Facioscapulohumeral Muscular Dystrophy
FSHD
International Research Consortium Research Workshop Meeting 2003

Tuesday, November 4, 2003
10 a.m. - 4 p.m.

The Westin Bonaventure Hotel and Suites
Los Angeles, California USA
Santa Barbara A/B Rooms

Chair: **Silvère van der Maarel, Ph.D.**
Organizers: **Daniel Paul Perez, Silvère van der Maarel, Ph.D.**

Sponsored by:

FSH Society, Inc.

Muscular Dystrophy Association (MDA USA)

Association Française Contre les Myopathies (AFM)

October 21, 2003

PREFACE

Dear Colleagues,

On behalf of the FSH Society, I welcome you to the Facioscapulohumeral Muscular Dystrophy (FSHD) International Research Consortium (IRC) Workshop 2003.

We are working hard toward a goal we know we can achieve. Today we are encouraging collaboration and mutual exploration from each and every participant. Our community is small in size and modest. Together with other colleagues who have contributed effort and time, we represent a substantially larger effort. I tell you this so you will understand the total dedication involved in every phase of this FSHD research program and community. We want our long range plan of solving the puzzle of FSHD to work and we know it can. And, given your solid participation and candid input – it will!

At last year's FSHD IRC workshop in Baltimore, Maryland, a request was made to create more time for discussion between the various attendees and groups. We received a number of high quality abstracts to be presented during this 2003 workshop. The 2003 FSHD IRC workshop was re-designed to allow for and to encourage scientific discussion and debate. We will follow the workshop format used at the World Muscle Society (WMS) in which platform presentations are complemented by active poster sessions. This format does allow more time for plenary discussions following the platform presentations and ensures time and attention to the poster sessions.

Platform lectures will be limited to 20 minutes. To encourage discussion, we request that presentations be approximately 12 minutes in length allowing for 8 minutes of discussion. To create more time for discussions, we ask that you do not include introductory slides of a redundant and familiar nature. We also ask that the moderators and presenters of each session stay on time to keep to our schedule.

Posters will be presented and viewed in three groups with approximately 15 people visiting each poster group/cluster on a rotating basis. Within each group, each presenter will have a few minutes to detail findings and explain the poster followed by a lively discussion by attendees.

Finally, as an attempt to initiate more scientific discussion to formulate better future research strategies for the FSHD field, we are adding a new module to this meeting. We asked four (4) members of this community of respected scientists in the field of FSHD research to make a provocative statement in the last hours of the workshop, so that we may initiate discussions. We have selected the four topics for the statement as follows: 4q-Specificity (statement by Rune Frants, Ph.D.); Pathways (statement by Sara T. Winokur, Ph.D.); Primary Mechanism (statement by Melanie Ehrlich, Ph.D.); and Model Systems (statement by Rossella Tupler, M.D., Ph.D.).

This meeting of the FSHD IRC could not have been organized without the support of our sponsors. I express my gratitude for the generous support of the FSH Society, the Muscular Dystrophy Association of the USA, and the Association Française Contre les Myopathies. In particular, I am indebted to Dan Paul Perez who took charge of most of the work organizing this meeting.

It is truly a pleasure to be meeting with the entire group and know that we will have a fast moving and interesting workshop!

Silvère van der Maarel, Ph.D.
Leiden University Medical Center, Leiden, The Netherlands

WELCOME
10:00 a.m.
OPENING REMARKS
Stephen J. Jacobsen, Ph.D., Chairman of the Board, FSH Society, Inc.
Howard L. Chabner, J.D., Board of Directors, FSH Society, Inc.

SLIDE SESSION I
10:00-11:00 a.m.
GENES and EXPRESSION
Silvère van der Maarel, Ph.D., Moderator
Leiden University Medical Center, Leiden, The Netherlands

10:00-10:20 a.m.

Shannon Venance, M.D., Ph.D.
University of Rochester School of Medicine and Dentistry, Rochester New York, USA
Expression profiling: Is there a role for vascular smooth muscle dysfunction in facioscapulohumeral muscular dystrophy (FSHD)?
SL Venance, D Henderson, J Sowden, S Welle, CA Thornton, R. Tawil. Neuromuscular Disease Unit, University of Rochester School of Medicine and Dentistry, Rochester NY.

10:20-10:40 a.m.

Alexandra Belayew, Ph.D.
University of Mons-Hainaut, Mons, Belgium
Functional study of the *DUX4* gene.
Eugénie Ansseau, Christel Mattéotti, Sébastien Sauvage, Aline Marcowycz,, Frédérique Coppée, and Alexandra Belayew
Laboratory of Molecular Biology, University of Mons-Hainaut, Mons, Belgium.

10:40-11:00 a.m.

Rossella Tupler, M.D., Ph.D.
University of Massachusetts Medical School, Worcester, Massachusetts, USA
Studying the over-expression of 4q35 genes in transgenic mice.
Davide Gabellini[1], Vladimiro Calvari[1], Ermelinda Porpiglia[1], Michael R.Green[1], Rossella Tupler[1,2]
[1]Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester, MA,USA.
[2]Biologia Generale e Genetica Medica, Via Forlanini 14, Pavia, Italy

POSTER SESSION & LUNCH
11:00 a.m.-1:00 p.m.
(Buffet Lunch Served at 12:00 p.m.)

GROUP 1

Rabi Tawil, M.D., Moderator
University of Rochester School of Medicine and Dentistry, Rochester New York

Anne Fernandez, Ph.D.
Institut de Génétique Humaine, Montpellier, France
A Comparative Proteomic Analysis of Muscle Degeneration Patterns in Fascio Scapulo Humeral Dystrophy.
Dalila Laoudj-Chenivesse, Gilles Carnac, Yegor Vassetzky, François Leterrier, René Bennes and Anne Fernandez
Institut de Génétique Humaine, UPR-CNRS 1142, 141 rue de la Cardonille, 34396 Montpellier, and
Association Francaise contre les Myopathies (AFM) 1 rue de l'Internationale, BP 59, 91002 Evry Cedex, FRANCE

Jane E. Hewitt, Ph.D.
University of Nottingham, Nottingham, United Kingdom
Identification of additional transcripts in the facioscapulohumeral muscular dystrophy region on human chromosome 4q35.
Jannine Clapp, Daniel J. Bolland, Andrew Jenkinson and Jane E. Hewitt
Institute of Genetics, Queen's Medical Centre, University of Nottingham, Nottingham, United Kingdom

Marcy C. Speer, Ph.D.
Duke University Medical Center, Durham, North Carolina,USA
D4Z4 binding proteins HMGB2, YY1, and nucleolin are excluded as candidate genes for FSHD1B.
K.L. Bastress, M. C. Speer, J. Stajich, J.R. Gilbert.
Duke University Medical Center, Durham, North Carolina, USA

Meena Upadhyaya, Ph.D.
Institute of Medical Genetics, Heath Park, Cardiff, United Kingdom
Association of the 4qA allele with Facioscapulohumeral Muscular Dystrophy (FSHD) in British and Turkish Populations.
Katie Wiseman, Mo MacDonald, Piraye Serdaglou, David N. Cooper, Meena Upadhyaya
Institute of Medical Genetics, Heath Park, Cardiff CF14 4XN, UK
Dept of Medicine, University of Istanbul, Istanbul, Turkey

GROUP 2

Silvère van der Maarel, Ph.D., Moderator
Leiden University Medical Center, Leiden, The Netherlands

Yi-Wen Chen, Ph.D.
Research Center for Genetic Medicine, Children's National Medical Center, Washington, DC, USA
Paired-like homeodomain transcription factor 1 (PITX1) RNA and protein are up-regulated in facioscapulohumeral muscular dystrophy (FSHD).
Yi-Wen Chen[1], Ling Li[1], Seung-Ah Chung[2], Ulla Bengtsson[2] and Sara T. Winokur[2]
[1]Research Center for Genetic Medicine, Children's National Medical Center, Washington, DC; [2].Department of Biological Chemistry, University of California, Irvine, CA

Frederique Coppée, Ph.D.
University of Mons-Hainaut, Mons, Belgium
Characterization of *DUX4c* and its role in facioscapulohumeral dystrophy (FSHD).
Aline Marcowycz, Eugénie Ansseau, Sébastien Sauvage, Christel Mattéotti, Sabrina Zorbo, Alexandra Belayew and F. Coppée.
Laboratory of Molecular Biology, University of Mons-Hainaut, Mons, Belgium.

