LETTERS TO JMG

Alterations of the Birt-Hogg-Dubé gene (*BHD*) in sporadic colorectal tumours

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Colorectal cancer (CRC) is the third most common cancer diagnosed in both men and women, and the second most common cause of cancer deaths in the United States. There were approximately 150 000 new cases resulting in 57 000 deaths in 2002.¹ CRC is one of the most studied cancer types and its underlying aetiology best elucidated. Colorectal tumorigenesis involves a multistep process including genetic and epigenetic alterations of numerous CRC related genes that may act as either oncogenes or tumour suppressor genes.^{2–5} The majority of sporadic CRCs are characterised by deletions of large chromosomal segments, which are thought to represent the loss of wild type tumour suppressor genes.^{6,7} About 15% of sporadic CRCs, on the other hand, show microsatellite instability (MSI), characterised by the insertion and/or deletion of simple repeat sequences and indicative of the involvement of defective mismatch repair.^{8,9}

Birt-Hogg-Dubé syndrome (BHD, OMIM 135150) is an inherited autosomal dominant syndrome characterised by a triad of cutaneous lesions consisting of fibrofolliculomas,

trichodiscomas, and acrochordons.¹⁰ A wide spectrum of neoplastic and non-neoplastic features has been described in BHD patients,¹¹ including diverse types of kidney tumours^{12–17} and spontaneous pneumothorax.^{12–16} BHD has also been reported to be associated with colonic polyposis and colorectal neoplasia,¹³ although a large study of 223 patients from 33 BHD families could not establish such a relation.²³ We recently reported a high incidence of colorectal polyps and carcinomas in patients with confirmed *BHD* germline mutations, indicating that the *BHD* gene may be involved in colorectal tumorigenesis.¹³ The *BHD* gene has been mapped to chromosome subband 17p11.2^{12,14} and recently identified to encode a novel protein named follicullin.¹⁵ Based on the presence of inactivating *BHD* mutations in BHD patients, and the detection of LOH in a significant proportion of BHD related tumours, the *BHD* gene was considered to be a tumour suppressor gene. A 44% frequency of frameshift mutations within a mononucleotide (C)₈ tract (nt 1733–1740) has been detected in BHD patients,¹⁵ and this repeat tract represents a *BHD* mutational hot spot.^{13,15} Other studies have reported the presence of frameshift mutations within intragenic mononucleotide tracts of the *TGFBR2* and *BAX* genes in CRC cell lines and tumours with high level MSI.^{24,25} The poly C tract of the *BHD* gene may therefore be a potential site of mutation in CRC characterised by MSI.

We have evaluated the role of the *BHD* gene in 47 unselected colorectal tumours (10 polyps and 37 carcinomas) by screening all coding exons of the *BHD* gene for mutations and analysing 46 of the tumours for LOH in the chromosome region surrounding the *BHD* locus. Furthermore, alterations in *BHD* promoter methylation profiles were determined in 23 cases of matched normal/carcinoma tissues where a sufficient quantity of DNA was available. We report the detection of two novel somatic missense mutations of the *BHD* gene and LOH in 81% of primary sporadic colorectal tumours with no change in promoter methylation profile. All mutations were detected in MSS tumours.

MATERIALS AND METHODS

Tissue samples and DNA extraction

Forty-seven matched samples (from 37 patients), of which 10 were colonic polyps with their matched carcinomas from the same patients, and 37 colorectal carcinomas, were obtained from the South Western Sydney Colorectal Tumour Bank (Liverpool Hospital, Australia). All tissue samples were collected prospectively with the informed consent of patients who underwent surgery in the South Western Sydney Area Health Service during the period 2000–2002. The lack of a family history of colorectal cancer or other familial cancer syndrome was ascertained by detailed questionnaire. This study was approved by the Institutional Review Board of the Van Andel Research Institute. Frozen sections (15 µm) were prepared from stored tumour specimens. The first, middle, and last slides (5 µm) were stained as reference slides. Manual microdissection was carried out on the unstained slides under low

Key points

- A high incidence of colorectal tumours was recently reported in patients with Birt-Hogg-Dubé syndrome (BHD), implicating a potential role for the *BHD* gene in colorectal tumorigenesis.
- We have screened the *BHD* gene for genetic (mutations and loss of heterozygosity (LOH)) and epigenetic (altered promoter methylation status) alterations in 47 unselected primary sporadic colorectal tumours (10 polyps and 37 carcinomas). One polyp and seven carcinomas showed microsatellite instability (MSI) while all other tumours were microsatellite stable (MSS).
- We identified two novel missense mutations, S79W and A445T, in two MSS carcinomas. Methylation status, examined by methylation specific PCR (MSP) analysis of 23 matched normal/carcinoma tissues, showed an absence of any *BHD* promoter methylation differences.
- Genotyping of microsatellite markers encompassing the *BHD* gene showed LOH in four of 10 (40%) polyps and 29 of 36 (81%) carcinomas. All four colon polyps showing LOH showed chromosomal loss in the corresponding carcinomas from the same patients. However, LOH was also present in the corresponding carcinomas of six other polyps that did not show LOH, suggesting the involvement of LOH in colorectal tumour progression.
- Our results suggest that the *BHD* gene is involved in the tumorigenesis of a subset of MSS sporadic colorectal carcinomas, and that allelic loss in the region close to the *BHD* gene may play a role in colorectal tumour progression.

Figure 1 Schematic map of microsatellite markers encompassing the *BHD* gene. The relative distances (in cM) between each marker and their relationship to the *BHD* locus are indicated.

power light microscopy (20–40×) by scraping of individual cell populations with a 28 gauge needle. DNA was isolated from microdissected tumour cells and specimens of normal colonic mucosa using the Qiagen DNeasy Mini system (Qiagen, Valencia, CA), according to the manufacturer's instructions. DNA was extracted from peripheral blood leucocytes using the DNA isolation kit for mammalian blood (Roche Molecular Biochemicals).

Analysis of microsatellite instability (MSI) status

Paired colorectal carcinoma, polyp, and constitutional DNA samples ($n=47$) were analysed using a panel of 10 microsatellite markers comprising mononucleotide (BAT25, BAT26), dinucleotide (D2S123, D5S346, D18S34, D3S1611), and tetranucleotide (D1S518, D7S1808, D3S2432, D10S1426) repeats. Amplification was performed in a final volume of 10 μ l containing 25 ng DNA, 20 pmol each primer, 16 μ mol/l dATP, 0.2 mmol/l remaining dNTPs, 0.4 μ Ci of α - 32 P [dATP], 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 1.5 mmol/l MgCl₂, and 0.5 U *Taq* polymerase (Amersham-Pharmacia Biotech). PCR was initiated by a five minute denaturation (94°C) followed by 34 cycles of denaturation (94°C, 45 seconds), primer annealing (55–65°C, 45 seconds), and extension (72°C, 45 seconds). PCR cycling was ended with a 10 minute extension (72°C) step. Radioisotope labelled PCR products were electrophoresed on 6% sequencing gels and visualised by autoradiography. Samples were classified as MSI-L (low level microsatellite instability) if instability was observed at 20–40% of loci assayed or MSI-H (high level microsatellite instability) if instability was observed at over 40% of loci assayed.²⁶

Mutation analysis

Mutation screening was performed on all 47 matched samples. The entire coding region of the *BHD* gene (exons 4–14) was screened. Primer sequences and PCR conditions were according to Nickerson *et al.*¹⁴ PCR was performed using a DNA Engine Tetrad (MJ Research, Waltham, MA). PCR products were analysed on standard 1.5% agarose gels stained with ethidium bromide (0.5 μ g/ml) before purification with Multiscreen PCR cleanup plates (Millipore, Molsheim, France). Sequencing reactions were performed using the Big Dye Terminator system (Applied Biosystems, Foster City, CA), purified through Sephadex G-50 (Amersham Biosciences, Uppsala, Sweden) and analysed on an ABI 3700 genetic analyser (Applied Biosystems). We aligned and analysed all sequences by Blast 2 analysis²⁷ and manually verified all sequences again. All sequence changes were verified by reamplification of the corresponding *BHD* fragment and sequencing of both DNA strands.

