

Sayeda Abu-Amero^{a, b}
Susan Price^c
Emma Wakeling^{a, b}
Philip Stanier^a
Richard Trembath^c
Michael A. Preece^{a, b}
Gudrun E. Moore^a

^a Queen Charlotte's and Chelsea Hospital,
London,

^b Institute of Child Health,
University of London,

^c Department of Clinical Genetics,
Leicester Royal Infirmary, Leicester, UK

Lack of Hemizyosity for the Insulin-Like Growth Factor I Receptor Gene in a Quantitative Study of 33 Silver Russell Syndrome Probands and Their Families

Key Words

Silver-Russell syndrome
Insulin-like growth factor I receptor
Hemizyosity
Gene dosage
Polymorphic markers
Single-stranded conformational
polymorphism

Abstract

Previous studies have shown that individuals with a deletion of 15q26.1→qter, which includes the insulin-like growth factor I receptor (*IGFIR*) gene, may exhibit phenotypic characteristics similar to those individuals with Silver-Russell Syndrome (SRS). Thirty-three SRS probands, with normal karyotypes, and their parents were investigated for the presence of both copies of *IGFIR* by gene dosage analysis of Southern blot hybridisation. All 33 SRS probands have both copies of *IGFIR*. Tetranucleotide repeat marker analysis for three locations on 15q also ruled out other deletions in these regions for those markers that were informative. Two important functional regions of *IGFIR* were also investigated for DNA mutations, using single-stranded conformational polymorphism analysis. No mutations were found in the cysteine-rich region involved in ligand binding (exon 3) or the ATP binding region (exon 16) which could contribute to the SRS phenotype. However, a silent mutation in the third position of one of the codons in the ATP region (3174 G→A, 1013 Glu→Glu) was found.

Introduction

Silver-Russell syndrome (SRS) is a clinical disorder where intrauterine growth retardation (IUGR) and poor postnatal growth lead to short adult stature [1–3]. In addition there is often a characteristic triangular facies, skeletal asymmetry and digital anomalies, including clinodactyly. Recently, evidence of a cognitive deficit has also been associated with SRS [4].

Although detailed clinical reports for SRS are available, there is still little known about its aetiology. No con-

sistent Mendelian or chromosomal basis of inheritance has been established for SRS and most cases are sporadic [5]. The most commonly reported aetiological finding involves either deletion for distal 15q or ring chromosome 15, with 18 documented cases [6–11]. Patients with ring chromosome 15 have IUGR, microcephaly, triangular face, hypertelorism, variable mental retardation and speech delay [8, 12]. Of primary interest for SRS is the insulin-like growth factor I receptor (*IGFIR*) gene, localised to 15q25–26. The r(15) patients exhibit hemizyosity for 15q distal markers at 15q26.3, 15q26.2 and/or

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1018–4813/97/0054–0235\$12.00/0

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Sayeda Abu-Amero
Queen Charlotte's and Chelsea Hospital, Goldhawk Road
London, W6 0XG (UK)
Tel. 0181-740 3533, fax 0181-383 1838
E-mail s.abu-amero@rpms.ac.uk

15q26.1 and hemizyosity for *IGFIR* [12, 13]. These findings are consistent with an SRS patient with a terminal deletion of 15q26.1 → qter, with hemizyosity for *IGFIR* [8]. However, 5 SRS patients who were diploid for distal 15q markers have also been reported suggesting that the loss of one copy of *IGFIR* may not contribute to the clinical manifestations in all cases of SRS [11].

In a cohort of 37 patients, there were 3 documented cases of SRS associated with maternal uniparental disomy (UPD) of chromosome 7 (mUPD 7) [14]. MUPD 7 has also been associated in 4 out of 35 patients with SRS or primordial dwarfism from several centres in Europe [15]. For the remaining SRS patients with copies of chromosome 7 from both parents it still remains possible that chromosome 7 may be implicated. Smaller deletions in imprinted regions may be involved, as has been found for many of the cases in Prader-Willi and Angelman syndrome, where mUPD 15 and pUPD 15 play only a small part (25 and 2% respectively) [16–18]. There are an additional 6 SRS individuals with other documented structural chromosomal abnormalities [19–24]. However, this still leaves a large majority of the patients with unexplained aetiology.