Denise A. Figlewicz, Ph.D.
Department of Neurology, University of Michigan, Ann Arbor, Michigan, USA
Myogenesis in Facioscapulohumeral Dystrophy (FSHD) and Control Myoblasts.
D.A. Figlewicz, I. Coltas, Y. Hong
Dept. Neurology, University of Michigan, Ann Arbor MI, USA

Mayana Zatz, M.D.,Ph.D.
Human Genome Research Center, Departamento de Biologia Universidade de São Paulo, Brasil
Homozygosity for Autosomal Dominant Facio-scapulohumeral Muscular Dystrophy (FSHD) does not result in a more Severe Phenotype.
MMO Tonini[1], RCM Pavanello[1], J Gurgel-Giannetti[2], RJ Lemmers[3], SM van der Maarel[3], RR Frants[3] & M Zatz[1] *
1) Human Genome Research Center, Departamento de Biologia, Universidade de São Paulo, SP- Brasil; 2) Division of Neuromuscular Diseases, Hospital das Clínicas , Universidade Federal de Minas Gerais , MG –Brasil; 3) Center for Human and Clinical Genetics, Leiden University Medical Center

GROUP 3

Rune R. Frants, Ph.D., Moderator
Leiden University Medical Center, Leiden, The Netherlands

Guiliana Galluzzi, Ph.D.
Catholic University, Rome, Italy & Center for Neuromuscular Diseases, UILDM, Rome, Italy
PFGE analysis of 4q35 rearrangements: sensitivity and specificity of EcoRI/BlnI test for FSHD.
G.Galluzzi, M.Rossi, L.Colantoni, B.Merico, P.Tonali, L.Felicetti, E.Ricci.
Department of Neurosciences, Catholic University, Rome, Italy
Center for Neuromuscular Diseases, UILDM-Rome, Italy

Valery Kazakov, M.D., Ph.D.
Department of Neurology, Pavlov State Medical University, St. Petersburg, Russia
About Nosological Place of the Facioscapuloperoneal (or facio-scapulo-limb, type 2) Autosomal Dominant Muscular Dystrophy.
Historical, Clinical and Molecular Genetic Study
V. Kazakov, D. Rudenko
Department of Neurology, Pavlov State Medical University, St. Petersburg, Russia

Peter Lunt
St. Michael's Hospital, Bristol United Kingdom
Cosegregation at 4q35 of 2 Bln1-resistant fragments suggests probable duplication of D4Z4 / D4F104S1 region in 2 unrelated FSHD families.
Peter Lunt1, Rabi Tawil2, Christoffer Jonsrud3, 'Athena Diagnostics Inc.' 4, Linda Tyfield5
1 St. Michael's Hosp., Bristol UK; 2Univ. Rochester Med. Centre, Rochester NY; 3Univ. Hosp., TromsÆ, Norway; 4'Athena Diagnostics Inc.' Worcester MA 01605; 5Southmead Hosp., Bristol UK.

Antonel Olckers, M.D., Ph.D.
Centre for Genome Research, Potchefstroom University for CHE, Pretoria, South Africa;
DNAbiotec Pty (Ltd), Pretoria, South Africa
Non-radioactive Detection of the Translocation Frequency Between 4q and 10q in the South African FSHD population
Olckers A1,2, Alessandrini M1; and Van der Merwe A1
1 Centre for Genome Research, Potchefstroom University for CHE, Pretoria, South Africa;
2 DNAbiotec Pty (Ltd), Pretoria, South Africa.

SLIDE SESSION II
1:00-2:00 p.m.
EPIGENTIC CAUSES
Denise Figlewicz, Ph.D., Moderator
Department of Neurology, University of Michigan, Ann Arbor, Michigan, USA

1:00-1:20 p.m.

Melanie Ehrlich, Ph.D.
Tulane Medical School, New Orleans, Louisiana, USA
Testing the Position-Effect Variegation Hypothesis for Facioscapulohumeral Muscular Dystrophy By Analysis of Histone Modification and Gene Expression in Subtelomeric 4q.
Melanie Ehrlich, Guanchao Jiang, Fan Yang, Petra G. van Overveld [1], Vettaikorumakankav Vedanarayanan [2], and Silvere van der Maarel [1]
Human Genetics Program and Department of Biochemistry, Tulane Medical School, New Orleans, LA 70112, USA; [1] Department of Human Genetics, Center for Human and Clinical Genetics, Leiden University Medical Center, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands; [2] Department of Neurology, University of Mississippi Medical School, Jackson, MS, 39216, USA

1:20-1:40 p.m.

Silvère van der Maarel, Ph.D.
Leiden University Medical Center, Leiden, The Netherlands
D4Z4 hypomethylation in FSHD causes the transcriptional upregulation of 4qter genes.
S. van der Maarel[1], P. van Overveld[1], R. Lemmers[1], L. Sandkuijl[1], L. Enthoven[2], S. Winokur[3], G-J. van Ommen[1], G. Padberg[4], R. Frants[1].
1) Department of Human Genetics, Leiden Univ Medical Center, Leiden, Netherlands; 2) Division of Medical Pharmacology, Leiden/Amsterdam Center for Drug Research, Leiden University Medical Center, Leiden, The Netherlands; 3) Department of Biological Chemistry, College of Medicine, University of California, Irvine CA, USA; 4) Department of Neurology, University Medical Center Nijmegen, Nijmegen, The Netherlands.

1:40-2:00 p.m.

Sara Winokur, Ph.D.
University of California, Irvine, California, USA
Nuclear localization of the FSHD gene region.
Peter Masny, MD[1], Ulla Bengtsson[1], Seung-Ah Chung[1], Jorge Martin[1], Silvere van der Maarel, PhD[2], Denise Figlewicz, PhD[3] and Sara Winokur, PhD[1]
[1]Department of Biological Chemistry, University of California, Irvine, CA
[2]Department of Human Genetics, Leiden University, Leiden, The Netherlands
[3]Department of Neurology, University of Michigan, Ann Arbor, MI

BREAK
2:00-2:15 p.m.
(Ice Cream & Sorbet Served)

SLIDE SESSION III
2:15-3:15 p.m.
INVITED SESSION
Rabi Tawil, M.D., Moderator
University of Rochester School of Medicine and Dentistry, Rochester New York

2:15-2:45 p.m.

Eric C. Schirmer, Ph.D.
Department of Cell Biology, The Scripps Research Institute, La Jolla, California, USA
Nuclear Envelope Proteins with Potential Involvement in Muscular Dystrophies.
Eric C. Schirmer, Laurence Florens, Tinglu Guan, John R. Yates III, and Larry Gerace
Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037

2:45-3:15 p.m.

