Table 1 FISH data

Probe	STS markers covered		Result	Cytogenetic position
YAC878C12	D11S1762/D11S1339	– D11S1167	+	q22.2
YAC876G04	D11S817	– Y734D08L	−	q22.3
YAC801E11	D11S384	– D11S1897	−	q22.3
YAC755B11	D11S1960	– Y296F0FR	−	q23.1
BAC442e11	D11S1340	– D11S4516	−	q23.2
Cos4746	MLL		+	q23.3
Cos4748	MLL		+	q23.3
YAC742F09	D11S461/D11S939	– HLR2	+	q23.3
Cos2072c1	(Subtelomere)		+	q25

(+) Hybridisation to both chromosomes 11. (−) Hybridisation only to normal chromosome 11.

second model, a single strand nick in the tip of the hairpin could result in a double strand break and then lead to illegitimate recombination. The second model could be consistent with the formation of the t(11;22).[13]

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Microdeletion in the *FMR-1* gene: an apparent null allele using routine clinical PCR amplification

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EDITOR—Fragile X syndrome is the most common chromosomal cause of inherited mental retardation. At the chromosome level, this syndrome is characterised by the presence of a fragile site at Xq27.3.[1] The incidence of this disorder is approximately 1 in 4000 and 1 in 7000 in males and females, respectively.[2 3] In most cases, the mutation responsible for fragile X syndrome is a CGG repeat expansion in the 5' untranslated region (UTR) of exon 1 of the *FMR-1* gene. People in the normal population have six to approximately 50 repeats.[4 5] Those with 50 to 200 repeats correspond to the premutation class. Repeats in this class are meiotically unstable and can expand to a full mutation.[4] The premutation class encompasses the "grey area" of 45-55 CGG repeats for which there is a variable risk of repeat expansion.[6] Subjects with a full mutation have repeat lengths in excess of 200, which are associated with hypermethylation of the CpG island immediately upstream of the *FMR-1* gene.[7–9] This methylation correlates with transcriptional suppression of the *FMR-1* gene, while the repeat expansion has been suggested to cause translational suppression by impeding the migration of the 40S ribosomal subunit along the 5' UTR of the *FMR-1* gene transcript.[9–11]

Fragile X syndrome has also been found to occur in a few patients without CGG repeat expansions. These mutation events fall into two classes, intragenic point mutations[12 13] and deletion events.[14–22] Of the latter class, five patients with microdeletions in the 5' UTR of the *FMR-1* gene transcript have been described.[23 24]

We report here a patient referred for fragile X testing who was found to carry an apparent null allele by PCR amplification of the CGG repeat region of the *FMR-1* gene. This patient was analysed further using a combination of primers flanking the CGG repeat region, together with FMRP studies, in order to characterise the nature of the molecular defect underlying this apparent null allele.

Case report

The proband was born to healthy, non-consanguineous parents at 40 weeks of gestation. There was no significant family history. He weighed 4500 g (>90th centile), head circumference was 37.5 cm (>90th centile), and length was 57.5 cm (>90th centile). There

was aspiration of meconium at delivery necessitating assessment in the neonatal unit. He appeared well initially but on the following day was noted to be irritable and hypotonic with an abnormal Moro reflex. A cranial ultrasound scan was normal. He required an inguinal hernia repair at a few weeks of age. His early development was felt to be normal. He had gastro-oesophageal reflux diagnosed at 8 months and was treated with ranitidine. He had mild plagiocephaly and a torticollis that required surgical correction at 18 months. He had persistent problems with drooling of saliva and tends to have an open mouthed expression. In the second year of life he had problems with recurrent ear infections requiring insertion of grommets and adenoidectomy. An assessment at the age of 3 years showed his speech and language development to be significantly delayed. His parents felt his comprehension was limited and he had difficulty retaining information. The delay had been noted earlier but had been attributed to his recurrent ear infections. Full assessment at that time showed that he had developmental delay in all areas. He had some behavioural problems with trichotillomania and obsessive traits. He did not play well with other children.