The insulin-like growth factor family is composed of insulin, IGFI and IGFII (insulin-like growth factors I and II respectively), their corresponding receptors and at least six binding proteins [25]. The ligands, receptors and binding proteins play a pivotal role in the regulation of growth and development, in both fetal and postnatal life [26, 27].

IGFIR mediates the action for both IGFI and IGFII, but having a much higher affinity for IGFI. *IGFII* and *IGFIIR* are expressed at the two-cell stage while the insulin receptor and *IGFIR* have been detected at the eight-cell stage in preimplantation mouse and human embryos [28, 29]. *IGFIR* is widely expressed after implantation especially in the developing nervous system and muscle [30], but decreases dramatically during postnatal development [31].

It is interesting to note that mice knockout experiments have shown that hemizyosity at *Igf1r* locus does not have any effect on growth in mice, although severe growth retardation (55% reduction in size compared to wild type mice), as well as developmental delay in ossification, CNS abnormalities and hypoplasia is observed for homozygous null mice [32, 33]. Both *Igf1* and *Igf2* have been shown to utilise *Igf1r* in early embryonic development, and the complete absence of the receptor would severely compromise the functioning of these ligands [33, 34].

Normal phenotype in mice hemizygous for *Igf1r* points to species difference in compensatory mechanisms for gene hemizyosity, suggesting that there may be up-regulation of the remaining *Igf1r*, which does not appear to be the case in humans. SRS is not a constant phenotype in deletion of 15qter, pointing to a heterogeneous disorder. Hemizyosity of *IGFIR* may be only a part of IUGR/SRS in deletion of 15qter as the loss of one copy of *IGFIR* was accompanied by loss of flanking chromosomal material, which may include other functional genes that play a role in growth and development. However, based on its pivotal role in embryonic and post-natal growth and differentiation, it is an ideal candidate for any growth retardation phenotype, and hence SRS.

Here we report findings on 33 SRS probands. These are a subset of the 37 patients reported by Preece et al. [14]; 4 were excluded from this report due to lack of DNA/blood. Since the studies were conducted in parallel, the 3 probands subsequently shown to have mUPD 7, were part of this investigation. Hemizyosity for *IGFIR* was investigated by quantitative analysis of Southern hybridisation. Tetranucleotide markers localised to the distal portion of 15q, were used to support Southern hybridisation data. Finally, SSCP analysis was undertaken to screen for any point mutations in two exons known to be critical to *IGFIR* function.

Materials and Methods

Subjects

33 SRS probands and both parents were included in this study. 28 probands fulfilled at least three of the following diagnostic criteria: low birth weight (>2 SDS below mean); short stature at the time of diagnosis (>2 SDS below mean; the range of heights is not given here, as at the time of this investigation, a number of the probands had undergone growth hormone treatment and hence were of almost normal height); characteristic facial features; and facial, trunk or limb asymmetry. The remaining 5 probands had consistent postnatal growth pattern and facial features but slightly higher birth weights (2.58–3.11 kg). Blood was obtained from the families and genomic DNA isolated [35]. Ethical approval was obtained for this study by the Joint Research Ethics Committee of Great Ormond Street Hospital and the Institute of Child Health (approval No. 1278).

The cohort consisted of 16 females and 17 males, ranging in age between 0.83 and 34.3 years at the time of investigation. Classical facial features, as described by Russell [1], were seen in 22, whilst 11 had a milder facial phenotype. Limb asymmetry of ≥ 1.0 cm associated with facial asymmetry were present in 12 individuals, and clinodactyly was seen in 24 individuals. At the time of birth of the probands, mean maternal age was 27.8 (range 17.8–37.8) years and mean paternal age was 30.6 (range 18.8–44.8) years.

Cytogenetic Studies

In probands karyotype was normal (performed at the North East Thames Regional Cytogenetic Centre).

Southern Blot Hybridisation

4 µg of total genomic DNA were digested with 24 u of *Hind*III (GIBCO BRL) for 6 h at 37°C and electrophoresed on 0.8% agarose gels overnight. Southern blotting and hybridisation were carried out by standard methods [36].

Filters were simultaneously probed with a 0.7-kb cDNA *Eco*RI fragment of the human *IGF1R* (IGF-1-R.8; American Type Culture Collection, Rockville, Md.) and *W21G*, a 1.6 kb-*Hind*III fragment located on 22q11.1–q11.2 (HGMP UK DNA Probe Bank).

