomethylation. We could not identify any mutations in the candidate genes tested for.

Conclusion: Our data suggest that in phenotypic FSHD hypomethylation is restricted to D4Z4 and that phenotypic FSHD and ICF do not share a defect in the same molecular pathway.

Introduction

Autosomal dominant facioscapulohumeral muscular dystrophy (FSHD) presents with progressive muscular weakness and wasting of the face, shoulders, and upper arms [1]. FSHD is caused by contraction of the polymorphic macrosatellite repeat D4Z4 on the subtelomere of chromosome 4q [2]. In patients with FSHD, this contraction is always associated with a specific variant of chromosome 4, termed 4qA [3, 4]. Few patients with FSHD, referred to as patients with phenotypic FSHD, show normal-sized D4Z4 repeats on both chromosomes 4. Interestingly, in both 4q-linked FSHD and phenotypic FSHD patients, the D4Z4 repeat shows reduced levels of DNA methylation. The D4Z4 hypomethylation is more prominent and present on both chromosomes 4 in phenotypic FSHD, whereas in 4q-linked patients it is restricted to the disease chromosome [5].

Low methylation levels of D4Z4 are also found in patients with the immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome [6]. Patients with ICF syndrome have recurrent respiratory and gastrointestinal infections, as a result of a reduction in serum immunoglobulin levels. Further, because of decondensation of juxtacentromeric heterochromatin regions, characteristic rearrangements of chromosomes 1,9, and 16 are found in phytohemagglutinin-stimulated lymphocytes of these patients. Patients with ICF show a wide variety of facial anomalies but do not present any myopathic symptoms [7].

ICF syndrome is caused in 60% of patients by mutations in the DNA methyltransferase gene *DNMT3B* [8,9]. Mutations in the catalytic domain of *DNMT3B* reduce its methyltransferase activity, resulting in hypomethylation of the classic repeat arrays satellite 2 (Sat2) and satellite 3 (Sat3), the NBL2 repeat, and the D4Z4 repeat [8]. Approximately 40% of patients do not have mutations in *DNMT3B* (ICF2). Nevertheless, in patients with ICF2, these repeats are also hypomethylated in addition to hypomethylation of α-satellite DNA [10]. As patients with phenotypic FSHD and patients with ICF share an epigenetic hallmark, we hypothesized that a more genome-wide impairment of DNA methylation, as seen in ICF, may explain the pathogenesis of phenotypic FSHD. To determine whether phenotypic FSHD is caused by a defect in the same molecular pathway as ICF, we compared patients with phenotypic FSHD and patients with ICF at the phenotypic and the epigenetic level.

Material and methods

Subjects

Seven Dutch phenotypic FSHD families were included in this study (Figure 1). Families 1, 2 and 3 were reported earlier on basis of their D4Z4 hypomethylation [5]. The other families are reported here for the first time. Although D4Z4 methylation is not very low in Families 6 and 7, these families were included on basis of their FSHD-like phenotype. All patients were referred under the diagnosis of FSHD on a clinical basis and all patients and their relatives were examined by one of the authors (M.W. and G.P.) familiar with FSHD. For this study, DNA was not available for all clinical examined family members. The clinical diagnosis of FSHD is based on asymmetric weakness and atrophy of the facial and shoulder girdle muscles with early scapular winging and elevation of the scapula when trying to anteflex the arms. Focal involvement of the deltoid muscle, severe and often asymmetric

upper arm weakness, early involvement of abdominal muscles, and asymmetric weakness of the foot-extensors is present in more advanced disease [1].

Patients with phenotypic FSHD are patients with the clinical diagnosis of FSHD but without contraction of the D4Z4 repeat array. A muscle biopsy was performed in all families, except Family 4 (Table 1). The biopsies showed mild aspecific dystrophic or nonspecific myopathic findings as varying of muscle fiber diameter with no indications of specific myopathies such as acid maltase deficiency or inclusion body myositis. Most biopsies were performed at a time when extensive immunohistochemistry was not available. Genetic testing or immunobiochemical analysis in muscle tissue to exclude dominant or recessive limb girdle dystrophies was performed in two patients (Table 1).