Analysis of loss of heterozygosity (LOH) status

LOH was performed on 36 matched normal/tumour tissue pairs, as well as 10 matched normal/polyp pairs. Allelic deletions of the chromosome 17p region flanking the *BHD* gene were assessed using microsatellite markers D17S1857, D17S740, D17S2196, and D17S620. The relative distances between each marker and their relationship to the *BHD* gene were calculated using the UCSC Genomic Bioinformatics site (fig 1). PCR conditions were according to Khoo *et al.*¹³ One μ l of each PCR product was added to a cocktail containing 5 μ l of DNase free, RNase free distilled water, 10 μ l of Hi-Di formamide and 0.2 μ l of ROX 400HD size standard. The mixture was denatured at 95°C for five minutes before loading into an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). Analysis of raw data and assessment of LOH were carried out using

Genescan v 3.7 and Genotyper v 3.7 software (Applied Biosystems). LOH was defined according to the following formula: LOH index = (T2/T1)/(N2/N1), where T was the tumour sample, N was the matched normal sample, and 1 and 2 were the intensities of smaller and larger alleles, respectively.²⁸ If the ratio was <0.67 or >1.3, the result was determined to be LOH. Initially, the two closest markers (D17S740 and D17S2196) were analysed for LOH. A designation of LOH was given when at least one of the markers had a ratio that was <0.67 or >1.3. If the LOH value was close to these thresholds (0.67 + 0.1; 1.3 – 0.1), a further two markers, D17S1857 and D17S620, were examined to confirm the LOH status.

Analysis of *BHD* promoter methylation profile

We examined the promoter methylation status of the *BHD* gene in 23 matched normal/carcinoma sample sets. DNA methylation status was determined by a methylation specific PCR approach (MSP).^{29–30} DNA was treated with sodium bisulphite, which converts all unmethylated cytosines to uracils, leaving methylated cytosines unchanged. Briefly, 2 μ g of DNA was denatured by incubation in 0.2 mol/l NaOH (37°C, 10 minutes). Cytosines were then modified in 3 mol/l sodium bisulphite (adjusted to pH 5.0; Sigma Chemical Co, St Louis, MO) and 10 mmol/l hydroquinone (Sigma) at 50°C for 16 hours. DNA samples were then purified through columns (Microcon YM-100, Millipore, Bedford, MA), treated again in 0.3 mol/l NaOH, precipitated with ethanol using glycogen as a carrier, and resuspended in 20 μ l DNase free, RNase free distilled water before storing at –20°C. The specific primers for methylated sequences were designed as follows: BHD-BISF-OF (5'-ATGTGGATAGGAAGTTTGTGGTTATATTT-3') as the forward primer, and BHD-BISF-OR (5'-ACAAAATCACCCCAAACCC-3') as the reverse primer. An aliquot of the bisulphite treated product (2 μ l) was amplified in a 25 μ l reaction containing 2 mmol/l MgCl₂, 0.24 mmol/l each dNTP (Invitrogen), 0.02 U *Taq* DNA polymerase (Invitrogen), and 0.1 μ mol/l of each primer. PCR conditions were 95°C for five minutes followed by 35 cycles of 94°C (30 seconds), 60°C (30 seconds), and 72°C (45 seconds). PCR was ended with a seven minute extension (72°C). A nested PCR was then performed using 1 μ l of the initial amplification reaction. The primers used were BHD-BISF-IF: 5'-GAAATGGTTTTTTTAGTATTTTGTGGTG-3' and BHD-BISF-IR: 5'-CCCAAAACCCCAAACCC-3', with conditions similar to those described for the preceding PCR amplification, with the exception that 40 amplification cycles were carried out. The PCR products were purified using Microcon YM-100 columns (Millipore). After amplification, 20 μ l of the 414 bp PCR product was incubated with 0.3 U of *Rsa*I (New England BioLabs Inc, Beverly, MA) for two hours at 37°C. λ DNA (0.3 μ g) and distilled water were used respectively as positive and negative controls. Products of restriction digestion (20 μ l) were electrophoresed on 2% agarose gels containing ethidium bromide, and visualised under UV illumination. The sizes of the *Rsa*I digestion products were 160 and 254 bp.

RESULTS

Tumour MSI status

Analysis of MSI status showed that eight of 47 tumours tested showed MSI (table 1). This represents approximately 17% of the sporadic colorectal tumour cases evaluated in this study. Five carcinomas (CRC-7, CRC-17, CRC-18, CRC-46, and CRC-52) showed a high frequency of MSI (MSI-H), while two carcinomas (CRC-23 and CRC-42) exhibited a low frequency of

Table 1 MSI and inactivation profiles of the *BHD* gene in sporadic colorectal carcinomas and polyps

Sample ID	MSI status	<i>BHD</i> mutation	LOH	Methylation
CRC-1	-	-	+	ND
CRC-2	-	-	+	ND
CRC-3	-	-	-	ND
CRC-4	-	-	ND	ND
CRC-6	-	-	+	-
CRC-7	+ (H)	-	+	ND
CRC-9	-	-	+	-
CRC-12	-	-	+	ND
CRC-13	-	-	-	-
CRC-14	-	-	+	ND
CRC-17	+ (H)	-	+	-
CRC-18	+ (H)	-	-	ND
CRC-19	-	-	+	ND
CRC-20	-	-	+	ND
CRC-22	-	-	+	ND
CRC-23	+ (L)	-	+	ND
23P	-	-	-	ND
CRC-28	-	S79W	+	-
CRC-30	-	-	+	-
CRC-31	-	-	+	-
CRC-34	-	-	+	ND
34P	-	-	+	ND
CRC-35	-	-	+	-
35P	-	-	-	ND
CRC-37	-	-	+	ND
37P	-	-	-	ND
CRC-38	-	-	-	-
CRC-42	+ (L)	-	+	-
42P	+ (L)	-	+	ND
CRC-43	-	-	+	-
43P	-	-	-	ND
CRC-44	-	-	+	-
44P	-	-	-	ND
CRC-45	-	-	+	-
CRC-46	+ (H)	-	-	-
CRC-48	-	-	+	-
CRC-49	-	-	+	-
CRC-50	-	-	+	-
50P	-	-	-	ND
CRC-52	+ (H)	-	+	-
CRC-54	-	A445T	-	-
CRC-55	-	-	+	-
CRC-56	-	-	+	-
56P	-	-	+	ND
CRC-59	-	-	+	-
59P	-	-	+	ND
CRC-60	-	-	-	-

P = polyp, - = absent; + = present; H = high level MSI; L = low level MSI; ND = not determined.