Quantitative Analysis of Southern Hybridisation

For each filter, incorporation of radioactivity in each band was measured for each individual by volumetric analysis using a PhosphorImager (Model 400; Molecular Dynamics). If the average reading for *IGF1R* and *W21G* of all parents on a filter are represented as IC and WC respectively, and the value of *IGF1R* and *W21G* for each proband represented as IP and WP respectively, then the ratio of *IGF1R* to *W21G* for the proband was calculated using the following formula:

$$IP / [(WP/WC) \times IC]$$

A ratio of 1.0 indicates that for each copy of *IGF1R* there is one copy of *W21G* and hence diploid number of the receptor, whereas a ratio of 0.5 indicates that there is only one *IGF1R* copy for two *W21G* copies and hence hemizyosity of *IGF1R*. For each proband two readings were obtained. Since there were no significant differences in the readings obtained from the first nine families using duplicate filters, and then reprobed filters, all subsequent readings were obtained by stripping filters in boiling 0.1% SDS for 30 min and reprobing as described, and the average of two readings taken.

Tetranucleotide Marker Analysis

Three sets of tetranucleotide primers (Research Genetics, USA) from distal 15q were used as genetic markers for genotyping. The primers were selected for distal location on 15q and high levels of heterozygosity (table 1). Radiolabelled PCR amplification were carried out in 25-µl reaction volume containing: 50 ng DNA plus (final concentration) ammonium buffer [16 mM (NH₄)₂SO₄, 67 mM Tris HCl pH 8.8, 0.1% Tween-20], 2 mM dNTP mix, 1.5 mM MgCl₂, 50 ng each primer, 0.1 µl α³²P dCTP (ICN) and 0.15 u BioTaq polymerase (Bioline). All PCR reactions were carried out in a Hybaid Omnigene thermal cyclor.

Reaction conditions for the three sets of primers are given below:

(94°C, 4 min) × 1: (94°C, 1 min; AT °C, 1 min; 72°C, 1 min) × 25: (72°C, 10 min) × 1, where AT is the annealing temperature for D15S642 (66°C), D15S657 (63°C) and D15S816 (59°C).

6 µl of PCR reaction were denatured and electrophoresed on 6% denaturing polyacrylamide gel (National Diagnostics).

Single-Stranded Conformational Polymorphism Analysis

Radiolabelled PCR reactions for single-stranded conformational polymorphism (SSCP) analysis were carried out as described above. SSCP was used to study two regions. Primer sequences for exon 3 (bp 685–996) (CF and CR) and for exon 16 (bp 3001–3228) (AF and AR), and their reaction conditions are shown below (exon sequences

Table 1. Levels of heterozygosity and localisation of tetranucleotide PCR primers with respect to D15S187 (0 cM) used for genotyping the 33 families

Marker	Heterozygosity	Location cM	Informativity %
D15S816	0.53	97.2	33.3
D15S657	0.72	102.9	24.2
D15S642	0.87	109.9	48.5

D15S657 and D15S816 are immediately proximal to *IGF1R* and D15S642 is distal (CHLS@http://www.chlc.org; 37).

are shown in bold, and intron sequences are shown in normal type) [38, 39]:

CF 5'-CTCTCCACAG**TGTGCCCAAG**-3'
CR 5'-ATACCT**CTGGCTGCCGTTGC**-3'
(94°C, 4 min) × 1: (94°C, 1 min; 65°C, 1 min; 72°C, 1 min) × 30: (72°C, 10 min) × 1
AF 5'-TCTTCTCCAG**TGTACGTTCC**-3'
AR 5'-GGAAC**TTTCTTACCACATG**-3'
(94°C, 4 min) × 1: (94°C, 1 min; 57°C, 1 min; 72°C, 1 min) × 30: (72°C, 10 min) × 1

7 µl of the PCR reactions were denatured and electrophoresed on 6% nondenaturing polyacrylamide gel (easigel, Scotlab), below 10°C for 16–20 h at 280–320 V.

Sequencing

Sequencing was carried out on a model 373 ABI sequencer.