James Marshall, Ph.D.
Sydney IVF, Sydney, Australia
Pre-implantation genetic diagnosis (PGD) for Facioscapulohumeral Muscular Dystrophy (FSHD) – a reproductive option using genetic linkage analysis.
J. Marshall, D. Leigh, S. McArthur and K. de Boer.
Sydney IVF, Sydney, Australia

DISCUSSION and CLOSURE
3:15-4:00/4:30 p.m.
Silvère van der Maarel, Ph.D., Moderator
Leiden University Medical Center, Leiden, The Netherlands

THE FOUR TOPICS
A statement will be made on four relevant topics to initiate and promote an active discussion:

4q-Specificity
A statement to be made by Rune Frants, Ph.D.

Pathways
A statement to be made by Sara T. Winokur, Ph.D.

Primary Mechanism
A statement to be made by Melanie Ehrlich, Ph.D.

Model Systems
A statement to be made by Rossella Tupler, M.D., Ph.D.

CONCLUDING REMARKS
4:30 p.m.-

Silvère van der Maarel, Ph.D.
Stephen J. Jacobsen, Ph.D.

NOTES:

Expression profiling: Is there a role for vascular smooth muscle dysfunction in facioscapulohumeral muscular dystrophy (FSHD)?

SL Venance, D Henderson, J Sowden, S Welle, CA Thornton, R. Tawil.

Neuromuscular Disease Unit, University of Rochester School of Medicine and Dentistry, Rochester NY.

The facioscapulohumeral muscular dystrophy (FSHD) genetic locus maps to chromosome 4q35 where a deletion of a critical number of 3.3 Kb D4Z4 tandem repeats results in phenotypic manifestation. The molecular and cellular pathogenesis of FSHD remains elusive despite awareness of the genetic lesion. Dysregulation of gene expression through position effects on and/or inappropriate de-repression of gene transcription have been proposed as possible mechanisms. Microarray techniques now permit characterization of differential patterns of expression in diverse biological conditions and is ideally suited for investigating transcriptional dysregulation in FSHD. We performed DNA expression profiling using Affymetrix third generation U133A/B GeneChip technology (~44000 probe sets estimated to cover >33,000 human genes) on muscle biopsy samples obtained from 10 genetically confirmed individuals with FSHD. Comparison was made to profiles obtained from biopsy samples from16 normal controls and 12 samples from genetically confirmed myotonic dystrophy type 1 (DM1). To reduce the possibility of spurious differences in expression profiles, all experimental and control samples were from male subjects, obtained by needle biopsy, from the same muscle (vastus lateralis) and all underwent identical processing. Analysis of the gene expression data included sequential filtering of genes, not expressed or with no change in level of expression, in either FSHD or normal muscle. Secondly, genes that were similarly expressed in myotonic dystrophy were filtered out. Finally, differentially expressed genes that had a ≥ 2 fold change, a false detection rate of $< 10\%$ and a p value of ≤ 0.01 were considered selective for FSHD.

Importantly, on the initial analysis, none of the 4q35 genes, including ANT1 and FRG1 showed selective increased expression in FSHD; the ANT1 and FRG1 results were confirmed by RT-PCR. However, 67 genes, whose protein products are at least partially characterized, were specifically and significantly over- (26 genes) or under-expressed (41 genes) in FSHD. Several intriguing patterns were evident. Skeletal muscle specific transcripts, including myosin heavy and light chains, tropomyosins, troponins I/C/T, and in particular desmin, a marker for muscle fiber regeneration, were down regulated. Transcripts involved in Notch signaling and myogenesis (including JAG1, NOV, PEN2, MEF2C, VGL2) were differentially expressed, further reflecting alterations in muscle differentiation. Interestingly however, there was upregulation of multiple transcripts specific to smooth muscle and endothelium, including myosin heavy and light chains, actin, transgelin, calponin, ryanodine receptor 3 and thrombomodulin. These findings in combination with the unexplained retinal vascular abnormalities evident in patients with FSHD raise the possibility of vascular derangement as a unifying hypothesis in disease pathogenesis. We propose a role for vascular smooth muscle differentiation and/or dysfunction, either as a primary or a secondary effect, in the pathogenesis of FSHD. Confirmation of these findings at the mRNA and protein level, as well as with a second set of samples from 10 female patients with FSHD, are currently underway.

Functional study of the DUX4 gene.

Eugénie Ansseau, Christel Mattéotti, Sébastien Sauvage, Aline Marcowycz,, Frédérique Coppée, and Alexandra Belayew

Laboratory of Molecular Biology, University of Mons-Hainaut, Mons, Belgium.

The FSHD candidate gene we are studying (DUX4; Gabriëls et al 1999, Gene 236, 25-32) is located within the D4Z4 unit itself. Shortening of the D4Z4 array would open an inhibitory chromatin and allow DUX4 expression in patient myoblasts. The DUX4 protein has a double homeodomain and locates to the cell nucleus, partly overlapping with emerin and lamin. Hundreds of 3.3-kb elements with homologous DUX genes not linked to FSHD are scattered in the human genome: their expression constitutes a background against which DUX4 expression is difficult to single out. DUX1 is a non pathological protein expressed in vivo from such gene (Ding et al 1998, Hum.Mol.Genet. 7, 1681-94).

A rabbit antiserum was raised against a peptide of the DUX4 carboxy-terminal (tail) domain lacking in DUX1 and probably in other DUX proteins. This serum was used to show DUX4 expression from its natural gene promoter: mouse myoblast C2C12 cells were transfected with a pGEM plasmid containing either a 13.5-kb EcoRI genomic fragment with two D4Z4/DUX4 units corresponding to a patient locus (Gabriëls et al 1999), or a 3.3-kb KpnI fragment containing a single DUX4 gene. As a positive control, we used the pCIneo-DUX4 expression vector (DUX4 ORF under control of a strong CMV promoter). Nuclear extracts of these cells were analysed by electrophoresis on SDS-PAGE 10% followed by Western blotting: in each sample a 52 kDa protein was detected with the apparent molecular weight of bona fide DUX4 produced by transcription/translation in vitro.

We could not identify DUX4 protein partners with the yeast two hybrid system (MatchMaker, Clontech), because it showed a strong transcriptional activity that could be mapped to its tail domain. In contrast, DUX1 gave no background activation and was used to screen a human muscle cDNA library. Among the 42 positives, 35 encoded desmin. Since the DUX1 double homeodomain is very similar to the DUX4 one, desmin might also interact with DUX4. This is being checked by GST pull down experiments. Desmin is a muscle intermediate filament protein that interacts with nuclear envelope proteins (Stromer and Bendayan 1990, Cell Motil Cytoskeleton. 17, 11-8). It is mutated in some muscular dystrophies and is a plausible DUX partner to be further evaluated.

Gabellini et al (2002, Cell 110, 339-348) identified a repressing cis-element in D4Z4 that interacted with YY1 and inhibited expression of a linked reporter gene in HeLa cells. This element precisely mapped within the DUX4 promoter, just 5' from the Sp1 site that mediated its basal expression (Gabriëls et al 1999). We inactivated the YY1 site according to Gabellini et al (2002) in our DUX4-LUC construct, only weakly affecting its transient expression in C2C12 cells. Because this mutation had created a new binding site, we used a different mutation to prevent YY1 binding, and observed a 1.5-fold activation in C2C12 cells. These experiments demonstrated a weak inhibitory role of YY1 on the DUX4 promoter activity in myoblasts. Additionally, a putative MyoD cis-element was found onto the DUX4 transcription start site. This element was shown to be repressing: its mutation increased DUX4-LUC expression 2-3 fold in C2C12 cells and co-transfection with a MyoD expression vector inhibited 4-fold the wild type DUX4-LUC construct. These observations suggested that DUX4 expression was to be expected in cells with low MyoD levels, i.e. myoblasts but not myotubes.