MSI (MSI-L). CRC-42 also showed a low level of MSI in its corresponding polyp (42P). All other tumours (39 of 47) were microsatellite stable (MSS).

BHD mutations

Screening of the *BHD* coding region identified two novel somatic mutations in exon 4 (c.691C>G) and exon 12 (c.1788G>A) of CRC-28 and CRC-54, respectively (table 1, fig 2). Both are missense mutations (S79W and A445T), leading to non-conservative amino acid changes. In both cases the carcinomas were MSS and tumours with *BHD* mutations represented approximately 7% of the MSS colorectal carcinomas tested (n=30). No mutations were detected in the (C)₈ repeat tract (nt 1733–1740), known to be a mutational hot spot within the *BHD* gene, in either the MSI or MSS tumours. *BHD* mutations were absent in all colon polyps.

LOH status

LOH at the chromosomal region surrounding the *BHD* locus was identified in 81% (29 of 36) of the sporadic colorectal carcinomas, and 40% (four of 10) of colon polyps (table 1). The

Figure 2 Detection of mutations within the *BHD* gene in sporadic colorectal cancer. Two novel somatic mutations of the *BHD* gene were detected in two MSS colorectal carcinomas. Each of the mutations is not present in the matched normal tissues. (A) c.691C>G (S79W) in CRC-28 and (B) c.1788G>A (A445T) in CRC-54.

four colon polyps with LOH were from the same people who showed LOH in their colorectal carcinomas (CRC-34, 34P; CRC-42, 42P; CRC-56, 56P; and CRC-59, 59P). CRC-28 showed LOH, along with somatic mutation S79W.

BHD promoter methylation

Methylation specific PCR analysis of the *BHD* promoter did not detect any promoter methylation profile differences in the 23 matched sets tested (table 1). Unfortunately, methylation profiles for the rest of the samples could not be determined owing to insufficient DNA being available.

DISCUSSION

Early studies have reported several cases of colorectal neoplasia in patients with *BHD*.^{19–22} However, one recent study²³ showed a lack of statistical significance when comparing the incidence of colon cancer in 111 *BHD* affected and 112 *BHD* unaffected subjects, as well as the occurrence of colon polyps

in 45 BHD affected and 38 BHD unaffected subjects, thus excluding any association between colonic neoplasia and BHD. Nevertheless, we recently reported six cases of colonic polyps and two cases of possible colon cancer in a BHD family with confirmed *BHD* germline mutations,¹³ indicating that the *BHD* gene is involved in the tumorigenesis of these BHD related colorectal tumours. In this study, we show that the *BHD* gene is also involved in a subset of sporadic colorectal cancers. Two cases of MSS colorectal cancer were found to harbour two novel somatic missense mutations, S79W and A445T, in exons 4 and 12, respectively. Interestingly, no frameshift mutation was identified in the hypermutable poly C tract, particularly in the MSI carcinomas, as this region is a potential site for insertion or deletion in cancers with defective mismatch repair. These results suggest that the *BHD* gene may be involved in a pathway of colorectal tumorigenesis that is distinct from the pathway of mismatch repair deficiency. However, the sample size of the MSI tumours is small in this series and further investigation is warranted.

The missense mutations detected were non-conservative amino acid substitutions (S79W and A445T) in the *BHD* gene product which could cause conformational changes in the structure of the protein, leading to dysfunction.³¹⁻³⁵ Protein phosphorylation, a modulator of protein function and stability, can occur at Ser, Thr, or Tyr residues and is mediated by specific protein kinases. In CRC-28, the change from Ser to Trp leads to the loss of a potential site of phosphorylation whereas the Ala to Thr change in CRC-54 leads to the gain of a potential phosphorylation site. These amino acid changes could lead to altered protein phosphorylation status with consequent functional changes.

LOH, which indicates the loss of one functional copy of a gene, has been used as a marker for diagnosis and prognosis of cancer. In this study, we identified LOH at microsatellite loci flanking the *BHD* gene in 40% of colon polyps and 81% of colorectal carcinomas. Together with the finding of LOH in matched normal/carcinoma samples of LOH negative polyps, we propose that LOH surrounding the *BHD* locus may be involved in colorectal cancer progression, although other tumour suppressor genes located on chromosome 17p, such as *p53*, should not be excluded. The *p53* gene is located approximately 9 cM telomeric to the *BHD* gene. Studies have shown that LOH at 17p may be essential for the malignant transformation of benign lesions in colorectal neoplasms.³⁶⁻³⁷ Therefore, the effects of LOH on *BHD* gene expression and regulation in colorectal tumours merits further investigation.

Sample CRC-28 was found to harbour a mutation in the *BHD* gene as well as LOH around the *BHD* region, which could represent two hits of the *BHD* gene in accordance with Knudson's classical two hit theory. Apparent biallelic alteration of the *BHD* gene appears to be uncommon and the high frequency of LOH in the rest of the tumours without mutations suggests several possibilities. First, loss of a single allele may be the preferred mode of inactivation of the *BHD* gene and that haploinsufficiency contributes to tumorigenesis. Second, there may be mutations present in the regulatory region of the *BHD* gene which were not tested in this study. Finally, there may be loss of other tumour suppressor genes in the vicinity of the *BHD* gene.

DNA methylation is an epigenetic alteration that interferes with transcriptional initiation. In general, methylation of CpG dinucleotides in the promoter regions of tumour suppressor genes leads to loss of tumour suppressor gene expression (silencing) and consequent function. Hypermethylation of tumour suppressor genes has been frequently reported in many tumour types. We recently identified the involvement of the *BHD* gene in sporadic renal tumours by showing frequent methylation of the *BHD* promoter in a wide spectrum of sporadic renal tumours.³⁸ In the present study, we did not detect any *BHD* promoter methylation profile differences in the 23 colorectal carcinoma cases where a sufficient amount of DNA

was available for the MSP assay. We conclude that epigenetic alteration of the *BHD* gene is not a common event in colorectal cancer.

In summary, we have shown that the *BHD* gene is mutated in a subset of MSS sporadic colorectal carcinomas, and allelic loss around the region of the gene may play a role in the progression of colorectal tumours.

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A gene locus for branchio-otic syndrome maps to chromosome 14q21.3-q24.3

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Branchio-oto-renal syndrome (BOR, OMIM 113650) is an autosomal dominant disorder characterised by the association of hearing loss (HL), structural ear anomalies, branchial arch defects, and renal anomalies.¹ The prevalence approximates 1:40 000 in the general population, and has been reported in about 2% of deaf children.² Age of onset for deafness varies from childhood to early adulthood.³ The clinical expression of BOR exhibits wide intra- and inter-familial variability. In addition, reduced penetrance for BOR has been assumed.⁴ The major feature of BOR, which occurs in 93% of patients, is HL, which can be conductive, sensorineural, or mixed. Besides the classical ear, kidney, and branchial arch anomalies, different developmental manifestations of BOR in other organ systems have been described. Among these, dysfunction of the lacrimal duct system is a common association.⁵⁻¹⁰ Thus, BOR represents a clinically and genetically heterogeneous disease complex that manifests predominantly during organogenesis. A gene locus for autosomal dominant BOR had been localised on chromosome 8q13.^{11, 12} Subsequently, mutations in the human homologue of the *Drosophila eyes absent* gene (*EYA1*) have been shown to be causative for BOR (OMIM 601653).¹³ Branchio-otic syndrome (BOS) (OMIM 602588) was initially described as a disorder distinct from BOR, featuring the same clinical symptoms as BOR with

Key points

- Branchio-oto-renal syndrome (BOR) is an autosomal dominant developmental disorder characterised by the association of hearing loss, branchial arch defects, and renal anomalies. Branchio-otic syndrome (BOS) represents a related disorder presenting with the same clinical features without renal anomalies.
- Recessive mutations in the human homologue of the *Drosophila eyes absent* gene (*EYA1*) have been shown to cause BOR and BOS. A locus (*BOS2*) for autosomal dominant BOS has been localised to chromosome 1q31.
- We performed a genome wide search for linkage in a large pedigree with BOS with more than 40 affected subjects and mapped a new gene locus (*BOS3*) to chromosome 14q21.3-q24.3. The highest multipoint lod score was $Z_{\max}=4.81$ ($\theta=$) for marker D14S980.
- Identification of the gene causing branchio-otic syndrome type 3 will offer new insights into the development and molecular mechanisms of hearing.