On examination by a clinical geneticist, the proband was found not have any phenotypic features suggestive of fragile X syndrome, although he did have early features of joint laxity. His head circumference was on the 50th-90th centile, his height on the 75th centile, and weight on the 50th centile. He had mild clinodactyly and fetal pads. He had mild facial asymmetry and a deep crease between his first and second toes. Examination was otherwise unremarkable. The case was referred to the laboratory for fragile X screening.

Materials and methods

CYTOGENETIC AND DNA ANALYSIS

Cytogenetic analysis of a folate deprived culture of lymphocytes was performed as previously described.[25] An estimation of the length of the CGG repeats, together with an analysis of the methylation status of the CpG island of the *FMR-1* gene, were performed by PCR amplification and Southern blot analysis, respectively. In the case of the latter, 5 μg of genomic DNA was digested with *Eco*RI and *Nru*I, electrophoretically separated, blotted onto a positively charged nylon membrane, and hybridised with approximately 10-20 ng of probe StB12.3, as described previously.[26] The hybridisation solution contained herring sperm DNA at 75 μg/ml to prevent non-specific binding of the probe. The blots were washed finally in 0.2 × SSC plus 0.1% SDS at 60°C. DNA controls included a normal male, a male with a full mutation (expanded CGG repeat with hypermethylation of the CpG island), a female with a premutation, and a normal female control. A radioactively labelled 1 kb ladder was included for sizing purposes.

PCR amplification of the CGG repeat region of the *FMR-1* gene using primers FMRA and FMRB was carried out in 15 μl reactions. Each reaction comprised 10% DMSO, 50% w/v glycerol, 60 pmol of each primer, 0.4 U of *Taq* DNA polymerase, 1 × PCR buffer with 0.32 mmol/l of dCTP, dATP, dTTP, and 1.5 mmol/l deaza GTP, 0.25 μl of 10 μCi μl α^{32}P dCTP, and 0.6 mg/ml genomic DNA. Non-radioactive PCR amplification using primers FMR1 and FMR2 was carried out using the GC rich kit of Roche Diagnostics Ltd according to the manufacturer's instructions. The sequences of the primers used in the amplification reactions were FMRA (5'-GACGGAGGCGCCCGTGCCAGG-3'), FMRB (5'-TCCTCCATCTTCTCTTCAGCCCT-3'), FMR1 (5'-ATAACCGGATGCATTTGAT-3'), and FMR2 (5'-AGGCCCTAGCGCCTATCGAAATGAGAGA-3'). Primers FMR1, FMRA, FMRB, and FMR2 were designed using the *FMR-1* gene sequence deposited in GenBank (Accession number X61378), with their 5' ends at base positions 2271, 2684, 2844, and 3106, respectively. The PCR cycling conditions comprised 95°C for two minutes followed by 30 cycles of 97°C for 30 seconds, 55°C for one minute, and 72°C for one minute. The reactions were held at 4°C following a final extension of 72°C for 10 minutes. Amplification products were electrophoresed in a 1% agarose gel, together with a 100 bp DNA ladder. In the case of radioactive amplification, the products were electrophoresed in a denaturing sequencing gel using a radioactively labelled M13 sequencing ladder for sizing purposes.

Amplification products were purified for sequencing using a PCR purification kit (Roche Diagnostics). Each amplicon was sequenced using the forward and reverse amplifying primers and an Applied Biosystems (ABI) sequencing kit. DNA was recovered by ethanol precipitation and subsequently washed in 70% ethanol before the addition of denaturation buffer and loading in an ABI PRISM™ 377 DNA sequencer. The electropherograms were subsequently assembled using SeqMan DNA software.

PROTEIN ANALYSIS

An EBV transformed B lymphoblastoid cell line was established from a peripheral blood sample of the proband. FMRP and eIF4e levels were determined in whole cell lysates in a slot-blot based assay, using purified flag tagged murine Fmrp[27] and purified human eIF4e[28] as standards. Sample proteins and standards were applied to nitrocellulose membranes with a Bio-Rad slot blot apparatus. Using standard protocols, FMRP and eIF4e were detected with mouse monoclonal primary antibodies mAb 1C3 for FMRP, kindly provided by Jean-Louis Mandel,[29] and anti-eIF4e (Transduction Laboratories) and HRP conjugated goat anti-mouse secondary antibody (Kirkegaard and Perry Laboratories). Signals were generated by Enhanced Chemi Luminescence (Amersham) and detected by exposure to Hyperfilm (Amersham). Signal intensities were quantified by analysis of digital scans using the program NIH Image 1.62b7f to plot signal profiles. Areas under the plot profile were calculated and used as signal intensities after subtracting out signals