In a way to evaluate DUX4 function, we transfected TE671cells with pCIneo-DUX4, extracted nuclear proteins 48 hours later, and detected a 3-fold increase in MEF2 and p53 concentrations. This observation

is in agreement with recent data of Winokur et al (2003, Neuromuscul Disord. 4, 322-33) demonstrating premature differentiation of FSHD myoblasts. In addition, a strong decrease in expression of genes involved in resistance to oxidative stress was detected in these transfected cells by RNA analysis on microarrays, as shown recently for FSHD myoblasts (Winokur et al 2003).

In conclusion, our data showed that DUX4 expression in non-affected muscle cells could impinge on the activity of genes known to be disturbed in FSHD myoblasts.

We thank the MDA (USA), AFM (France), and ABMM (Belgium) for funding. Graduate student fellowships to E.A. and C.M. came from the FRIA (Belgium), and to S.S. and A.M. from the AFM.

Studying the over-expression of 4q35 genes in transgenic mice.

Davide Gabellini1, Vladimiro Calvari1, Ermelinda Porpiglia1, Michael R.Green1, Rossella Tupler1,2

1Program in Gene Function and Expression
University of Massachusetts Medical School
Worcester, MA, USA

2Biologia Generale e Genetica Medica
Via Forlanini 14
Pavia, Italy

Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common hereditary myopathies. The disease has been causally related to deletion of tandemly arrayed 3.3 kb repeat units (D4Z4) on chromosome 4q35 possibly affecting chromatin organization and nearby gene expression. Consistently, we have observed that three genes mapping at 4q35, FRG2, FRG1 and ANT1, are over-expressed in the FSHD affected muscle. We have also discovered that within D4Z4 unit a 27 bp DNA element has a protein-binding activity. Through biochemical purification, we have isolated three proteins, YY1, HMGB2, and nucleolin, forming a multi-protein complex that binds D4Z4. We have been also able to demonstrate that this multi-protein complex is involved in silencing of gene located at 4q35. Our data suggest that D4Z4 elements contribute to 4q35 gene silencing through the interaction with a transcriptional repressing complex. We propose that the reduction of D4Z4 tandemly repeated units to a critical threshold might induces the over-expression of 4q35 genes and, as a consequence, triggers FSHD pathogenesis.

Over-expression of 4q35 genes might lead to the dystrophic phenotype either by giving rise to a series of biological events in muscles eventually leading to muscle weakness or, more simply, by having a toxic effect or altering one structure of the muscle cell.

To verify the proposed pathogenic model in vivo, we have generated transgenic mice over-expressing ANT1, FRG1, and FRG2. For this purpose, ANT1, FRG1, and FRG2 have been cloned into an expression vector carrying the human skeletal actin (HSA) promoter that is expressed in all skeletal muscle fibers of the adult mouse, with minor fiber to fiber variation.

We have obtained 8 founders expressing the HSA-FRG1 transgene, 7 founders expressing the HSA-FRG2 transgene, and 1 founder expressing the HSA-ANT1 transgene. We have expanded the mouse colonies over-expressing the HSA-FRG1 and HSA-FRG2 transgenes and we are expanding the mouse colony of the HSA-ANT1 transgene.

The transgene expression profiles were analyzed at either the mRNA level or the protein level in skeletal muscle, heart, brain, kidney and liver. The HSA-FRG1, HSA-FRG2, and HSA-ANT1 transgenes were expressed uniquely in skeletal muscle tissues. Detailed analysis of selected mouse strains is in progress to determine if pathological signs are present in muscle tissues of transgenic animals.

We expect functional analysis of genes in the FSHD chromosomal region to provide relevant information to understand the molecular basis of FSHD. Furthermore, animal models reproducing some features of FSHD will be a valuable and endless source of biological material to study the biological effects of 4q35 gene over-expression in muscle. Eventually they can be used to gauge the efficacy of therapeutic treatments.

A Comparative Proteomic Analysis of Muscle Degeneration Patterns in Fascio Scapulo Humeral Dystrophy

Dalila Laoudj-Chenivesse, Gilles Carnac, Yegor Vassetzky, François Leterrier, René Bennes and Anne Fernandez

Institut de Génétique Humaine, UPR-CNRS 1142, 141 rue de la Cardonille, 34396 Montpellier, and Association Francaise contre les Myopathies (AFM) 1 rue de l'Internationale, BP 59, 91002 Evry Cedex, France

Facio Scapulo Humeral Dystrophy (FSHD) is a degenerative neuromuscular disease that has been linked to deletion of tandemly repeated 3.3 kb units (D4Z4) in the subtelomeric region of chromosome 4 (4q35). This decreased number of repeats has been proposed to result in inappropriate over-expression of few genes immediately upstream of the D4Z4 repeats. Among these genes, one with a known function is the heart and muscle-specific isoform of the adenine nucleotide translocator (Ant1). Using comparative proteomic two dimensional profiling, we have identified specific changes in protein expression of FSHD muscle. The first category of protein alterations identified shows quantitative changes in muscle fiber-specific proteins including myosin light chains and tropomyosin isoforms. These changes were accompanied by altered fiber-type distribution and shape and were not found in 2D protein profiles from Duchenne Muscular Dystrophy (DMD) muscles. The second group of alterations identified concerned proteins involved in oxidative stress and lipid peroxidation that are indicative of mitochondrial dysfunction and apoptosis. Consistent with the hypothesis that the Ant1 gene present at the 4d35 locus could be inappropriately over-expressed, we found increased levels of Ant1 protein in all FSHD muscle samples. We propose that increased expression of Ant1 leads to mitochondrial dysfunction that result in substantial alterations in muscle fiber metabolism, fiber type ratio and eventually atrophy of FSHD muscle fibers. This model predicts an aggravation with age and intensive muscle use that could be compensated or slowed down by the use of anti-oxidants.

Identification of additional transcripts in the facioscapulohumeral muscular dystrophy region on human chromosome 4q35

Jannine Clapp, Daniel J. Bolland, Andrew Jenkinson and Jane E. Hewitt

Institute of Genetics, Queen's Medical Centre, University of Nottingham, Nottingham, NG7 2UH, UK

We have used a combination of bioinformatics and RT-PCR to produce a detailed analysis of the gene complement and expression profiles for the distal 5.5Mb of 4q. Thus far, 29 transcripts have been identified, many of which are expressed in fetal or adult skeletal muscle and may also be candidates for a role in FSHD. The majority of the genes are clustered in the proximal 2.5Mb of 4q35. We have examined in detail the 3Mb interval between the cadherin gene FAT and FRG1 at the distal end of this region as this has been especially poorly characterized. Interestingly, we have found at least two transcripts in this interval that appear to represent spliced, non-coding RNAs. To investigate the evolutionary history of the FSHD region and to help gene identification strategies, we have carried out comparative mapping studies in mouse and puffer fish. In the homologous region on mouse chromosome 8, gene content, distribution and density are similar to human. However, FRG2 and D4Z4 are not conserved in the mouse. The Fat-Frg1 interval is 0.5Mb larger than the human equivalent region. Although homology of synteny is less extensive in Takifugu and Tetraodon, the Fat-Frg1 linkage group is conserved in both these genomes with the two genes separated by 100-120kb in both puffer fish, suggesting there may be important regulatory sequences in this interval.