Table 1 Clinical data of affected subjects from the BOS kindred

		HL						
		SND		CD				
Subject	AO	R	L	R	L	EP	BC	LD
II.2	ND	ND	ND	ND	ND	-	-	-
II.3	ND	ND	ND	ND	ND	+	-	-
III.3	ND	ND	ND	ND	ND	-	-	-
III.5	ND	ND	ND	ND	ND	-	-	-
III.9	~18	H + to +++*	H + to +++++*	-	-	-	-	+
III.10	~10	H + to +++++*	H + to +++++*	-	-	+	-	+
III.12	~18	ND	ND	ND	ND	-	-	-
III.14	~6	ND	ND	ND	ND	+	-	-
IV.2	~20	H + to ++*	H + to +++*	-	-	-	-	-
IV.5	ND	ND	ND	ND	ND	-	-	-
IV.8	ND	ND	ND	ND	ND	+	-	-
IV.9	~3	H + to +++*	H + to +++*	-	-	-	-	+
IV.12	~1	A +++++	A +++	-	-	-	-	-
IV.13	~10	+++	+++	-	-	-	-	-
IV.14	ND	+	+++++	-	-	-	-	-
IV.16	~20	H + to +++++*	H + to +++++*	ND	ND	-	-	-
IV.17	~22	++++	++++	-	++++	-	-	-
IV.18	~13	H + to +++++*	H + to +++++*	-	-	-	-	-
IV.19	ND	ND	ND	ND	ND	-	-	-
IV.20	~10	ND	ND	ND	ND	-	-	-
IV.21	ND	ND	ND	ND	ND	-	-	-
V.2	~7	H + to ++	L +	-	-	-	-	-
V.3	ND	ND	ND	ND	ND	+	-	-
V.4	ND	ND	ND	ND	ND	-	-	-
V.5	ND	ND	ND	ND	ND	-	+	-
V.6	~3	H +	H +	-	-	-	-	-
V.7	ND	H ++	H ++	L +	L +	-	-	-
V.8	ND	+	+	-	-	-	+	-
V.10	ND	ND	ND	ND	ND	-	-	-
VI.1	1	H + to +++	H + to +++	+	+	-	-	-
VI.2	<1	A +++++*	A +++++	-	-	+	+	-
VI.3	3 weeks	H + to +++	H + to +++	-	-	-	-	-
VI.4	7	H +	-	-	-	-	-	-

AO = age of onset, HL = hearing loss, SND = sensorineural deafness, CD = conductive deafness, + = mild, ++ = mild-moderate, +++ = moderate, ++++ = moderate-severe, +++++ = severe, * = progressive, L = low frequency, M = middle frequency, H = high frequency, A = all frequencies, EP = ear pits, BC = branchial cysts, LD = lacrimal duct stenosis, ND = no data.

*Seventeen additional patients had hearing loss (fig 1), but detailed clinical data were not available.

the exception of renal anomalies.¹ The large variety of clinical phenotypes and the description of mutations in the *EYA1* gene for BOR and BOS patients¹³⁻¹⁵ show that BOR and BOS can represent allelic defects of the *EYA1* gene. The identification of a second gene locus in a large BOS pedigree on chromosome 1q31 established the presence of genetic locus heterogeneity for BOS.⁴ No linkage to this locus has been published for BOR families and the gene defect is still to be identified. The issue of genotype/phenotype relationships regarding clinical features of BOR or BOS remains unsolved. We describe here a genome wide search for linkage in a large pedigree with BOS, in which linkage to the *EYA1* locus on chromosome 8q13 had been excluded, resulting in a new locus (*BOS3*) on chromosome 14q.

METHODS

Blood samples and clinical data for a large multigeneration family with over 40 affected subjects with BOS were obtained after informed consent was given by patients and unaffected relatives. The ethnic origin of the family was Anglo-Saxon Australian. Clinical examinations and renal ultrasound were

performed in 32 affected family members. Twenty blood samples were collected (14 from affected subjects, six from unaffected relatives or partners) and DNA was extracted for molecular analysis. All 32 affected subjects had deafness (100%). In 17 affected family members, precise audiometric data were available. Diagnosis was sensorineural HL in 14 of them (82%) and mixed HL in three of them (18%). The affected frequencies varied from low to high frequencies as well as the presence of HL in all frequencies. Severity varied from mild to severe HL being still progressive in six cases. Among subjects IV.14, IV.17, and V.2 differences in the HL between the right and left ear were found. In IV.14 sensorineural HL was mild in the right ear whereas it was moderate to severe in the left ear. In IV.17, in addition to moderate to severe sensorineural HL on both sides, moderate to severe conductive HL was present only on the left side. In V.2 high frequency HL in the right ear differed from low frequency in the left ear. Age of onset was very variable with an average of 9.5 years, ranging from 3 weeks to 22 years. Eight subjects (25%) had branchial arch defects, three with branchial cysts, and six with ear pits as external ear manifestation (table 1).

Figure 1 Haplotypes on chromosome 14q12-q23 of the BO family. Haplotypes are shown for the subjects where DNA was available (indicated by an arrow) or haplotypes could be inferred. Thirteen microsatellite markers are shown on the left from cen to qter (top to bottom). Filled upper right quadrant indicates diagnosis of hearing loss, filled lower right quadrant ear pits, filled upper left quadrant lacrimal duct stenosis, and filled lower left quadrant branchial cysts. Haplotypes are interpreted as differently coloured bars. Paternal haplotypes are drawn to the left, maternal ones to the right. Segments of haplotypes which could not unambiguously be assigned to the paternal or maternal haplotype are represented by a thin line. Inferred haplotypes are indicated in parentheses. The black haplotype cosegregates with the affected status. Note that marker D14S1013 is flanking the *BOS3* locus on its centromeric borders, as defined through a recombination in II.2, and that marker D14S53 is flanking the *BOS3* locus at its q terminal border as defined by a recombination in V.5. Flanking markers are underlined.

Figure 2 Multipoint lod scores for the *BOS3* locus on chromosome 14q21.3-q24.3 versus the 13 markers shown in fig 1. Relative position is given in cM according to the Genethon map.¹⁸ The two markers D14S1013 and D14S53 flanking the *BOS3* region (fig 1) are underlined. cen, centromeric orientation; qter, q terminal orientation.