Family members were assigned as nonaffected when they did not show any symptoms or signs of FSHD on examination at 20 years or older. A summary of the clinical findings, including clinical severity scores, and D4Z4 allele sizes and D4Z4 methylation levels of all individuals is presented in Table 1 [11, 12]. All phenotypic FSHD cases showed normal-sized D4Z4 alleles, as determined by pulsed field gel electrophoresis [13].

Genomic DNA was isolated from peripheral blood lymphocytes using a standard salt extraction protocol [14]. DNA from a FSHD1 patient with a D4Z4 contraction (C1) and a nonaffected relative (C2) were included as negative controls. Also, DNA was isolated from both ICF type 1 (I1) and ICF type 2 (I2) EBV-transformed lymphoblastoid cell lines (LCLs) as positive controls.

Southern blot analysis using methylation-sensitive restriction enzymes

Digestions were performed using the following restriction enzymes: *Eco*52I, *Bsp*119I, *Hha*I, and *Hpa*II (MBI Fermentas, Germany). For *Hpa*II digestion, its non-methylation-sensitive isoschizomere *Msp*I (MBI Fermentas, Germany) was used as a control for complete digestion. In short, 2 μg of genomic DNA was digested with 20 units of the appropriate restriction enzyme according to the manufacturer's specifications. DNA digests were separated on a 0.8% agarose gel and blotted overnight on a hybond-XL membrane (Amersham Bioscience, Piscataway, NJ). Probes were labeled by random priming with 32P-dCTP using the Megaprime DNA labeling system (Amersham Pharmacia Biotech, Piscataway, NJ). The probes targeted against the NBL2 repeat and the α-satellite repeats on chromosomes 3, 9, 11, 13/21, 16, and 18 were obtained through standard PCR, followed by ligation of the PCR products into the pCR2.1-TOPO vector

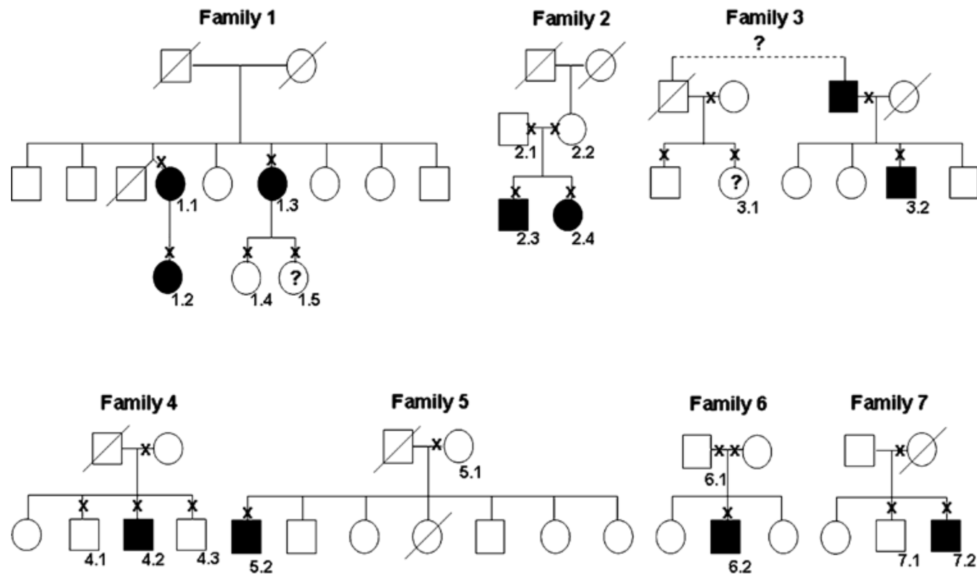

Figure 1

Seven Dutch families with phenotypic facioscapulohumeral muscular dystrophy (FSHD) included in the study.