D4Z4 binding proteins HMGB2, YY1, and nucleolin are excluded as candidate genes for FSHD1B.

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Facioscapulohumeral muscular dystrophy is a disease of skeletal muscle, with symptoms including both facial and shoulder girdle weakness and progression to involve the pelvic girdle and extremities in the majority of cases. For nearly all of the cases of FSHD, the molecular basis of the disease can be identified as a deletion of the D4Z4 repeat on the end of the long arm of chromosome 4. However, in 5-10% of FSHD families there is no linkage to 4q35. This type of FSHD is referred to as type 1B (FSHD1B; OMIM 158901) or non-chromosome 4-linked facioscapulohumeral muscular dystrophy in the small group of families where there is no short 4q35 D4Z4 fragment. Efforts to identify linkage to other areas of the genome have been of limited success in families where non-linkage to chromosome 4 has been established. A region on chromosome 15 that is consistent with linkage was seen in 2 FHSD1B families, however follow-up studies on genes in that region have yet to yield results. Recently, the three identified proteins, which bind the D4Z4 repeats of chromosome 4q35, have become candidate genes for FSHD1B. The D4Z4 binding proteins include YY1, a known repressor/activator, HMGB2, involved in the modulation of chromatin structure, and nucleolin, another transcription factor. We investigated these genes using denaturing high performance liquid chromatography (DHPLC) as candidate genes for FSHD1B. The screen included a total of 33 affected individuals from 6 non-chromosome 4-linked families. Samples were organized into 14 pools with 5 cases each and contained at least one control, one 4-linked patient sample, and a non-chromosome 4-linked FSHD sample. All variations detected were followed up by sequence analysis. Coding sequences and promoter region was analyzed for HMBG2 and no sequence variations were detected. For YY1, all 5 exons were analyzed and a polymorphism was detected in exon 1. There is a 3 bp INDEL, which results in the deletion of one histidine from a tract of 11 (H75del), seen in 3 control samples and 2 non-chromosome 4-linked FSHD patients. In nucleolin, several SNP's were seen, including the non-synonymous change P515H, however all polymorphisms either occurred in the control samples or were known SNPs. These results suggest that HMBG2, YY1, and nucleolin are unlikely to represent the genes responsible for FSHD in these families

Association of the 4qA allele with Facioscapulohumeral Muscular Dystrophy (FSHD) in British and Turkish Populations

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Facioscapulohumeral muscular dystrophy is an autosomal dominant disorder caused by the deletion of D4Z4 repeat units specific to the subtelometric region of chromosome 4. The disease, characterised by the slow and progressive weakening of the facial, scapular and humeral muscles affecting one in 20,000, reaches full penetrance by the age of 20.

Recently, a polymorphism has been identified in the β-satellite repeats, distal to the D4Z4 repeat units on chromosome 4, that has two allelic forms (4qA and 4qB).

Allele 4qA was found to co-segregate with FSHD-specific short D4Z4 alleles in the Dutch population. This 4qA polymorphism is also associated with the D4Z4 repeats at 10q. In the normal population, both polymorphic alleles (4qA, 4qB) are present in equal frequency. In order to ascertain whether the 4qA allele segregates with the small FSHD allele in different populations, we have studied a panel of 65 unrelated FSHD individuals, of whom 50 were British and 15 were of Turkish origin. Fifty unaffected individuals of British and Turkish origin were also examined as controls.

High molecular weight DNA was extracted from blood samples, and p13E-11 short fragments were identified by digestion with the restriction enzymes EcoRI, XapI and double digestion with both EcoRI and BlnI. DNA was resolved using pulsed field gel electrophoresis. Southern blots were hybridised with radiolabelled p13E11 probe and visualised by autoradiography.

To study the distribution of alleles 4qA and 4qB, DNA samples were digested with the restriction enzyme HindIII, and the Southern blots were hybridised consecutively with probes specifically designed to illuminate alleles 4qA and 4qB (probe A and B). In all 65 affected individuals, allele 4qA was always found to segregate with the FSHD specific small fragments identified with probe 13E-11. By contrast, an equal frequency of alleles 4qA and 4qB was observed in the unaffected controls.

This study confirms the specific association of the allele 4qA with the FSHD disease allele in both British and Turkish populations. These findings will not only refine the molecular diagnosis for FSHD in the patients with borderline short EcoRI/BlnI fragments but also emphasise that allele 4qA may well have some functional relevance in the patho-physiology of FSHD.

Paired-like homeodomain transcription factor 1 (PITX1) RNA and protein are upregulated in facioscapulohumeral muscular dystrophy (FSHD)

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To identify differentially expressed genes specific to facioscapulohumeral muscular dystrophy (FSHD), we have performed expression profiling of FSHD muscles using the Affymetrix Human Genome U133 set representing approximately 44,000 transcripts. By comparing profiles of FSHD muscles (n=9) to normal controls (n=4) and disease controls including Duchenne muscular dystrophy (DMD) (n=5), juvenile dermatomyositis (JDM) (n=10) and dysferlin deficiency (n=5), we identified 668 FSHD genes that were specifically dysregulated in FSHD (377 up- and 219 down-regulated genes). The most significantly up-regulated gene in the FSHD but not other muscle disorders was the paired-like homeodomain transcription factor 1 (PITX1) (11 fold). We confirmed the change of PITX1 RNA and protein levels by real-time quantitative RT-PCR and immunohistochemistry of FSHD muscles using a PITX1 antibody. The immunohistochemistry data showed that PITX1 was up-regulated in the myonulei of FSHD muscles. In vitro studies demonstrate that PITX1 is detected in both control and FSHD myoblasts with either a punctate staining or diffuse nuclear pattern. Over-expression of PITX1 in C57/BL10 mice by electroporation causes muscle necrosis. PITX1 has been implicated in patterning of lateral plate mesoderm derivatives for specification of hindlimb identity and has hormone-regulated activity. In chick embryo, wing buds in which Pitx1 was misexpressed developed into limbs with some morphologic characteristics of hindlimbs, and the muscle pattern was transformed to that of a leg. Our recent data generated by genome-wide expression profiling showed that PITX1 gene is the most significantly differentially expressed gene in biceps (forearm) muscle of FSHD patients. In addition the change was not observed in other muscular dystrophy (DMD, JDM, and dysferlinophathy) suggesting the disease-specific up-regulation of PITX1 might play an important pathophysiological role in the disease.

Characterization of DUX4c and its role in facioscapulohumeral dystrophy (FSHD)

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FSHD is linked to a partial deletion of the D4Z4 repeat array in 4q35. Shortening of the repeat array is thought to destabilize a heterochromatin structure leading to over-expression of neighboring genes. It was indeed shown recently that the closer a gene is to the D4Z4 locus the more it is repressed in control myoblasts but upregulated in FSHD ones (Gabellini et al, 2002, Cell 110, 339-348). Our group has characterized a gene (DUX4, double homeobox gene) within each D4Z4 unit and has proposed it as a candidate for FSHD. A very similar subtelomeric region with a D4Z4-like array is present on 10q26, but partial deletions of this array don't lead to FSHD. The homology between chromosomes 4 and 10 extends 50 kb proximal from the repeat array, and ends at an inverted and truncated member of the D4Z4 repeat family (D4S2463; Lyle et al, Genbank 1995), next to the FRG2 gene. We found that this element comprises a gene similar to DUX4, and present on chromosome 4 only. We named this gene DUX4c for centromeric. We suggest that DUX4c is up-regulated in a way similar to FRG2 in FSHD myoblasts (Gabellini et al, 2002), and hypothesize that DUX4c could be implicated in the development of the FSHD phenotype together with DUX4.