In three affected subjects (9%) lacrimal duct stenosis was diagnosed as a common feature associated with BOR/BOS. No congenital renal anomalies were found, although two adult affected sibs had renal carcinomas, which most likely was coincidental. The absence of congenital renal anomalies suggests a diagnosis of BOS rather than BOR in this family. Genomic DNA was isolated, by standard methods,¹⁶ either directly from blood samples or after Epstein-Barr-virus transformation of peripheral blood lymphocytes.¹⁷ DNA was available for haplotype analysis in 14 affected and six unaffected subjects for the genome wide search for linkage. In the other subjects haplotypes were inferred if possible (fig 1). A total of 380 microsatellite markers from the Genethon final linkage map¹⁸ with an average spacing of 11 cM were used. For further fine mapping on chromosome 14q21.3-q24.3, six additional markers, with an average distance of 3.5 cM, were used. Order and sex averaged distances (in parentheses) between these markers from centromeric to q telomeric are as follows: D14S599 (2.9 cM), D14S306 (2.8 cM), D14S1013 (3.2 cM), D14S748 (4.5 cM), D14S587 (4.1 cM), D14S980 (2.9 cM), D14S274 (3.9 cM), D14S592 (8.0 cM), D14S588 (2.9 cM), D14S1002 (4.5 cM), D14S1025 (3.7 cM), D14S53 (5.1 cM), and D14S606.¹⁸ Semiautomated genotyping was performed with a MegaBACE-1000 analysis system. Data were analysed by Genetic Profiler Software, version 1.1. Two point lod score calculations were performed by the LINKAGE program package,¹⁹ with the help of the LINKRUN computer program (T F Wienker, unpublished data), using an autosomal

dominant model with 100% penetrance and a gene frequency for BOS of 0.0001. The "lodmax - 1 support interval" was defined as the genetic map positions intersecting the lod score curve at $Z_{\max}=1$.²⁰ For haplotyping and computation of multipoint lod scores, the program SIMWALK²¹ was used, assuming equal allele frequencies. Because of the reduced penetrance described in BOR/BOS, the calculations were performed on basis of an "affecteds only" strategy.

RESULTS

Before starting the genome wide search for linkage, the *EYA1* gene locus was excluded by linkage and mutational analysis. By evaluating the results of the genome wide search, the locus for BOS on chromosome 1q31 was also excluded for this kindred (data not shown). From the total genome search for linkage, only for one locus was cosegregation of the haplotype pattern in all affected subjects found for markers D14S587, D14S592, and D14S588 on chromosome 14q21.3-q24.3, yielding a maximum two point lod score of $Z_{\max}=3.27$ ($\theta=0$) for marker D14S587 (table 1). Further fine mapping with an additional six markers confirmed the locus. Haplotype analysis showed clear evidence that the disease allele cosegregated with all affected subjects and was absent from unaffected subjects (fig 1). A recombination event in II.2 defined marker D14S1013 as proximally flanking, and a recombination in V.5 identified marker D14S53 as distally flanking the critical genetic region within a 37.7 cM interval on chromosome 14q21.3-q24.3. Multipoint analysis of the 11 markers resulted in a $Z_{\max}=4.81$ at marker D14S980 at relative position 50.9 (fig 2). The 95% confidence interval at $Z_{\max}=1$ ²⁰ extends over a 33.9 cM interval between the markers D14S1013 and D14S53 within the set of 11 microsatellites. Marker D14S980 also showed the highest two point lod score value $Z_{\max}=4.11$ ($\theta=0$) (table 2).

DISCUSSION

Here we have reported a third gene locus for BOS, *BOS3*, which maps to chromosome 14q21.3-q24.3. According to the UCSC Genome Browser, the interval between markers D14S1013 and D14S53 spans a physical distance of approximately 33 Mb, relative marker positions are 41 383 995 and 74 328 130, respectively. A recombination in the healthy subject III.8 could define marker D14S587 as proximally flanking. As reduced penetrance for BOS is known, this does not represent a secure border. Further fine mapping with more affected members of this pedigree and examination of other familial cases with BOS will help to refine this region. In contrast to the pedigree

Table 2 Two point lod scores generated in the BOS kindred at various recombination fractions for markers at the *BOS3* locus

Marker	Two point lod scores at recombination fraction $\theta=$								$Z_{\max}(\theta)$
	0	0.001	0.01	0.05	0.1	0.2	0.3	0.4	
D14S599	-4.991	-3.645	-1.891	-0.659	-0.237	0.02	0.053	0.028	0.053 (0.3)
D14S306	-4.220	-2.777	-0.984	0.192	0.529	0.585	0.381	0.127	0.585 (0.2)
D14S1013	-0.135	0.624	1.494	1.926	1.892	1.492	0.953	0.419	1.926 (0.05)
D14S748	1.902	1.898	1.853	1.656	1.410	0.934	0.508	0.182	1.902 (0.0)
D14S587	3.268	3.261	3.202	2.933	2.590	1.881	1.170	0.518	3.268 (0.0)
D14S980	4.114	4.107	4.038	3.726	3.321	2.461	1.544	0.638	4.114 (0.0)
D14S274	2.163	2.157	2.097	1.830	1.492	0.831	0.286	0.005	2.163 (0.0)
D14S592	2.672	2.666	2.612	2.371	2.067	1.459	0.876	0.368	2.672 (0.0)
D14S588	1.962	1.958	1.922	1.756	1.544	1.108	0.679	0.298	1.962 (0.0)
D14S1002	3.359	3.353	3.295	3.033	2.696	1.995	1.275	0.583	3.359 (0.0)
D14S1025	2.254	2.249	2.201	1.988	1.717	1.166	0.634	0.214	2.254 (0.0)
D14S53	0.324	1.064	1.925	2.335	2.272	1.816	1.208	0.568	2.335 (0.05)
D14S606	-4.205	-2.769	-0.981	0.188	0.517	0.568	0.388	0.173	0.568 (0.2)

described by Kumar *et al*⁴ linked to chromosome 1q31, where HL was diagnosed in 50% of the affected subjects, deafness seems to be a major feature in this pedigree. As 25% of the patients show an association with branchial arch defects, a non-syndromic form of deafness is unlikely. The diagnosis of lacrimal duct stenosis, a common association of BOR and BOS, further confirms the diagnosis of BOS. The HL varied in form, severity, frequency, and the age of onset among the different family members and even between the ears of one patient, a characteristic feature of BOR and BOS. The low percentage of branchial arch defects compared to previously described families with BOS and BOR can be explained either by the known variable expressivity or by the genetic heterogeneity of BOS.

Genes encoding proteins involved in renal and otic morphogenesis and organogenesis are good candidates. *EYA1* deficient mice have been shown to lack ears and kidneys and show abnormal apoptosis of organ primordia.²² Another member of the *EYA* gene family, *EYA4*, is responsible for late onset deafness.²³ *EYA2* and *EYA3* are excluded from the *BOS3* locus on chromosome 14, as they are localised on chromosomes 20 and 1, respectively. Gene loci for non-syndromic forms of autosomal dominant²⁴ and autosomal recessive deafness²⁵ were mapped to the critical interval on chromosome 14q21.3-q24.3. Whether the autosomal dominant form is an allelic variant of BOS requires the identification of the causative gene. Identification of the gene causing BOS3 in this pedigree and other patients with BOS will lead to new insights into the pathophysiology and development of auditory function.

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Data access. URL for data in this article are as follows: Genethon map: <http://www.cephb.fr/ceph-genethon-map.html>. Online Mendelian Inheritance in Man (OMIM): <http://www.ncbi.nlm.nih.gov/Omim> UCSC Genome Browser: <http://genome.ucsc.edu/>.