Results

Quantitative Analysis of Southern Hybridisation

Filters containing *Hind*III digests of proband and parent genomic DNA were hybridised simultaneously with *IGF1R* and *W21G* probes, giving two distinct bands of approximately 1.7 and 3.5 kb respectively (fig. 1). The ratio of *IGF1R* to *W21G* for the probands are summarised in figure 2, showing that the ratios of *IGF1R* to *W21G* follows a normal distribution with ratios close to one. The range of average readings obtained was 0.84–1.28, with a mean of 1.04 (SD 0.10). These data indicate no evidence of hemizyosity of *IGF1R* in this group of 33 SRS probands.

Tetranucleotide Marker Analysis

All 33 families were informative for at least one of the tetranucleotide markers, although only one family was informative for all the markers. In all cases where the markers were informative, Mendelian inheritance was

Fig. 1. Southern hybridisation of four families. Total genomic DNA was digested with *Hind*III and electrophoresed on 0.8% agarose gel and blotted. Filters were probed simultaneously with *IGF1R* (1.7 kb, lower band) and *W21G* (3.5 kb, upper band) and exposed to X-ray film overnight. Order: mother, proband and father.

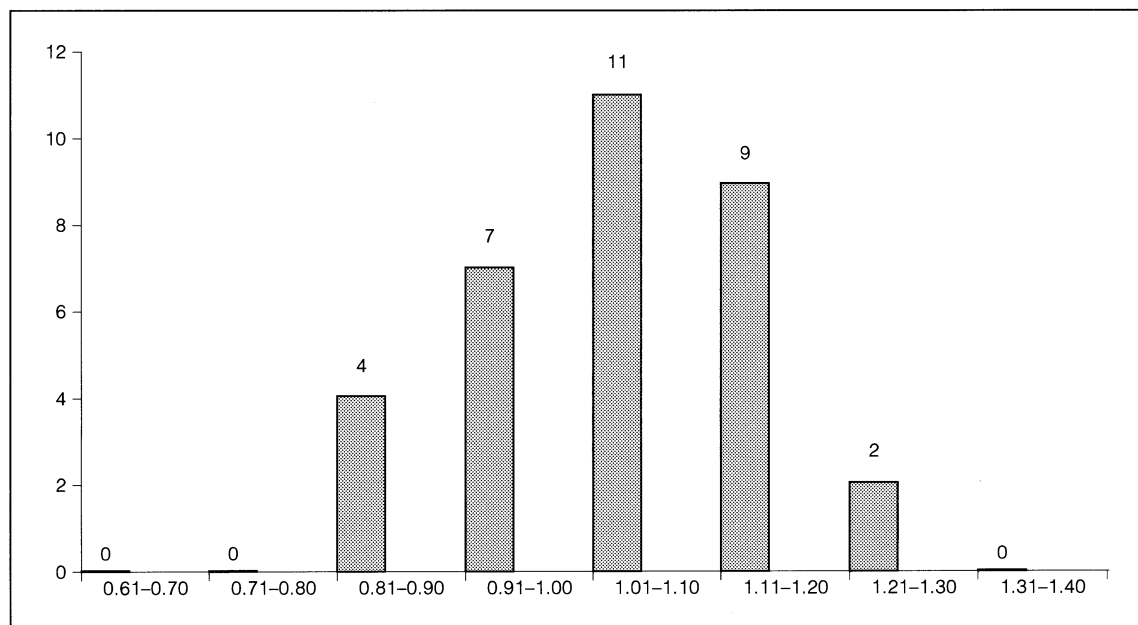
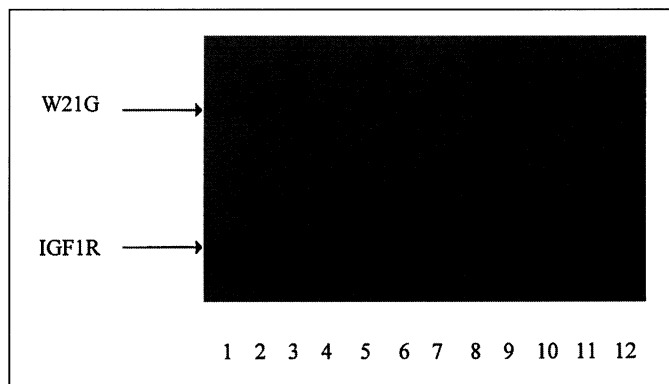


Fig. 2. Histogram representing the *IGF1R* to *W21G* ratio for 33 probands as determined by quantitative analysis of Southern hybridisation. Individuals all have ratios close to one, with a mean of 1.04 and sample standard deviation of 0.103, indicating the presence of two copies of the *IGF1R* gene.

demonstrated for the families, supporting no deletion of the region around *IGF1R* (fig. 3).