DUX4c only differs from DUX4 in the promoter region, and in the carboxy-terminal domain of the encoded proteins that are 374- and 424- residue long, respectively. We have fused the DUX4c promoter to the luciferase gene and analyzed its function by transient expression in mouse muscle C2C12 cells. The luciferase activity is 7-fold higher than a promoter-less vector, showing the functionality of the DUX4c promoter.

We have subcloned the DUX4c ORF in a pCIneo plasmid allowing the expression of a 44 kDa protein by transcription/translation in vitro in a reticulocyte lysate. We have developed a rabbit antiserum against a DUX4c peptide (residues 350-365, 50% identity with DUX4). The specificity of this antiserum has been demonstrated by immunoprecipitation of the DUX4c protein produced by transcription/translation in vitro. This antiserum has been used on nuclear extracts of myoblast primary culture derived from patients with FSHD, and it detected a protein with the apparent molecular weight of DUX4c. The intracellular DUX4c location was determined by immunofluorescence with this rabbit antiserum on human rhabdomyosarcoma TE671 cells transfected with pCIneo-DUX expression vectors. DUX4c localizes in the nucleus mostly at the nuclear envelope like other DUX proteins studied.

We have subcloned the DUX4c ORF in the pGBT9 vector to produce a GAL4 DBD-DUX4c fusion protein and evaluated its activity in the yeast simple-hybrid system. DUX4c presented no transcriptional activity, in contrast to DUX4, that was a very potent transactivator. This activity maps to the carboxy-terminal domain and we would like to construct chimeric DUX4/4c proteins to map it precisely.

Recent data of Lemmers (2003, Neurology 22, 178-83) have shown a deletion extending from D4Z4 to DUX4c in two families with FSHD, suggesting that this gene is not implicated in the disease. However, it is present in most patients and its activation could influence the variable FSHD phenotype since DUX4c could bind by its homeodomains the DUX4 target genes but would have different effect on their transcriptional level.

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Myogenesis in Facioscapulohumeral Dystrophy (FSHD) and Control Myoblasts

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The insulin-like growth factor, IGF-I, is known to play a role both in myoblast proliferation and in maintenance of muscle following differentiation. The effects of IGF-I are mediated by different intracellular signaling pathways for proliferation vs. differentiation. Previously, we have observed phenotypic differences in myoblasts cultured from FSHD skeletal muscle biopsies, including morphologic changes and loss of replicative capacity. FSHD myoblasts leave the cell cycle earlier, fuse more readily, and upregulate some genes associated with myogenesis. Because of the role of IGF-1 in myoblast proliferation, we are investigating the IGF-1 signaling pathways in FSHD myoblasts. Using real-time PCR, we have found a significantly decreased expression of the IGF-I receptor in FSHD myoblasts. However, following differentiation, expression of the IGF-1 receptor is equivalent in FHSD and control myotubes. These data support our hypothesis that significant biochemical differences are found at a very early stage in muscle development in FSHD. IGF-1 is currently a candidate for clinical trial in other muscular dystrophies; whether or not it might be expected to provide a benefit for FSHD patients will be more clear following comprehensive investigation of the signaling pathways and availability of intracellular docking proteins, IRS-1 and IRS-2, in FSHD patients' myoblasts.

Homozygosity for Autosomal Dominant Facio-scapulohumeral Muscular Dystrophy (FSHD) does not result in a more Severe Phenotype.

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Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant disorder with an incidence of 1 in 20,000 births. It is characterized by progressive weakness of facial, shoulder girdle and upper arm muscles, with remarkable clinical variability and frequent clinical anticipation. Asymptomatic carriers seem to be concentrated in some genealogies. FSHD, mapped to 4q3, is associated with a deletion in a D4Z4 repeat array. Here we describe the first case of homozygosity in a 29-year-old patient, from a highly inbred family with a high proportion of asymptomatic carriers (11 among 17 who inherited the FSHD fragment). The proband inherited two 4qA type 24Kb FSHD fragments from both parents who are first-degree cousins. His father was asymptomatic but his 53-year old mother is clinically affected. Three among 4 probands' sibs carry one FSHD allele. Two of them are asymptomatic but his 18-year old brother has muscular weakness and a phenotype of FSHD. A female cousin also has a classical FSHD course. In short, we are reporting for the first time that homozygosity for FSHD is compatible with life and that two same origin FSHD fragments do not cause a more severe phenotype. It was proposed that FSHD would be caused by upregulation of genes, located proximal to D4Z4, due to a transcriptional derepression inversely related to the size of the deletion .Under this hypothesis it is difficult to explain why our homozygous proband is not more severely affected than his brother, who carries only one deleted allele, suggesting a more complex mechanism.

PFGE analysis of 4q35 rearrangements: sensitivity and specificity of EcoRI/BlnI test for FSHD

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Facio-scapulo-humeral muscular dystrophy (FSHD) remains a gene-orphan disease, however a considerable progress has been made in developing a reliable molecular test since a DNA rearrangement at 4q35 locus has been unequivocally associated with the disease. The test consists in the detection, after hybridization with probe p13E-11, of a shortened EcoRI BlnI-resistant fragment (10 to 40 kb in FSHD patients, > than 40 kb in normal individuals) originated through a deletion event. While the sensitivity of this molecular approach is very high (over 96%), its specificity has never been adequately considered. We studied 157 families by PFGE, that allows to visualize all 4q alleles, irrespective of their size. In 126 families (group A) a diagnosis of FSHD had been performed on the base of a clinical picture, according to ENMC Consortium criteria. The remaining 31 (group B) did not fulfill the diagnostic criteria for FSHD. 219 affected and 155 unaffected individuals from the 126 FSHD families belonging to group A and 87 individuals from the 31 belonging to group B were analyzed. In addition, we studied 72 unrelated normal individuals.

Group A: an EcoRI BlnI-resistant fragment, 13 to 41 kb in size, was detected in 121 out of the 126 families. Unexpectedly, in 41 individuals belonging to 17 families we found 23 distinct EcoRI fragments, BlnI-resistant (23 to 41 kb in size), in addition to those causally associated with the disease. It is also worth-noting that 22 out of the 41 subjects carried exclusively a 23-41 kb fragment different from that associated with the disease in the family and none was affected by FSHD.

Group B: eight EcoRI, BlnI-resistant fragments in the range 22 to 41 kb were found in individuals belonging to 7 out of the 31 families examined.

A BlnI-resistant EcoRI fragment sized 30 to 41 kb was detected in 6 out of 72 normal individuals.

Because of the existence of overlap of 4q35 EcoRI fragments between patients and normal individuals, a diagnosis of FSHD should not be based on molecular grounds alone in families not fulfilling the diagnostic criteria for FSHD.

About Nosological Place of the Facioscapuloperoneal (or facio-scapulo-limb, type 2) Autosomal Dominant Muscular Dystrophy.
Historical, Clinical and Molecular Genetic Study

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Duchenne in 1855 described of FSHD with extension of the weakness from the facial, shoulder girdle and upper arm muscles with subsequent involvement of the trunk, pelvic girdle and thigh muscles (gradually descending variety). Latter, Erb (1882) and Landouzy–Dejerine (1884) described another variety of MD in which after weakness of the facial and shoulder-girdle muscles the peroneal group (anterior tibial) was involved (descending with a "jump" variety). Davidenkov in 1962 confirmed the existence of this very special type of MD.