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Mitotic recombination mediated by the *JJAZF1* (*KIAA0160*) gene causing somatic mosaicism and a new type of constitutional NF1 microdeletion in two children of a mosaic female with only few manifestations

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Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder with an estimated birth incidence of 1 in 2500 and marked variability of expression. The hallmark symptoms of the fully manifested disease encountered in nearly all patients are cutaneous neurofibromas, café au lait spots, axillary freckling, and Lisch nodules. Other common manifestations are bone dysplasias, scoliosis, vasculopathy, and learning disabilities. NF1 patients also suffer from an increased risk of specific tumour types like plexiform neurofibromas, neurofibrosarcomas, optic gliomas, other CNS tumours, pheochromocytomas, juvenile xanthogranuloma, and juvenile myeloid leukaemia. Mutations of the *NF1* gene at 17q11.2 encoding neurofibromin are the molecular basis of the disease. Neurofibromin contains a GTPase activating domain and is a negative regulator of Ras GTPases. Homozygous inactivation of neurofibromin is associated with a dysregulation of Ras mediated signalling pathways and tumorigenesis in NF1 patients.¹ More than 70% of the germline mutations are protein truncating and are distributed throughout the coding region.^{2–4} No distinct genotype-phenotype correlation concerning type and position of the mutations has been established, apart from patients with microdeletions of the *NF1* gene region, which are associated with a more severe clinical phenotype and facial dysmorphism. This was recognised very early and confirmed by several studies.^{5–9} Molecular characterisation of the deletion boundaries showed that non-allelic recombination between two highly homologous sequences separated by ~1.5 Mb eliminates 14 genes together with the *NF1* gene during germ cell development.^{10–14} These 60–85 kb spanning low copy repeats are derived from segments of the *WI-12393* gene and contain sequences with homology to chromosome 19. The structure of the *NF1* gene region at 17q11.2 is further complicated by other duplicated sequences, such as pseudogene exons of the *SMURF2* and the *KIAA0160* genes.^{10–15} Up to now, homologous recombination between these duplicated sequences during mitotic cell divisions has not been reported. Here, we describe two brothers with severe NF1. Their mother, who does not fulfil the diagnostic criteria for NF1, only has a few inconspicuous café au lait spots and lacks any neurofibromas and other tumours. She shows somatic mosaicism for a large deletion of the *NF1* gene region in peripheral blood leucocytes and skin fibroblasts. We determined the mechanism underlying the deletion and the size of the deleted interval at the cytogenetic and molecular level with respect to the duplicated sequences in the *NF1* region.

MATERIAL AND METHODS

Patients

The 60 year old female investigated in this study (II.2, fig 1) has four café au lait spots on her thighs and forearms. Some minor freckling was found in the left axilla, but she did not have dermal neurofibromas or Lisch nodules. Her two sons,

however, suffer from a severe form of NF1 (patients III.1 and III.2). In early childhood, they had multiple café au lait spots distributed all over the body and noticeably large hands and feet. In the younger brother, retarded psychomotor development was documented. Later in life, coarse facial features with hypertelorism were noticed, in addition to macrocephaly, axillary freckling, mild scoliosis, and multiple neurofibromas, which were too numerous to count. Multiple melanocytic naevi and low set nipples were additional features. The intelligence quotient of the sons was judged by their mother as normal, but was specifically tested only in her oldest son and was found to be in the normal range. Both attended regular school. Patient III.1 suffered from a malignant glandular schwannoma in the left gluteus which metastasised. At the age of 29 years, he died from intracranial bleeding before genetic investigations were started. His younger brother, patient III.2, who

Key points

- Large deletions of the *NF1* gene region at 17q11.2 have been observed in 5–20% of all NF1 patients and are most frequently about 1.5 Mb in size. In the majority of these NF1 patients, a segment of a similar size carrying 14 functional genes is eliminated during meiosis by homologous recombination between low copy repeats (LCRs) of the *WI-12393* gene and sequences with homology to chromosome 19.
- Here we report on two brothers suffering from a severe form of NF1 and their mother, who does not fulfil the general diagnostic criteria for NF1. Marker and FISH analyses showed mosaicism for a large microdeletion of the *NF1* gene region in the mother. The microdeletion was transmitted to her younger son and probably to her older son as well, who died from complications of the disease some years ago.
- By contrast to previously characterised microdeletions, we located the breaks in another duplicated segment of the *NF1* region, the *KIAA0160* gene and its pseudogene. The disrupted *KIAA0160* gene is closely flanked by the *WI-12393* gene, which is retained, and by *WI-12393* related LCR sequences on the distal and proximal side, which have been shown to be involved in homologous recombination in other patients with *NF1* microdeletions.
- We conclude that somatic recombination between the *KIAA0160* gene, which has also been called *JJAZF1*, and its pseudogene caused gonadosomatic mosaicism of the large deletion in the mother. Furthermore, marker analysis showed that mitotic intrachromosomal recombination is the mechanism underlying this deletion.

Figure 1 Genotype analysis of 11 polymorphic markers flanking the *NF1* gene region and two markers within the *NF1* gene (D17S1849, D17S1166) in the family of the index patient III.2. The haplotypes of patient III.2 were determined by analysing two hybrids, Nos 4 and 15, carrying only the deleted chromosome 17 and two hybrids, Nos 8 and 17, with the normal chromosome 17. Patient III.2 is hemizygous for markers D17S635, D17S1849, D17S1166, and D17S1800. Markers in parentheses located within the deleted interval of proband II.2 are present as single copies in the majority of peripheral blood leucocytes.

is still alive, had sphenoid wing dysplasia. At the age of 11 years, progressive dislocation of his hips and genu valgum were diagnosed, which had to be surgically corrected several times.

FISH analysis

Chromosome spreads were prepared from peripheral blood lymphocytes and skin fibroblasts of proband II.2 and blood lymphocytes of her son (the index patient III.2) according to standard methods. Skin fibroblasts of the mother were obtained by a punch biopsy of the left axillary region. BAC clones used as FISH probes were purchased from the BAC/PAC Resource Center (www.chori.org/bacpac) and have previously been characterised.^{10–15} PCR products used as FISH probes, DJ1686/1863, DJHK10/11, and DJ1576/1578, were amplified with primers listed in table 1 using the Expand Long Template PCR System (Roche Molecular Biochemicals). The respective products were cloned with the TOPO TA Cloning System (Invitrogen) and labelled for FISH analysis. Two colour FISH was performed using BAC-DNA or the cloned PCR products labelled either with biotin-16-dUTP or digoxigenin-11-dUTP (Roche-Diagnostics, Mannheim, Germany). Hybridisation signals were visualised by FITC-avidin and biotinylated

anti-avidin (Vector, Burlingame, USA) or with anti-digoxigenin antibodies and in a second step with anti-mouse antibodies conjugated to Texas-Red (Dianova, Hamburg, Germany). Slides were counterstained with diamidinophenylindole (DAPI) and mounted with Vectashield antifade solution (Vector, Burlingame, USA).