SSCP Analysis of the Cysteine-Rich Region

PCR amplification of the cysteine-rich region gave a band of 327 bp which corresponds to the 312-bp exon 3 plus flanking intron nucleotides incorporated into the primers [38, 39].

The three families in figure 4 are representative of all 33 probands' and their parents' banding pattern for this region. The lack of any band shift in the PCR products indicates no mutations in this region [40].

SSCP Analysis of the ATP Region

A band of 255 bp was observed corresponding to the 228-bp exon 16 and flanking intron sequence incorporated into the primer design. 28 of the 33 families had identical banding patterns [38, 39].

Band shifts were observed for SSCP in this region, where individuals in five families had one or two bands (fig. 5). Sequence analysis showed that individuals with single bands were of two types. The first group were homozygous G at position 3174, in agreement with the published sequence [38, 39]. The second group of individuals with a single band had a base transition (G → A) at

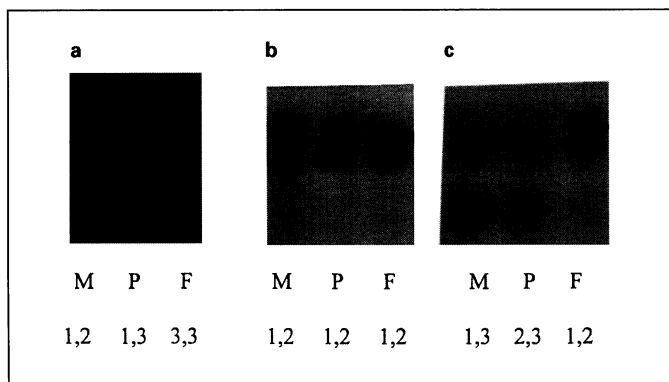


Fig. 3. 6% Denaturing polyacrylamide gel electrophoresis of PCR products obtained for polymorphic PCR primers D15S816 (a), D15S657 (b), and D15S642 (c) located on distal 15q. M = mother, P = proband and F = father.

this position, and were homozygous for this change. Individuals with two bands were heterozygous; the transition was in the third base of a codon, GAG → GAA, where both code for glutamine.

Discussion

Previous reports for *IGFIR* hemizyosity as a possible aetiological cause for SRS have all been supported by visible cytogenetic deletions [6–11]. There is no report of SRS individuals with normal karyotype who are hemizygous for *IGFIR*. In this investigation, the largest to date for hemizyosity of *IGFIR*, both chromosomes 15 were cytogenetically normal. This did not rule out the possibility that *IGFIR* was deleted, as conventional karyotyping methods have a range limit of 1–2 Mb and *IGFIR* covers only 100 kb [38]. Quantitative analysis of Southern hybridisation data together with polymorphic PCR markers, point to the presence of two copies of *IGFIR* for all 33 SRS probands, thereby ruling out the possibility that hemizyosity at this locus could contribute to the observed phenotype in this group.

IGFIR is a heterotetrameric, transmembrane glycoprotein, composed of two α - and two β -subunits linked by disulfide bonds. *IGFIR* shows a high degree of primary and secondary structural similarity to the insulin receptor. Ligand binding in the cysteine-rich domain [41] of the α -subunits at the extracellular surface stimulates intracellular, tyrosine-specific protein kinase activity which leads to β -subunit autophosphorylation and subsequent phos-

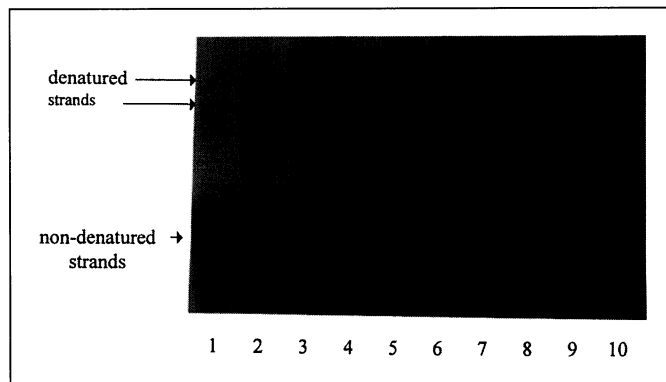


Fig. 4. SSCP analysis of exon 3 of the *IGFIR* gene. Lane 1, non-denatured control sample; lanes 2–10, 3 of 33 families investigated, order mother, proband and father. The upper two bands represent the two denatured alleles for this region and the third lowest band is an alternative conformation of one of these alleles. Note that all individuals have identical banding patterns.