Closed circles/squares represent affected individuals; open circles/squares represent unaffected family members. All patients were referred under the diagnosis of FSHD on a clinical basis and all patients and their relatives were examined by one of the authors (M.W., G.P.) familiar with FSHD, as represented in the figure by x. Family 1 consists of three patients (1.1, 1.2, and 1.3) and two nonaffected family members (1.4 and 1.5). Family member 1.5 showed an asymmetric mouth and her eyelashes on the right side did not disappear while closing her eyes tightly, which was insufficient clinical certainty of FSHD. Family 2 consists of an affected sibling pair (2.3 and 2.4) and two healthy parents (2.1 and 2.2); the mother in this family also shows D4Z4 hypomethylation but no muscular symptoms. Patients 3.1 and 3.2 have the same family name and grew up in the same region of the Netherlands. The parents of both patients lost contact with their family, but they are possibly related to each other. Patient 3.1 showed disputable signs of facial weakness and weakness of the right shoulder girdle muscles weakness without wasting Family members of Patient 3.2, such as his affected father, were not available for our examination. In Family 4, one individual is affected (4.2), while his two brothers are unaffected (4.1 and 4.3). Both Families 5 and 6 consist of an affected child (5.2 and 6.2) and an unaffected parent (5.1 and 6.1). Patient 5.2 previously presented with a scapuloperoneal syndrome and at age of examination his pelvic girdle muscles were affected as well. Family 7 consists of an affected (7.2) and an unaffected (7.1) brother.

Indi-vidual	Status	4-1 (kb)	4-2 (kb)	10-1 (kb)	10-2 (kb)	*BsaA*I (%)	*Fse*I (%)	CSS	Age (at examination) (years)	F	S	E	P	Muscle biopsy
1.1	A	50A	90B	25A	75A	21	5	10	56	+	+	+	+	+
1.2	A	50A	135B	75A	125A	20	4	9	34	+	+	+	+	–
1.3	A	48B	90B	25A	90A	49	41	6	52	+	+	+	–	+
1.4	N	90B	128B	25A	150A	52	53	0	31	–	–	–	–	–
1.5	?	90B	130B	25A	150A	58	63	0	30	?	–	–	–	–
2.1	N	96A	173B	35A	85A	58	68	0	63	–	–	–	–	–
2.2	N	75B	170A	55A	65A	35	16	0	59	–	–	–	–	–
2.3*	A	96A	170A	65A	85A	26	12	7	38	+	+	+	+	+
2.4	A	96A	170A	65A	85A	44	19	7	35	+	+	+	+	+
3.1	?	38B	70B	15A	45B+	30	31	2	42	±	±	–	–	–
3.2	A	48A	120B	15A	70A	24	13	4	38	+	+	+	–	+
4.1	N	70A	160B	47A	75A	62	56	0	46	–	–	–	–	–
4.2	A	50A	70A	38A	47A	36	15	6	54	+	+	+	+	–
4.3	N	85B	160B	47A	75A	60	55	0	56	–	–	–	–	–
5.1	N	65A	75B	60B+	90A	70	47	0	79	–	–	–	–	–
5.2	A	65A	110A	90A	100A	33	17	7	53	+	+	+	+	+
6.1	N	170B	300A	23A	55A	33	25	0	74	–	–	–	–	–
6.2*	A	65A	170B	50A	200A	31	34	8	46	+	+	+	+	+
7.1	N	48B	90B	65A	185A	36	46	0	62	–	–	–	–	–
7.2	A	48B	94B	25A	33A	44	42	7	55	+	+	+	+	+

Table 1

Summary of clinical and genetic findings in seven Dutch families with phenotypic facioscapulohumeral muscular dystrophy.

Clinical status, D4Z4 allele sizes, D4Z4 methylation levels (BsaAI and FseI), clinical severity score (CSS) on a scale from 0 to 10, age at examination, involvement of facial muscles (F), shoulder girdle muscles (S), extensors of the foot (E), and pelvic muscles (P) (+ = affected muscle; – = nonaffected muscle), and whether a muscle biopsy was taken (+ = muscle biopsy was taken; – = no muscle biopsy was taken). Numbers in the table correspond with numbers in Figure 1.

* *In Individuals 2.3 and 6.2, genetic testing or immunobiochemical analysis in muscle tissue was performed. DNA of Patient 2.3 showed no mutations for proximal myotonic myopathy (PROMM). Muscle tissue of Patient 6.2 was screened negative for Becker muscular dystrophy and for limb-girdle muscular dystrophy type 2A and type 2B. + Trisomic cases; individuals carry three 4q-type repeat arrays, of which one is located on chromosome 10q.*