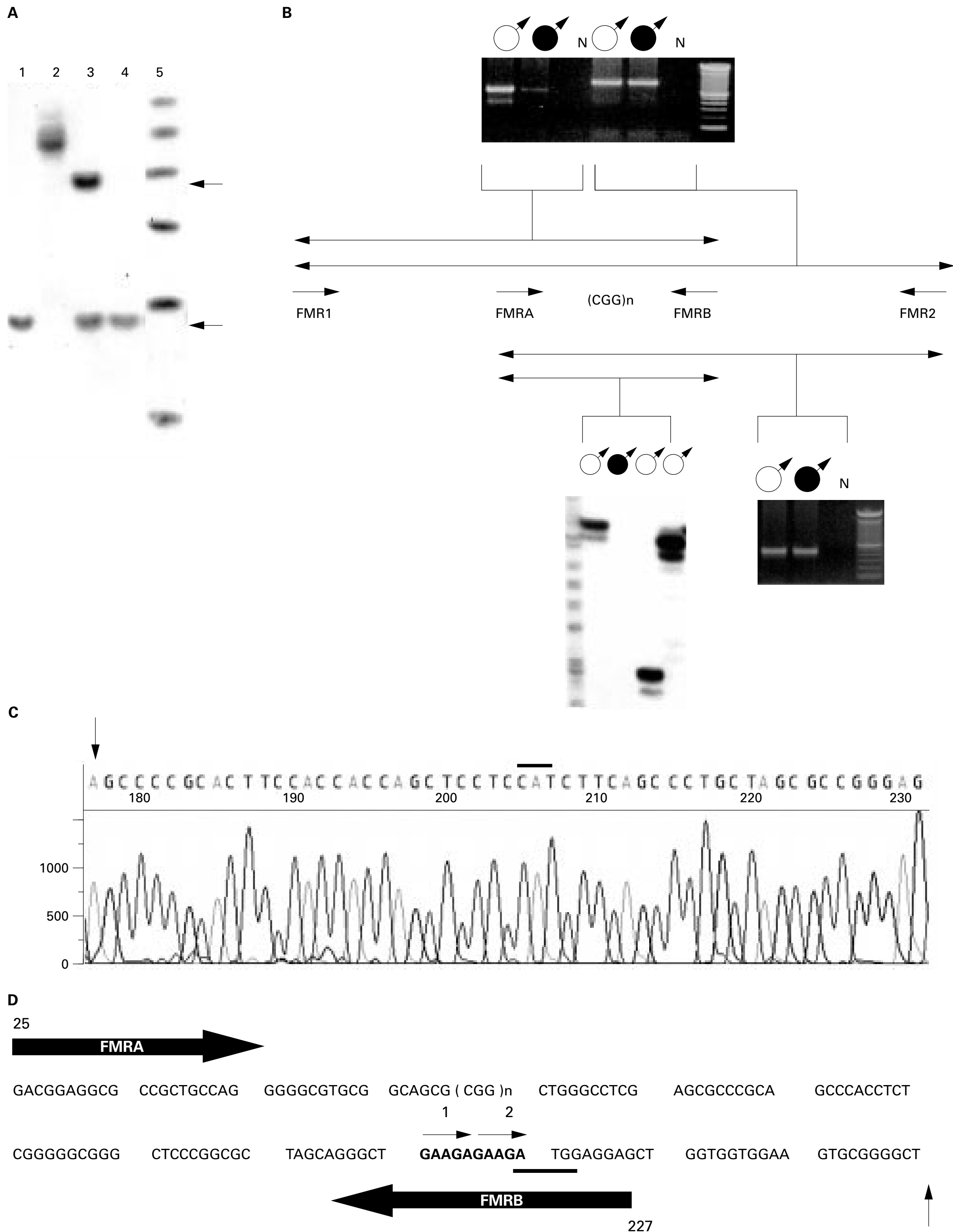

Figure 1 DNA analysis of the CGG repeat region of the FMR-1 gene. (A) EcoRI plus NruI digested genomic DNA from a normal male (lane 1), a male with a full mutation (lane 2), a normal female (lane 3), and the proband (lane 4) was probed with StB12.3. The 1 kb ladder is shown in lane 5, with the lengths of the unmethylated and methylated alleles in a normal subject indicated on the right hand side of the panel. (B) PCR amplification products encompassing the CGG repeat region of the FMR-1 gene are shown. The proband and normal males are represented by the filled and open symbols, respectively, while negative PCR controls are indicated by the letter N. The radioactively labelled products corresponding to PCR amplification using primers FMRA and FMRB were electrophoresed in a denaturing sequencing gel with a labelled M13 sequencing ladder, while the other amplification products were separated in 1% agarose gels with 100 bp ladders. (C) Electropherogram of the sequence of the proband's FMR-1 gene encompassing the ATG initiation codon (indicated by a horizontal bar). The sequence is shown in the 3′ to 5′ direction. The vertical arrow indicates an arbitrary start site for the sequence presented in (D). (D) Partial sequence of the FMR-1 gene (GenBank accession number