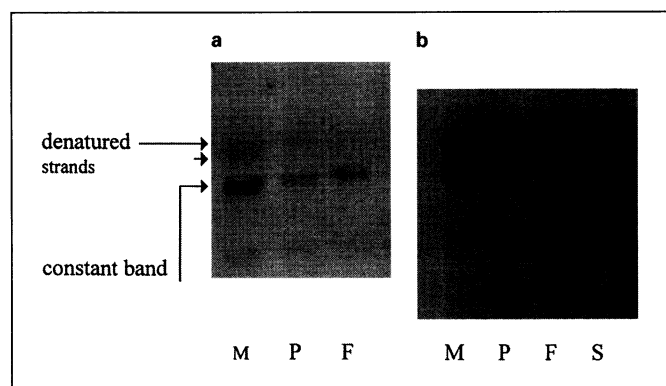


Fig. 5. SSCP analysis of exon 16 of the *IGFIR*. In family 1 (a), the parents have two bands and the proband has one band. In family 2 (b), the parents have one band and the proband and sibling have two bands. The third band is constant. M = mother, P = proband, S = sibling and F = father.

phorylation of cytoplasmic components of an IGF-I-specific signal transduction cascade [38, 42, 43].

In the two exons examined by SSCP, exon 3 and 16, no mutation was detected that would play a role in disabling *IGFIR*. Exon 3 is the cysteine-rich domain involved in ligand binding. It has been shown that IGF1, IGFII and at a much lower affinity, insulin, bind to this region, and the specificity of ligand binding is not only determined by the number of cysteine residues but also their distribution in the protein binding region and the flanking amino acids [41]. It was proposed that a mutation that interfered with ligand recognition would impair *IGFIR* functioning, so

that only the non-mutated allele would be capable of ligand binding and hence activation of the tyrosine kinase domain. However, no mutations were detected.

The second region that was investigated using SSCP was exon 16, located in the tyrosine kinase domain. This is the most highly conserved region compared to the insulin receptor (80–95%; [39]) highlighting its functional importance. There are four potential ATP binding sites, Gly 976, 978 and 981 and Lys 1003, in the tyrosine kinase domain [44]. Disruption of any of these binding sites would effectively down-regulate subsequent phosphorylation of other target proteins normally receptive to IGF1/IGFII mediated signalling via IGFIR. It is unlikely that the novel mutation found at nt 3174 (G→A) has phenotypic implications. Since there is no change in the amino acid and hence the protein structure, the transition does not alter the function of this region. This is the first time that a polymorphism, showing Mendelian inheritance, has been described in the coding region of *IGFIR*.

Despite the lack of any evidence for hemizyosity of *IGFIR* being involved in a possible aetiology for SRS in our cohort of 33 individuals, it does not suggest that it should be completely excluded as a candidate gene. Other

well documented cases with deletion of one copy of *IGFIR* show evidence of IUGR, and in a subset, the SRS phenotype. Since this receptor plays such a pivotal role in early embryonic development, it is important to include it in any investigation concerned with prenatal and postnatal growth retardation, in addition to other candidate genes involved in cell growth, proliferation, and development. Although no smaller deletions or base mutations were found in *IGFIR* for these individuals, only two out of the total of 21 exons encoding the functional receptor were investigated by SSCP, and mutation in the other exons could also affect receptor functioning.

Acknowledgements

S.A.A. and E.W. were funded by the MRC and Children Nationwide. S.A.A. is now a Dunhill Medicine Trust Fellow. S.P. is grateful for support from the Child Growth Foundation and Serono Laboratories. We would also like to thank Professor Peter Scambler (ICH) for use of the PhosphorImager and Lisa Lowry (RPMS) for sequencing the PCR products. We are grateful to Mr. L. Butler, Director of the NE Thames Regional Cytogenetics Unit, for the karyotype analyses and to our clinical colleagues who referred families for study.

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