and transformation of the vector into Top 10 chemically competent *E coli* (Invitrogen Carlsbad, CA). The primers for the NBL2 probe were described previously, whereas the primers for the various α-satellite repeats were designed using Primer3 software (Whitehead Institute for Biomedical Research) based upon the probes used in earlier studies [6, 15]. Primer sequences are available upon request. For sequence verification, all probes were sequenced by the Leiden Genome Technology Center (LUMC, Leiden, The Netherlands). The oligonucleotide probes for classic satellites 2 and 3 were as described previously [10]. Hybridizations were carried out overnight at 65 °C with 100 μg/ml fish sperm DNA (Roche, Basel, Switzerland) in formamide hybridization mixture, except for the hybridization with the NBL2 probe, which was performed in NaPi/PEG/SDS hybridization mixture [16]. After washing in SSC/SDS buffers with a diminishing gradient, digestion patterns were visualized using a phosphor-imaging screen (Storm, Amersham Bioscience). For the Sat 2 and Sat 3 oligonucleotide probes, hybridization and washing was carried out at 45 °C in SSPE/SDS buffer.

Pericentromeric abnormalities involving chromosomes 1, 9, and 16

Cytogenetic analysis was performed on lymphocytes from peripheral blood cultures after stimulation with phytohemagglutinin for 96 hours. A minimum of 50 GTG-banded metaphases were examined for pericentromeric abnormalities [17].

Hypogammaglobulinemia

White blood cell counts and morphologic differentiations were performed on a hematology analyzer (Advia, Bayer). The immunoglobulins (IgG, IgA, and IgM) were quantified by an Immage nephelometer from Beckman Coulter. All reagents were obtained from Beckman Coulter. Immunophenotyping studies were performed on heparinized blood specimens. Immune fluorescent staining was performed on 100 μl whole blood with fluorochrome-labeled antibodies against CD3, CD4, CD8, CD19, CD56, and CD45. All antibodies were purchased from Beckman Coulter (Miami, FL), except CD56 (Becton, Dickinson and Company, San Jose, CA). Next, all erythrocytes were lysed for 10 minutes in the dark by means of icecold 155 mM NH4Cl (pH 7.4) according to the lyse/no wash method. Staining of cells was determined in four-color analysis using a Beckman Coulter Epics XL flow cytometer (Beckman Coulter, Hialeah, FL). All measurements were performed on a minimum of 50,000 cells. Data (collected in list mode) were analyzed using the EXPO32 ADC software (Beckman Coulter).

UniGeneID	Gene name	Function	Chromosome
Hs.202672	DNMT1	Maintenance DNA methyltransferase	19p13.2
Hs.515840	DNMT3A	*de novo* DNA methyltransferase	2p23
Hs.643024	DNMT3B	*de novo* DNA methyltransferase	20q11.2
Hs.592165	DNMT3L	Stimulation of DNMT3B	21q22.3
Hs.459049	MTHFS	Component of folate cycle	15q24.3
Hs.594444	LMNA	Component of nuclear lamina	1q21.2-q21.3
Hs.368410	CBX2	Component of PRC1 complex	17q25.3
Hs.632724	CBX5	Component of heterochromatin	12q13.13
Hs.522639	SUV39H1	Histone methyltransferase	Xp11.23

Table 2

Candidate genes for phenotypic facioscapulohumeral muscular dystrophy.

The coding regions of several candidate genes playing a role in chromatin structure were sequenced in three patients with phenotypic facioscapulohumeral muscular dystrophy.

Mutational analyses of candidate genes

The coding regions of several candidate genes for phenotypic FSHD were sequenced (Table 2). Each exon was amplified by PCR using primers in flanking introns. Primer sequences are available upon request. PCR was performed for 35 cycles using SilverStar DNA polymerase (Eurogentec, Seraing, Belgium) in 1.5 mM MgCl2 PCR buffer (pH 9.0). After an initial denaturation at 94 °C for 3 minutes, PCR cycles were as follows: 40 seconds at 94 °C, 40 seconds at 55 °C, and 1 minute at 72 °C, to be concluded with a final extension at 72 °C for 5 minutes. Sequencing reactions were performed by the Leiden Genome Technology Center (LUMC, Leiden, The Netherlands).

Statistical analysis

Independent-sample *t* tests were performed to compare serum IgG, IgA, and IgM levels between the patients with phenotypic FSHD and their nonaffected family members ($P<0.05$ was considered as significant).