X61378) indicating the GAAGA direct repeats (in bold type and numbered horizontal arrows) and the ATG initiation codon (underlined). The location of the FMRA and FMRB primers are shown as horizontal arrows, together with their position with respect to the transcription start site. The nucleotide sequence derived from the electropherogram is shown starting at an arbitrary site, indicated by an arrow.

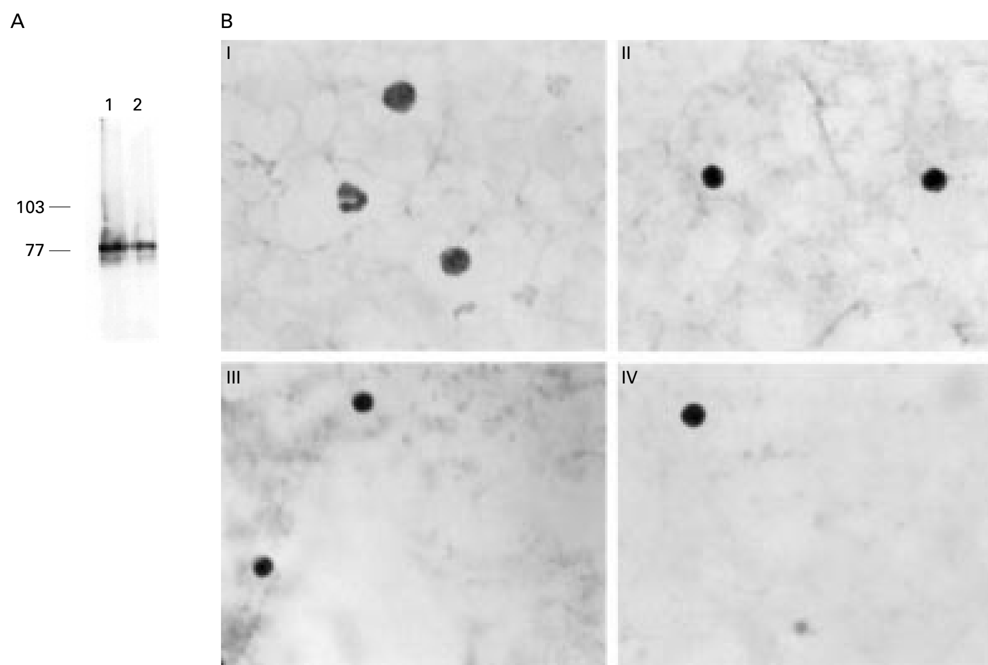

Figure 2 FMRP analysis. (A) Western blot analyses of FMRP expressed by the proband and a normal male are shown in lanes 1 and 2, respectively. Molecular weight standards (expressed in kDa) are indicated on the left hand side of the panel. (B) Immunohistochemical staining of FMRP in lymphocytes of the proband (I), the proband's carrier mother (II), a negative control (III), and a positive control (IV). FMRP staining is seen in the cytoplasm, with the nuclei stained with Nuclear Fast Red.

from the background and from the secondary antibody controls as appropriate. Standard curves were generated using data from the purified proteins, which then allowed the quantitation of protein levels in the samples. Quantitation data was calculated as the molar ratio of FMRP:eIF4e. Purified FMRP was obtained from Keith Wilkinson and eIF4e was from Curt Hagedorn, both of Emory University.