We studied the pattern of muscle involvement in patients with autosomal dominant descending with a “jump” type of MD who were examined (V.K) in 1969 and then in 1993–1997 included new patients (always 142 patients from 21 families). All these patients were in different phases of the disease. Six pedigrees (25 patients) were re-examined (V.K.) after 24 and even 28 years. Thus, the succession and rate of involvement of separate muscles and muscle groups in the myodystrophic process as well as the degree of affection of the individual muscles and the sequence of the distribution of the process were traced in the various phases of the disease during many years. The diagnosis of MD in each pedigree was supported by needle EMG, motor and sensory nerve conduction velocities, muscle biopsy (in one patient from the pedigree), CT and MRT of muscles. Molecular genetic analysis of 12 Russian AD FSLD2 families in 35 affected members showed the probe p13E-11 detected EcoRI/BlnI of DNA fragments size (DFS) between 13 – 35 kb (or equal 37) (double digestion) cosegragated with the disease and linked with 4q35. These data permit to conclude that a facioscapuloperoneal and (facio)scapuloperoneal MD is the same nosological entity. The best and more correct name for it is: “facioscapulolimb MD, type 2 (FSLD2), descending with a “jump” with initial facioscapuloperoneal or (facio)scapuloperoneal phenotypes, autosomal dominant with DFS ranged 13-35 kb (Duchenne de Boulogne)”. Among the observed cases we did not come across any having the autosomal dominant gradually descending variety of FSHD called "facio-scapulo-limb muscular dystrophy, type 1 (FSLD1) with initial facioscapulohumoral phenotype, autosomal dominant with DFS less than 38 kb (Erb, Landuzy and Dejerine)’’. Data show that FSLD2 is a very special type of MD with «hard» static and dynamic patterns of muscle involvement, with a mild course of the disease and slight/severe affection of the isolated facial muscles. The probe p13E-11 can be used for detecting DFS between 13-35 kb (or equal 37) for FSLD2 are assigned with chromosome 4q35. This data well confirm of historical study of the problem (the establishing of the main reason of the famous discussion between Erb and Landouzy-Dejerine) and Davidenkov’s opinion about nosology of the scapulo-peroneal amyotrophy.

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Cosegregation at 4q35 of 2 Bln1-resistant fragments suggests probable duplication of D4Z4 / D4F104S1 region in 2 unrelated FSHD families.

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In 2 families referred to our molecular genetic diagnostic service in Bristol for molecular testing (with p13E-11) for FSHD, we found co-segregation of a 10kb (7kb Bln1-resistant) fragment with a second Bln1-resistant fragment of < 38kb, consistently in males and females across at least 2 generations. Polymorphic marker analysis strongly favours both fragments in each family being linked together at 4q35 (rather than one coming from 10q26).

In the first family, from Norway, the Bln1-resistant fragments (25/22kb & 10/7kb) co-segregate with a variable-age-at-onset FSHD, which interestingly is also consistently associated with a sensorineural hearing loss.

In the second family, from Bristol UK & Rochester NY, the co-segregating Bln1-resistant fragments (38/35kb & 10/7kb) appear not to be associated with any symptomatic presentation. The 79-year-old late-adult-onset index case has a different Bln1-resistant fragment (34/31kb), which is assumed to be the allele responsible for FSHD in her and in a minimally symptomatic 62-year-old nephew. Polymorphic marker analysis at 4q35 supports this being on a different copy of chromosome 4 from the two cosegregating fragments. Two of the subjects in family 2 who demonstrate the cosegregating fragments have undergone PFGE study. Both of these subjects were found to show 5 fragments (3 Bln1-resistant, 2 Bln1- sensitive), thereby strongly supporting the possibility of a duplication of both D4F104S1 and D4Z4 loci in this region.

We can speculate that an unusually duplicated D4F104S1 locus might be inserted adjacent to the single D4Z4 repeat previously reported to lie proximal and in reverse orientation compared with the other D4Z4 repeats. Whether this single repeat normally has any mechanistic influence or relevance to FSHD is unknown.

A putative duplication of D4F104S1 and/or D4Z4 at 4q35 may not necessarily be pathogenic, but could certainly complicate diagnostic testing where this occurs.

Non-radioactive Detection of the Translocation Frequency Between 4q and 10q in the South African FSHD population

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The D4Z4 locus displays high homology to several other regions in the human genome, but most significantly to chromosome 10q26 (Deidda et al., 1995). This similarity has been proposed in the past to contribute to and serve as a model for explaining the occurrence of translocation events between these two regions. Reported translocation frequencies ranging from 20% (Van Overveld et al., 2000) to 32% (Matsumura et al., 2002) evidently illustrate the high frequency of this DNA rearrangement. The objectives of this study were to optimise the Bgl II – Bln I dosage test via a non-radioactive protocol and to verify the presence of translocation events between the 4q and 10q arrays of affected and unaffected individuals selected from South African FSHD families.

Results obtained from this study suggest that 4q – 10q plasticity is indeed observed in the South African FSHD population. However, a lower translocation frequency of 8.7% was obtained when compared to previously reported population studies. Since a relatively small cohort was investigated in this pilot study, additional analyses with a significantly larger sample size are in progress.

To date there have been no reports of non-radioactive detection in FSHD. Non-radioactive protocols eliminate the complications associated with radioactive based detection strategies. Unlocking the potential of molecular technologies such as this holds great promise for improvement of FSHD diagnostic protocols in the future.

Testing the Position-Effect Variegation Hypothesis for Facioscapulohumeral Muscular Dystrophy By Analysis of Histone Modification and Gene Expression in Subtelomeric 4q

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Key to understanding the molecular genetics of facioscapulohumeral muscular dystrophy (FSHD) is elucidation of how a short, FSHD-causing array of tandem 3.3-kb repeats (1-10 copies) at 4q35 communicates with the gene(s) that are its immediate downstream target. Much circumstantial evidence indicates that this direct or indirect cis interaction between a transcription regulatory region of the FSHD gene(s) and a short D4Z4 array at 4q35 establishes the cascade of events leading to the disease phenotype in the nuclei of certain skeletal muscle fibers. The most popular model for how the 4q35 D4Z4 array-shortening causes FSHD is that it results in a loss of postulated D4Z4 heterochromatinization, which spreads proximally, leading to over-expression of FSHD genes in cis. This would be similar to a loss of position-effect variegation (PEV) in Drosophila. In considering this hypothesis, it should be noted that three essential starting points of the model are unknown, namely, whether the D4Z4 array at 4q35 in unaffected individuals is highly condensed, whether this condensation spreads, and whether FSHD patients have too little of this condensation. Another model for FSHD invokes the loss of sequestration of 4q35 sequences in an inactive chromatin territory within the interphase nucleus when the 4q35 allelic region contains only a short D4Z4 array instead of a normal large array (11-100 D4Z4 repeats). This might lead to a decrease of wide-spread, regional gene silencing at 4q35 allelic regions in FSHD nuclei and could also be accompanied by the loss of heterochromatinization. To test for the putative heterochromatinization in the vicinity of the D4Z4 arrays, we quantitated chromatin immunoprecipitation with an antibody for acetylated histone H4 that discriminates between constitutive heterochromatin and unexpressed euchromatin. Contrary to the above models, H4 acetylation levels of the p13E-11 region adjacent to the 4q35 and 10q26 D4Z4 arrays in normal and FSHD lymphoid cells were like those in unexpressed euchromatin and not constitutive heterochromatin. In addition, results of analysis of histone H4 acetylation at 4q35 in human chromosome 4-containing somatic cell hybrids were not in accord with the PEV hypothesis or other models of regional chromatin condensation and gene silencing. Also, control and FSHD lymphoblastoid cells and peripheral blood mononuclear cells displayed similar H4 hyperacetylation (like that of expressed genes) at the 5' regions of 4q35 candidate genes FRG1 and ANT1. Lastly, contrary to the loss-of-PEV model or other models involving regional inactivation of gene expression at 4q35, there was no position-dependent increase in transcript levels from these genes in FSHD skeletal muscle samples compared with controls. Our results favor a new model for the molecular genetic etiology of FSHD, such as, differential long-distance cis looping that depends upon the presence of a 4q35 D4Z4 array with less than a threshold number of copies of the 3.3-kb repeat. Supported in part by FSH Society Grant FSHS-MB-06 and NIH Grant R21 AR48315.