Results

Hypomethylation is restricted to the D4Z4 repeat array in phenotypic FSHD.

To determine whether an overall impairment of DNA methylation in repeat arrays is also present in phenotypic FSHD, the non-D4Z4 repeats known to be hypomethylated in ICF were investigated by Southern blot analysis using methylation-sensitive restriction enzymes. The methylation status of the NBL2 repeat, the classic satellite repeats Sat2 and Sat3, and the α-satellite DNA repeat on chromosomes 3, 9, 11, 13/21, 16, and 18 was studied in three Dutch phenotypic FSHD families, both in patients and their healthy relatives (Figure 1; Families 1, 2, and 3). As a negative control, the methylation status from a 4q-linked FSHD patient and a nonaffected relative were studied. As a positive control, DNA of two patients with ICF (both ICF1 and ICF2) was included in the study. Significant hypomethylation of the NBL2, Sat2, and Sat3 repeat arrays was only detected in the two patients with ICF, while hypomethylation of the α-satellite DNA repeat array was restricted to the patient with ICF2, as reported previously [10]. Non-D4Z4 repeats were not hypomethylated in patients with phenotypic FSHD. Their methylation levels were comparable to their unaffected family members and the 4q-linked FSHD patient and a nonaffected relative. In Figure 2, results of the NBL2 Southern blot analysis for Families 1 and 2 and the α-satellite on chromosome 11 Southern blot analysis for Families 2 and 3 are shown. All results, including the results for the other repeat arrays, are summarized in Table 3.

No heterochromatic abnormalities involving chromosomes 1, 9, and 16 in phenotypic FSHD

To search for pericentromeric abnormalities in patients with phenotypic FSHD, 50 phytohemagglutinin-stimulated lymphocyte metaphases were studied for the presence of characteristic rearrangements involving chromosomes 1, 9, and 16. All members of the seven phenotypic FSHD families, including the nonaffected relatives, were studied. Abnormalities involving the heterochromatic regions of chromosomes 1, 9, and 16, such as whole-arm deletions, pericentromeric breaks, multibranched chromosomes, isochromosomes, and translocations that can be found in patients with ICF, were not detected in either patients with phenotypic FSHD or their healthy family members [7].

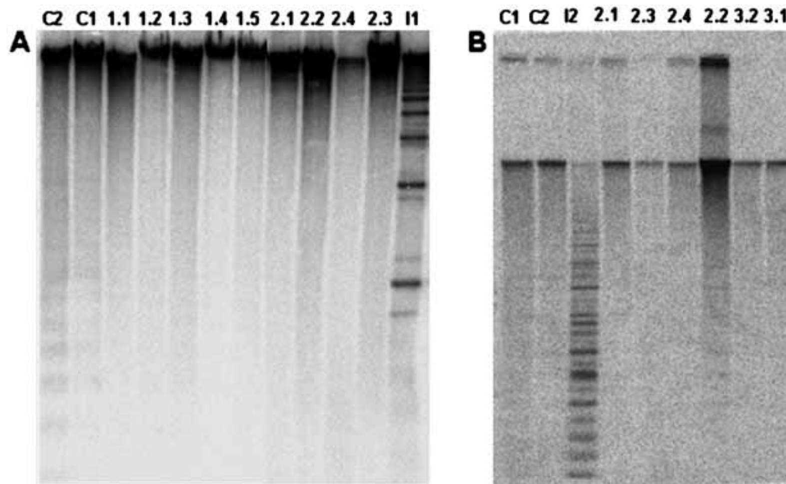

Figure 2

DNA methylation analysis in families with phenotypic facioscapulohumeral muscular dystrophy (FSHD).

A. *NBL2 Southern blot analysis in phenotypic FSHD Families 1 and 2. Numbers in figure correspond with numbers in figure 1. NBL2 repeat array is only significantly hypomethylated in I1, a patient with ICF1.*

B. *α-satellite on chromosome 11 Southern blot analysis in phenotypic FSHD Families 2 and 3. Numbers in figure correspond with numbers in figure 1α-satellite 11 repeat array is only significantly hypomethylated in I2, a patient with ICF2.*

No hypogammaglobulinemia in patients with phenotypic FSHD

Patients with ICF have recurrent infections as a result of hypogammaglobulinemia, a reduction in serum immunoglobulin levels (IgG, IgM, and IgA levels), with B cells. To investigate the occurrence of hypogammaglobulinemia in patients with phenotypic FSHD, several biomarkers, including serum immunoglobulin levels, were measured in blood of all patients with phenotypic FSHD included in this study and in their nonaffected relatives. The serum immunoglobulin levels were within the normal range in all phenotypic FSHD cases; no significant differences within these immunoglobulin levels between patients and healthy control individuals were found (Figure 3). Also, the other tested biomarkers were within normal range in the patients with phenotypic FSHD and no significant differences were observed between patients and controls (data not shown).