In the case of western blot studies, total proteins were isolated from EBV transformed B lymphoblasts of the proband, as well as from a normal male control. The proteins were electrophoresed in a 7.5% non-denaturing polyacrylamide gel, and transferred to nitrocellulose and hybridised using mAb 1C3 as described above.

In the case of immunohistochemical staining of FMRP from blood smears, a modification of the method of Willemsen *et al*[30] was used. Blood smears were counterstained with Nuclear Fast Red and 100 lymphocytes were examined for each person, together with positive and negative control blood samples. Less than 42% of lymphocytes are FMRP positive in affected males, whereas for carrier females this figure is 83%; the specificity of this assay is 100% for males and 41% for females.[31]

Results

Cytogenetic analysis of the proband's chromosomes indicated an apparently normal 46,XY karyotype. Southern blot analysis showed a positively hybridising 2.8 kb DNA fragment, suggesting a normal sized CGG repeat length in the *FMR-1* gene (fig 1A). PCR amplification of this locus using previously published primers FMRA and FMRB[32] yielded no product from the proband's genomic DNA. However, amplification products were obtained using primers FMRA and FMR2 (643 bp) and FMR1 and FMR2 (1 kb, fig 1B). The latter product was sequenced and showed a deletion of a 5 bp direct repeat, GAAGA, either immediately upstream, or encompassing the first base, of the ATG initiation codon of the *FMR-1* gene (fig 1C, D). The mother of the proband was found to be heterozygous for this deletion event (data not shown). The deletion leaves the ATG codon unchanged and in phase with the remaining open reading frame of the *FMR-1* gene.

FMRP quantitation, western blot analysis, and immunohistochemical studies were undertaken using the patient's lymphoblasts to determine the effect of the deletion event on translation initiation (fig 2). In order to assess the level of FMRP in the patient's lymphoblasts, quantitation studies were undertaken using the protein eIF4e as an internal control. The latter protein is the cap binding protein in eukaryotic translation initiation[33] and is the rate limiting factor in translation initiation.[34–36] FMRP levels were normalised with respect to eIF4e levels as a loading control. In seven cell lines from males with normal CGG allele lengths, the mean molar ratio of FMRP:eIF4e is 0.218 (standard deviation of 0.009). In the case of the cells from the proband, the molar ratio was 0.214, and thus the level of FMRP is not reduced compared to normal cell lines. In the case of the western blot analysis, normal sized FMRP was detected (fig 2A). Immunohistochemical staining of lymphocytes from the proband and his carrier mother showed FMRP staining in 80% and 98% of 100 lymphocytes examined, respectively (fig 2B).

Discussion

The proband reported here carries an apparent null allele with respect to the primer pair FMRA and FMRB, which are used routinely for amplifying the CGG repeat tract of the *FMR-1* gene. This case suggests that caution should be exercised regarding predictive testing for fragile X syndrome that relies solely on PCR amplification of the *FMR-1* gene using one primer pair only. This reliance has been suggested as a first level predictive screen for fragile X syndrome in the general population.[37] The need for caution with respect to single PCR amplifications of trinucleotide repeats has also been described with regard to predictive testing for the Huntington's disease (HD) gene.[38] Our data underline the need for complementing PCR analysis with Southern blotting or, at minimum, PCR amplification of the CGG repeat region with two primer pairs.

Direct sequencing of amplification products using primers that map further upstream and downstream of FMRA and FMRB identified a 5 bp microdeletion near, or encompassing, the initiation codon of the *FMR-1* gene. It appears that this deletion affects the annealing of the FMRB primer leading to inefficient amplification using this primer. The proband represents one of only a few cases that have been reported to have microdeletions in the *FMR-1* gene.[23 24] In these other cases, which were found in subjects with fragile X syndrome, the microdeletions ranged from 116 bp to 567 bp and were located in the 5' UTR of the *FMR-1* gene. The deletions were expected to lead to a lack of the *FMR-1* gene product, which was confirmed in some patients.[23] A mispairing model for the generation of a 486 bp deletion was described by Schmucker *et al*,[24] which involved chi-like elements flanked by direct tandem repeats. In the case reported here, end joining, strand slippage, or indeed homologous recombination are possible molecular mechanisms that could account for the 5 bp deletion event.