D4Z4 hypomethylation in FSHD causes the transcriptional upregulation of 4qter genes

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The autosomal dominant myopathy facioscapulohumeral muscular dystrophy (FSHD) progressively and variably affects the muscles of the face, shoulder and upper arm. FSHD is caused by contraction of the polymorphic low copy D4Z4 repeat array on 4qter. Several observations suggest an epigenetic etiology in FSHD. First, the subtelomere of chromosome 10q harbors a highly homologous polymorphic repeat and size reductions of this repeat are non-pathogenic. Second, (partial) exchanges between the homologous repeat arrays on 4q and 10q are frequently observed but these exchanged repeats are only pathogenic when the contracted form resides on chromosome 4. Finally, contractions of the D4Z4 repeat array on 4qter per se are not sufficient to cause disease since pathogenic arrays are only associated with one of two allelic variants (4qA) of 4qter located distal to D4Z4. To obtain evidence for an epigenetic mechanism underlying FSHD pathogenesis, we studied the DNA methylation of D4Z4. We demonstrate that contraction of D4Z4 in FSHD patients causes hypomethylation of D4Z4. Providing biological significance for this finding, we show that in cultured cells, forced hypomethylation of D4Z4 results in transcriptional upregulation of 4qter genes at distance of D4Z4, similar to the inappropriate gene expression observed in FSHD muscle. Finally, also FSHD pheno-copies, patients clinically identical to FSHD but without contraction of D4Z4, turn out to be hypomethylated at D4Z4. Together, these results strongly suggest that the cascade of epigenetic events causing FSHD involves hypomethylation of D4Z4, which in turn results in transcriptional upregulation of 4qter genes.

Nuclear localization of the FSHD gene region

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Facioscapulohumeral muscular dystrophy (FSHD) remains one of the few neuromuscular disorders for which the causative pathophysiologic pathway is unclear. RNA profiling of FSHD muscle and cell culture assays of FSHD myoblasts indicate a defect in the process of myogenesis. However, upon microarray expression analysis, none of the unique genes within the FSHD gene region were found to exhibit an altered pattern of expression, nor was there a gradient of expression extending proximally from D4Z4. Our data does not, therefore, support a position effect on 4q35 gene expression in FSHD. In consideration of other models for this disease, we have examined the nuclear positioning of the FSHD region (distal chromosome 4q) within the interphase nucleus. We have developed a program, Telomere Profiler, to capture, measure, quantitate and perform statistical analysis on the relative positions of 1q, 4q, 5q, 10q and 18q telomere probes within FSHD and control myoblast nuclei. The relative position of several telomeres within myoblast nuclei demonstrates that 4q is most closely associated with the nuclear periphery. Use of an "all telomere" TTAGGG probe confirms that 4q is relatively unique in localizing to the nuclear periphery, as most telomeres are found distributed throughout the nucleoplasm in myoblasts. The peripheral localization of 4qtel is dramatically disrupted in a lamin A/C deficient fibroblast cell line, while remaining intact in normal fibroblasts. Consideration of FSHD as a nuclear envelope disease warrants further investigation. Rather than a primary defect in nuclear envelope proteins, FSHD may arise through improper anchoring of chromatin (D4Z4) to the nuclear envelope. Such a model would account for the generalized disruption in myogenic gene expression patterns in FSHD, as is the thought to be the case for several other neuromuscular disorders (EDMD, LGMD1B, DCM, CMT2) involving nuclear envelope proteins.

Nuclear Envelope Proteins with Potential Involvement in Muscular Dystrophies

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In recent years 13 human diseases, eight of them dystrophies, have been related to proteins of the nuclear envelope (NE). These are generally referred to as the laminopathies (Burke and Stewart, 2001). Alterations in NE proteins could potentially yield phenotypes associated with dystrophies by influencing gene expression, differentiation, apoptosis, or nucleo-cytoskeletal contacts. To identify the full complement of NE proteins, we engaged in a multidimensional subtractive proteomic analysis which identified 67 novel transmembrane proteins. The eight proteins of this set that were cloned and expressed targeted to the NE, confirming the validity of the approach. A substantially higher than random percentage of the genes encoding these proteins map within genomic regions that have been partially mapped for various dystrophies. We are confident that some of the new NE proteins will indeed prove to underlie these dystrophies, as several map to variants of dystrophies (e.g. limb-girdle muscular dystrophy, Charcot-Marie-Tooth disease) already associated with other NE proteins.
Another of the proteins, NET19, maps to 4q35.2 roughly 360 kbp upstream of the D4Z4 deletion associated with Facioscapulohumeral muscular dystrophy (FSHD). Roughly 270 kbp upstream of FRG1 and 4.5 Mbp downstream of ANT1 (Gabellini et al., 2002), NET19 is clearly within the region of genes over-expressed in FSHD associated with D4Z4 deletions. While there are many genes whose expression is affected in this region, we are examining whether NET19 contributes to the FSHD phenotype because the atrial tachycardia (Shen and Madsen, 1991) and limb girdle muscles (Munsat 1994) sometimes affected are also characteristic of several laminopathies.

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Preimplantation genetic diagnosis (PGD) for Facioscapulohumeral Muscular Dystrophy (FSHD) – a reproductive option using genetic linkage analysis.

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Objectives: FSHD is the third most common hereditary muscle disease with over ninety-five percent of patients affected with FSHD carrying a chromosomal rearrangement within the subtelomeric 3.3-kb repeat elements (D4Z4) at 4q35. Diagnostic testing for FSHD involves multiple restriction enzyme digests and southern blotting -methods not suitable for single cell analysis. We designed a PGD test using familial STR linkage, providing an alternative reproductive option for some couples.

Materials/Methods: PGD, included day 3 embryo (single blastomere) or day 5 blastocyst (3-5 cells) biopsy, a multiplexed direct PCR amplification, and day-5 transfer. Couple 1 had a documented family history of FSHD, whilst couple 2 presented as a de novo mutation with material from a prior positive prenatal test available for analysis. New information in the genome database identified 3 STR markers in the FSHD region.

Results: In the first couple, analysis revealed four normal and two affected embryos. One embryo was transferred but no implantation occurred, subsequent transfer of two cryo-stored embryos resulted in a singleton pregnancy. For the second couple, PGD in the first cycle yielded two affected embryos and one normal embryo, transfer of which did not result in a pregnancy. A second stimulated cycle produced seven affected and two non-affected embryos, both of which were transferred. A singleton pregnancy resulted in the birth of an unaffected male.

Conclusion: PGD using STR linkage analysis offered two couples an alternative to prenatal testing for establishing a pregnancy and successfully avoiding a disease gene. This approach enabled analysis for the presence of a mutation unable to be tested for directly in single cells.