Individual	NBL2	Sat2	Sat3	α-sat-3	α-sat-9	α-sat-11	α-sat-13/21	α-sat-18
C1	+	+	+	+	+	+	+	+
C2	+	+	+	+	+	+	+	+
I1	–	–	–	+	+	+	+	+
I2	–	–	–	–	–	–	–	–
1.1	+	+	+	+	+	+	+	+
1.2	+	+	+	+	+	+	+	+
1.3	+	+	+	+	+	+	+	+
1.4	+	+	+	+	+	+	+	+
1.5	+	+	+	+	+	+	+	+
2.1	+	+	+	+	+	+	+	+
2.2	+	+	+	+	+	+	+	+
2.3	+	+	+	+	+	+	+	+
2.4	+	+	+	+	+	+	+	+
3.1	+	+	+	+	+	+	+	+
3.2	+	+	+	+	+	+	+	+

Table 3

Methylation status of non-D4Z4 repeat arrays (NBL2, Sat2, Sat3, and α-satellite on chromosomes 3, 9, 11, 13/21 and 18) in three phenotypic FSHD families.

Numbers in table correspond with numbers in Figure 1. + = methylation of repeat; – = significant hypomethylation of repeat.

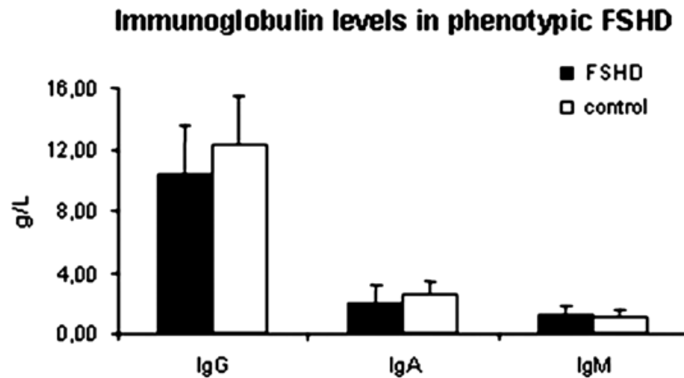

Figure 3

Serum immunoglobulin levels

Serum immunoglobulin levels in g/L in patients with phenotypic FSHD and their unaffected family members (shown as mean ± SD).

DNMT1, DNMT3A, DNMT3B, DNMT3L, MTHFS, LMNA, CBX2, CBX5, and *SUV39H1* are unlikely candidate genes for phenotypic FSHD