Changes in the sequence of DNA upstream of an initiation codon can dramatically influence translation efficiency.[39] Fragile X males with a full mutation have complete absence of FMRP. However, in the case described here, FMRP was detected of apparently normal size and at normal levels in the lymphocytes of the proband.

This study leads to the suggestion that the proband does not have fragile X syndrome and that the 5 bp deletion in this patient's *FMR-1* gene is not causative of his phenotype. The FMRP detected in this patient appears to be qualitatively and quantitatively normal. Therefore, the comprehensive screening of genes implicated in disorders that are similar to fragile X syndrome may help resolve the cause of this patient's phenotype.

We acknowledge Dr Hugh Lees of Waikato Tauranga for bringing this case to our attention and the technical assistance of Jane Iber. We further acknowledge the financial assistance of Laboratory Services of Auckland Hospital for running expenses, and the University of Auckland Research Committee and the Lottery Grants Board of New Zealand for funding an Applied Biosystems Model 377 DNA Sequencer.

- We report here a case that was referred for testing for fragile X syndrome. The patient was found to carry an apparent null allele by routine clinical PCR, but with CGG repeats that fall within the normal range.
- DNA sequencing showed that the patient carried a microdeletion of a 5 bp direct repeat immediately upstream, or encompassing, the translation initiation codon of the *FMR-1* gene.
- Protein studies indicated that the patient expressed the protein product of the *FMR-1* gene (FMRP), and that this expression was at near normal levels in the patient's lymphoblasts.

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Non-invasive evaluation of arterial involvement in patients affected with Fabry disease

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Editor—Fabry disease (FD) (OMIM 301500) is an X linked recessive disease resulting from deficiency of the lysosomal hydrolase α-galactosidase A.[1] The enzymatic defect leads to the widespread deposition of uncleaved neutral glycosphingolipids in the plasma and lysosomes, especially in vascular endothelial and smooth muscle cells. Initial clinical signs include skin lesions (angiokeratoma), excruciating acral pain, and benign corneal opacities. Progressive glycosphingolipid deposition in the microvasculature of hemizygous males subsequently leads to failure of target organs and to ischaemic complications involving the kidneys, heart, and brain.[2,3] Much interest is currently shown in emerging therapies for FD and recent studies have reported that genetic engineering has removed many of the obstacles to the clinical use of enzyme replacement and that infusions of purified α-galactosidase A are safe and biochemically active.[4,5] However, clinical and laboratory indicators of benefit are lacking, given the slow course of the disease. This emphasises the need for non-invasive surrogate endpoints to delineate target organ damage and to monitor the efficacy of enzyme replacement therapies.

Methods and results

In the present study, we determined intima-media thickness (IMT) at the site of the radial artery, a distal, muscular, medium sized artery, in a cohort of 21 hemizygous male FD patients, with a mean age of 32 years (SD 13, range 13-56 years), compared with 21 age and sex matched normal controls. All patients were diagnosed with FD by the presence of both clinical signs and a markedly decreased α-galactosidase A activity in leucocytes (<4 nmol/h/mg protein, normal values 25-55 nmol/h/mg protein). No patient had end stage renal disease. Measurements of the radial artery parameters were obtained with a high precision echotracking device (NIUS 02, SMH, Bienne, Switzerland) as previously described.[6,7] Briefly, the radiofrequency signal was visualised and the peaks corresponding to the blood-intima and media-adventitia interface were electronically tagged and followed over several cardiac cycles. Internal diameter and wall thickness were then measured with a precision of about 10 μm. Four to six measurements were averaged.[6,7] Radial artery IMT was measured 2 cm upstream from the wrist.

Compared to controls, FD patients had considerably higher IMT values at the site of the radial artery (fig 1). IMT was twice as high in