Genotyping and generation of hybrid cell lines

Markers analysed were D17S1873, D17S1841, D17S975, D17S1863, and D17S635 proximal to the *NF1* gene and markers D17S1800, D17S1880, D17S907, D17S1833, D17S1788, and D17S1867 distal to the *NF1* gene. Markers D17S1849 and D17S1166 within the *NF1* gene were also investigated. Genotyping was performed with 6FAM labelled primers and capillary electrophoresis on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). For PCR experiments, we used DNA isolated from blood lymphocytes and hybrid cell lines carrying the deleted chromosome of patient III.2 (hybrid line Nos 4 and 15) and from hybrids with the normal chromosome 17 of III.2 (Nos 8 and 17) to identify the haplotypes. The hybrid cell lines were generated by PEG mediated fusion of a mouse cell line with peripheral blood lymphocytes of patient III.2 (GMP Genetics, Suffolk, UK). Hybrid cell lines were genotyped with the standard set of markers routinely used by GMP Genetics. Marker analysis in all other members of the family was performed using genomic DNA from peripheral blood.

Analysis of markers within the genomic interval between the *KIAA0160* gene and its pseudogene

Dinucleotide repeat marker IVS27AC28.4 in intron 27b of the *NF1* gene was amplified with Cy5 labelled PCR primers as described by Lazaro *et al.*¹⁶ and analysed on an ALF-Express Sequencer (Amersham Pharmacia). The single nucleotide polymorphism SNP2 located in the *KIAA1821* gene was amplified with primers described by Eisenbarth *et al.*¹⁷ Resulting PCR products were digested with the restriction enzyme *MboI* and analysed by agarose gel electrophoresis.

Fine mapping of the deletion breakpoints

The region of the deleted interval and the adjacent segments were analysed by PCR using polymorphic and non-polymorphic STS markers and DNA from a hybrid cell line carrying only the deleted chromosome 17 of patient III.2. PCR products flanking the deletion boundaries, DJ1911/1910, DJ1561/1562, and DJ1948/1936, were amplified with primers DJ1911 (5' CTGCACATGGCATTGTTTGATT 3') and DJ1910 (5' AAACCTCTTGTGCCCTACTC 3'), DJ1561 (5' GTCCAATAAGCAAGAAAAGAGCAA 3') and DJ1562 (5' TGGGGAAGAAAA-CATCAGTCAA 3'), DJ1948 (5' GAGAAAATGAAAGGAGAG-CAAGAA 3') and DJ1936 (5' TCTTAGTGCCTCTGGGAGCAA 3'). PCR products were directly sequenced by cycle sequencing on an ABI 377 sequencer (Applied Biosystems) and analysed using the GCG software (Genetics Computer Group) to determine their origin and to evaluate whether sequences are derived from one or two distinct loci.

Table 1 Oligonucleotides to amplify regional PCR products used as FISH probes

Primer	Sequence (5'→3')	Amplified from BAC (Acc No)	Position on BAC	Size of PCR product (bp)
DJ1576	GACTGAATTGTATGATTTTGCC	640N20	67241	12 453
DJ1578	AATTACTGGG GGCTTCAGGAT	(AC090616)	79694	
DJ1686	CAGGTTATAGGGAAGGAGGAC	640N20	98021	6074
DJ1863	AGCAGCGGTAAGCAATGATG	(AC090616)	104095	
DJHK10	CATGGGGCTTCTAAACCAGA	307A16	4242	4131
DJHK11	GCCTCTGGAGAGGGAGAAGA	(AC003041)	8373

Figure 2 (A) Schematic presentation of the *NF1* gene region at 17q11.2 and the localisation of STS markers as well as FISH probes. The deletion extent was determined by STS analysis of hybrid cell lines carrying only the deleted chromosome 17 from patient III.2 and by FISH on metaphase chromosomes prepared from the index patient (III.2) and his mother (II.2). The horizontal bar represents chromosome 17. The position of the *NF1* gene, the functional *KIAA0160* gene, the functional *WI-12393* gene, and their pseudogene exons (ps) is indicated by arrows. STS markers and FISH probes (DJ1576/1578, DJHK10/11) are indicated by marks below the horizontal line. Deleted markers and probes are highlighted by a frame. BAC clones used for FISH are depicted by horizontal bars. The low copy repeats flanking the *NF1* gene region consist of fragments of the *WI-12393* gene and segments with high homology to chromosome 19 sequences, which are indicated by the grey rectangles. (B) Fine mapping of the deletion boundaries within the *KIAA0160* and *WI-12393* genes and their pseudogenes are shown by vertical rectangles with numbers based on the functional genes. The position of segments amplified with primers DJ1948/1936, DJ1561/1562, and DJ1910/1911 as well as the location of the FISH probes DJ1576/1578 and DJ1686/1863 are indicated beneath the horizontal line representing chromosome 17. As determined by FISH, PCR analysis, and sequencing of these products, the proximal deletion breakpoint maps between exons 5 and 9 of the *KIAA0160* pseudogene. The distal deletion boundary is located between exons 5 and 9 of the functional *KIAA0160* gene.

RESULTS

Deletion detection by haplotype analysis and FISH

Analysis of polymorphic markers showed a large deletion of the *NF1* gene region in the index patient III.2, as hemizygosity for markers D17S635, D17S1849, D17S1166, D17S1800 was observed in the DNA of peripheral blood lymphocytes (fig 1). To confirm these findings, FISH was performed with BAC 14206 (AC079915), which covers the proximal region of the *NF1* gene.¹⁵ A deletion of the corresponding region on one chromosome 17 was observed in all blood lymphocytes of patient III.2 (n=50). Upon this finding, the mother (patient II.2) of the index patient was also investigated by FISH. In her blood lymphocytes, the deletion was detected in 70% of the metaphases analysed (n=50), whereas in fibroblast cultures, the deletion of BAC 14206 was noted only in 15% of all metaphases (n=20) (data not shown).

Delineation of the deletion boundaries by FISH

To determine the extent of the deletion, we performed FISH with further BACs that have previously been mapped to the *NF1* gene region (figs 2 and 3).¹⁰⁻¹⁵ On metaphase chromosome spreads of the index patient III.2 and his mother, BACs 785C15, 3047G17, 499I20, 805L22, and 542B22 are deleted on one chromosome 17. Using BACs 2349P21 and 307A16 as FISH probe (fig 3A, B), a reduced signal was observed on one chromosome 17 homologue suggesting that these BACs span the deletion breakpoints on the affected chromosome. Convincing reduction of signal intensities was not observed using BACs 271K11 and 474K4 as FISH probes, which span the *WI-12393* gene derived LCRs (fig 2A). To confine the dele-

tion boundaries more precisely, FISH was performed with the cloned PCR products DJ1576/1578 amplified from BAC 2349P21 and DJHK10/11 amplified from BAC 307A16 (fig 2, fig 3C, D). Both probes are deleted on one chromosome 17 homologue. FISH probe DJ1686/1863, spanning exon 1 of the functional *WI-12393* gene, hybridised to both chromosomes 17q11.2 and is therefore not deleted. These findings strongly suggest that the proximal deletion breakpoint maps to the region of the *KIAA0160* pseudogene and the distal breakpoint to the functional *KIAA0160* gene (fig 2B).