In search for a second disease locus that may play a role in the pathogenesis of phenotypic FSHD, mutational analyses of several candidate genes (Table 2) with a role in chromatin structure or methyl cycle were performed in three patients with phenotypic FSHD from three different families (Figure 1; 1.1, 2.4, and 3.2). *DNMT1*, *DNMT3A*, and *DNMT3B* are all genes that encode a DNA methyltransferase, either predominantly functioning in maintenance of DNA methylation during cell division or acting as a *de novo* DNA methyltransferase. As mentioned before, 60% of ICF cases can be explained by the presence of a mutation in *DNMT3B* [8]. *DNMT3L* encodes a catalytically inactive DNA methyltransferase. However, by association with *DNMT3B* it can stimulate DNA methylation [18]. Interestingly, two *DNMT3B* mutations have been reported that do not affect the methylation activity of the enzyme itself, but rather diminish the interaction with DNMT3L, also leading to the ICF syndrome [19]. MTHFS (5,10-methenyl-tetrahydrofolate synthetase) catalyzes the transformation of 5-formyl-tetrahydrofolate to other reduced folates and as such has an important function in the initial step of the folate cycle, thus having an influence on DNA methylation [20]. Moreover, this gene is located in a candidate interval that was identified some years ago in a possible non-4q-linked FSHD family [21]. The protein lamin A/C, encoded by the *LMNA* gene, is a component of the nuclear lamina, which provides nuclear stability and may also interact with chromatin. Mutations in *LMNA* may lead to several diseases affecting a wide variety of organs, such as muscle, fat, bone, nerve, and skin [22]. More importantly, this protein is required for the proper localization of the FSHD 4qter region to the nuclear envelope [23]. As interconnectivity between the Suv39h-HP1 histone methylation system and Dnmt3b in mammals was demonstrated before, the exonic regions of the genes *CBX2* (chromobox protein homolog 2), *CBX5* (chromobox protein homolog 5), and *SUV39H1* (suppressor of variegation 3-9 homolog 1) were screened for mutations [24]. CBX2 is a component of the polycomb repressive complex 1 (PRC1). This complex has a function in the maintenance of the transcriptional repressive state of genes during development. It acts via chromatin remodeling and histone modification by mediating the monoubiquitination of histone H2A [25]. CBX5, better known as HP1α, is an important component of heterochromatin, as it may recognize and bind to histones H3 and H1 [26]. SUV39H1 is a histone methyltransferase that methylates lysine 9 of histone H3. As a consequence, transcriptional repression may occur by the recruitment of the

HP1 proteins [27]. In addition to earlier reported single nucleotide polymorphisms (data not shown), no mutations were detected in the exonic regions of all abovementioned candidate genes, excluding them as likely candidate genes for phenotypic FSHD.

Discussion

Patients with phenotypic FSHD present with hypomethylation of the D4Z4 repeats similar to the D4Z4 hypomethylation observed in patients with the ICF syndrome. Patients with ICF do not present a myopathic phenotype. As patients with ICF die at a very young age, before the usual onset of FSHD in the second decade of life, this may explain the lack of symptoms of muscular dystrophy in these patients. However, the occurrence of severe progressive infantile FSHD argues against this explanation. As D4Z4 hypomethylation has been found on both 4qA and 4qB chromosome ends of patients with ICF, the lack of muscular dystrophy cannot be explained by the specific association of ICF with the 4qB-type allelic variant [28]. We therefore aimed to investigate the commonalities and differences between FSHD and ICF at the clinical, molecular, and epigenetic level.

Unlike in 4q-linked FSHD, the hypomethylation found in patients with phenotypic FSHD is present on both D4Z4 alleles and not associated with a contraction of the D4Z4 repeat array. In a previous study, we identified five phenotypic FSHD cases with hypomethylation of D4Z4 [5]. Consistent with 4q-linked FSHD, these patients carry at least one 4qA chromosome. In the current study, we identified additional family members and independent cases on the basis of their phenotype. With the exception of Individuals 1.3 and 7.2, all cases with a phenotype consistent with FSHD including recognizable facial weakness carry at least one 4qA allele and show hypomethylation of D4Z4, with the restriction enzyme *FseI* being most informative. Individuals 1.5 and 3.1 also have 4qB-type alleles only, but there was insufficient clinical certainty of FSHD in these cases. Individual 1.5 only showed an asymmetric mouth and her eyelashes on the right side did not disappear while closing her eyes tightly and Individual 3.1 showed disputable signs of facial weakness and weakness of the right shoulder girdle muscles without wasting. One individual (5.2) with a distinct scapuloperoneal phenotype and no facial involvement is also hypomethylated and carries a 4qA allele. These individuals at the both ends of the clinical and genetic spectrum emphasize our inability to clearly define the boundaries of the FSHD spectrum. Moreover, the lack of evidence for

hypomethylation, most notably in Individual 7.2 and to a lesser extent in Individual 6.2, which have a phenotype consistent with FSHD, substantiates these issues.

As patients with phenotypic FSHD and patients with ICF share a similar epigenetic hallmark in D4Z4 hypomethylation, we hypothesized that a more overall impairment of DNA methylation of specific repeats, as in ICF, may also be observed in phenotypic FSHD, providing clues to its pathogenesis. We therefore compared several epigenetic and clinical features between patients with FSHD and patients with ICF. Southern blot analyses using methylation-sensitive restriction enzymes showed no hypomethylation of the NBL2 repeat, Sat2 repeat, Sat3 repeat, and α-satellite repeat on chromosomes 3, 9, 11, 13/21, 16, and 18 in three phenotypic FSHD families. The lack of hypomethylation of non-D4Z4 repeat arrays in patients with phenotypic FSHD is in agreement with the absence of *DNMT3B* mutations in these patients. However, in some 40% of patients with ICF, the so-called patients with ICF2, *DNMT3B* mutations have not been detected either, leaving the possibility open that both phenotypic FSHD and ICF2 are caused by a defect in the same gene or same molecular pathway [9]. Considering the very different clinical symptoms observed in phenotypic FSHD and ICF, the additional hypomethylation of several repeat arrays may well explain these phenotypic differences. However, at this moment, little is known about how mutations in the *DNMT3B* gene and how hypomethylation of several repeat arrays can cause the phenotype observed in patients with ICF. ICF-specific deregulation of several genes having a role in immune function was reported. However, these changes cannot be explained by significant differences in promoter methylation [29].

Compared to the Sat2 repeat, Sat3 repeat, and α-satellite repeat, D4Z4 is of a different type, being a non-satellite repeat. Besides the D4Z4 repeat array, NBL2 is the only other non-satellite repeat that is reported to be hypomethylated in ICF, making perhaps NBL2 the most interesting target for investigation in phenotypic FSHD. However, no hypomethylation of the NBL2 repeat array was observed in patients with phenotypic FSHD. Since D4Z4 hypomethylation is more prominent in patients with ICF compared to patients with phenotypic FSHD, this could also be the case for the NBL2 repeat array. As Southern blot analysis using methylation-sensitive restriction enzymes is usually not very sensitive to determine hypomethylation, the NBL2 repeat array was also studied with sodium bisulphite PCR sequencing (data not shown). However, the results from this assay were comparable to the results obtained by Southern blot analysis; no hypomethylation of the NBL2 repeat was detected in patients with phenotypic FSHD.

In addition to the comparative analysis of patients with phenotypic FSHD and patients with ICF at the epigenetic level, two studies were performed to compare these patients at the genotypic and phenotypic level. As D4Z4 hypomethylation is more subtle in phenotypic FSHD compared to ICF, subclinical values with relation to pericentromeric abnormalities and serum immunoglobulin levels could have gone unnoticed in patients with phenotypic FSHD. However, examination of 50 GTG-banded metaphases in each patient with phenotypic FSHD revealed no pericentromeric abnormalities. Further, serum immunoglobulin levels and other immunologic biomarkers were within normal range in all phenotypic FSHD cases and no significant differences between patients with phenotypic FSHD and healthy control individuals were identified. These results suggest that there is little or no phenotypic overlap between FSHD and ICF, not even at the subclinical level. Therefore, it seems very unlikely that both conditions are caused by a disturbance in the same molecular pathway.

Finally, no mutations were detected in the exonic regions of the genes *DNMT1, DNMT3A, DNMT3B, DNMT3L, MTHFS, LMNA, CBX2, CBX5,* and *SUV39H1,* making them less likely candidate genes for phenotypic FSHD. However, since only the exonic regions of these genes were analyzed in this study, intronic mutations affecting splicing and mutations in the promoter region remain possible. Before studying this in more detail, more substantial evidence for the involvement of one of these candidate genes and the occurrence of phenotypic FSHD has to be generated.

In this study, we showed that the hypomethylation seen in patients with phenotypic FSHD is restricted to the D4Z4 repeat array. Apart from the D4Z4 hypomethylation, no further commonalities between patients with phenotypic FSHD and patients with ICF were identified, either at the epigenetic level or at the phenotypic level. At this moment, the cause of the D4Z4 hypomethylation in FSHD, both 4q-linked FSHD and phenotypic FSHD, remains unknown. As hypomethylation of D4Z4 has also been observed in non-penetrant gene carriers, we hypothesize that D4Z4 hypomethylation is necessary but not sufficient to explain the occurrence of FSHD [5]. Apparently, other, possibly epigenetic, factors at D4Z4 differ between FSHD and ICF and as such control for the development of FSHD. For future research, the mechanism of D4Z4 methylation needs to be investigated in more detail to identify candidate genes that may be disrupted in phenotypic FSHD.

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