Identification of the deletion boundaries by PCR

PCR analysis of several STS using DNA from hybrid cell lines that carry only one chromosome 17 homologue of the index patient III.2 allowed us to confirm the presence of a large deletion (fig 2A). The markers that are framed in fig 2A were absent in the hybrid lines 4 and 15, but could be amplified from DNA of hybrids 8 and 17. To narrow down the boundaries more precisely, PCR products located within duplicated *KIAA0160* and *WI-12393* regions were amplified from the hybrid cell lines carrying the deleted chromosome 17 homologue and were sequenced. The location of these three primer pairs, DJ1948/1936, DJ1561/1562, and DJ1910/1911, is indicated in fig 2B. Sequence analysis of amplified products and comparison with the known sequences for 2349P21 (AC127024), 778K9 (AC023266), 307A16 (AC003041), and 640N20 (AC090616) enabled us to determine the presence or absence of the functional *WI-12393* gene and the distally located *WI-12393* pseudogene fragment, as well as the functional *KIAA0160* gene and its pseudogene sequences. The

Figure 3 FISH analysis to investigate the deletion boundaries on metaphase chromosomes in the index patient III.2. BAC and PCR probes from the 17q11.2 region labelled with biotin and detected by FITC (green) were cohybridised with BAC 1D5 (red) as reference, which maps to 17p13. The BACs 2349P21 (A) and 307A16 (B) showed a reduced signal on one chromosome 17 (arrowhead) and thus cover the proximal and distal deletion boundaries, respectively. FISH analysis of probe DJ1576/1578 (C) and DJHK10/11 (D) showed the complete absence of the corresponding sequences on one chromosome 17 homologue marked by an arrow.

PCR product DJ1910/1911 amplified from total DNA of the hybrid cell line 4 carrying only the deleted chromosome 17 contained sequences from both the functional *WI-12393* gene and its distally located pseudogene. This result clearly indicates that the deletion does not include these regions and that the breakpoint lies centromeric to intron 2 of the *WI-12393* gene. The DJ1910/1911 segment occurs only within the LCR sequence on the distal side of the *NF1* gene and is not present in the proximal LCR.¹⁵ Examination of the PCR fragment DJ1948/1936, however, showed that this product was exclusively derived from the *KIAA0160* pseudogene fragment located on BAC 2349P21. The sequence of the DJ1948/1936 product was unique and did not contain the functional *KIAA0160* gene which on the other hand could be coamplified from the hybrids with the normal chromosome 17. By contrast, analyses of the PCR product DJ1561/1562 showed sequences that were solely amplified from the functional *KIAA0160* gene, which is located on BAC 640N20 and 307A16, whereas the PCR product from the normal chromosome was mixed up with sequences from the pseudogene. Therefore, we conclude that the distal and proximal breaks are located within a duplicated segment between exons 5 and 9 of the *KIAA0160* gene and its pseudogene, respectively.

Mechanism underlying the deletion

To determine the recombination mechanism resulting in the deletion, polymorphic markers were also analysed in the grandmother, I.2, of the index patient. Haplotypes were determined in patient III.2 using the hybrid cell lines 4, 15 and 8, 17 carrying only the deleted or normal chromosome of the index patient III.2. The phases of the haplotypes of the grandmother I.2 were inferred on the most parsimonious assumption that the haplotype of II.2 is not the result of a recombination event

between grandmaternal haplotypes within the interval analysed (fig 1). According to our findings, intrachromosomal recombination between highly homologous *KIAA0160* sequences was associated with the deletion.

To show that the deletion occurred somatically in proband II.2, we investigated further markers in the genomic interval between the *KIAA0160* pseudogene and the functional *KIAA0160* gene and observed heterozygosity of proband II.2 in fibroblast cultures for the dinucleotide repeat marker IVS27AC28.4 in intron 27b of the *NF1* gene and for the single nucleotide polymorphism SNP2 located in the *KIAA1821* gene, flanking the *NF1* gene in the 3' direction (fig 4). The heterozygosity of these markers in normal cells of proband II.2 excludes the possibility that the germline deletion occurred during meiosis in the grandmother, and was partially corrected by mitotic recombination or segmental conversion of the deleted chromosome during early embryogenesis in somatic cells of proband II.2.

DISCUSSION

The *NF1* gene has one of the highest mutation rates in humans ($\sim 1 \times 10^{-6}$ /gamete/generation) and about 50% of all *NF1* patients have no family history of the disease. This high mutation rate cannot be simply explained by the enormous size of the *NF1* gene which spans 350 kb. It has been suggested that the high proportion of sporadic *NF1* patients mirrors not only new mutations in the germline of a parent but also postzygotic *NF1* gene mutations associated with gonadosomatic mosaicism in the transmitting unaffected parent (fig 5).¹⁸ Somatic mosaicism is clearly implicated in the pathogenesis of segmental neurofibromatosis,¹⁹ but only documented at the cytogenetic level for one patient.²⁰ With regard to point mutations, somatic mosaicism of the *NF1* gene is difficult to

Figure 4 (A) Heterozygosity of the dinucleotide repeat marker IVS27AC28.4 located in intron 27b of the *NF1* gene was observed in DNA isolated from primary fibroblast cultures. These cultures contained only a minor population of about 15% cells with del(17)(q11.2) (lanes 4 and 5, alleles of 207 and 209 bp). By contrast, the EBV cell line of patient II.2 mainly consists of cells (85-90%) carrying the deletion and thus only one allele of 207 bp was detected by this method (lane 6). In lane 1, an external size standard is given. (B) Heterozygosity of the single nucleotide polymorphism SNP2 in patient II.2. SNP2 is located in the *KIAA1821* gene, which directly flanks the *NF1* gene in the 3' direction. The 388 bp spanning PCR product was digested with *MboI* before gel electrophoresis. As indicated in the diagram, the PCR fragment contains two constitutional *MboI* sites and one polymorphic *MboI* site (arrow). In lanes 1 and 10, standards are loaded with the band sizes given in base pairs on the left. Control DNA from C1 (lane 2) is homozygous for the allele lacking the *MboI* restriction site, the control C2 (lane 3) is heterozygous. In lanes 4 to 6 the *MboI* digested PCR fragments from patient II.2 were analysed and the results for different DNA preparations of the EBV cell line are shown. The percentage of cells carrying the deletion varied between 80-90% in different subcultures. Despite this high percentage of hemizygous cells present in the cultures, heterozygosity is observed at a disproportionate level. Most probably, single stranded DNA molecules without the *MboI* restriction site that are not extended during the final PCR cycles form uncleavable heteroduplexes with alleles containing the *MboI* site. These heteroduplexes presumably increase the amount of the uncleaved 268 bp band artificially. In conclusion, the fibroblasts of patient II.2 (lanes 7 and 8) are heterozygous, although they only contain 15% of cells with the deletion, whereas lymphocytes of the index patient III.2 are hemizygous for the marker SNP2.

detect and to analyse in tissues, but may be one of the factors contributing to the relatively high number of sporadic cases and to the considerable variability of clinical signs and symptoms in NF1 patients.

Interstitial microdeletions, however, can be approached by FISH more easily and thus somatic mosaicism has been detected in a few NF1 patients at the cytogenetic level.^{8, 20-26} Large deletions of the *NF1* region have been reported in six

Figure 5 The different origins of sporadic forms of NF1.

patients who all showed generalised NF1 symptoms not restricted to a particular body region. Four patients had deletions of the whole *NF1* gene as shown by FISH analysis²³⁻²⁶ and two were ascertained by marker analysis.^{21, 22} Moreover, Tinschert *et al*²⁰ described a patient with segmental NF1 who is mosaic for an interstitial *NF1* deletion. Breakpoint boundaries in all these seven cases have not been analysed so far and thus the underlying mechanism of mitotic rearrangements remains unclear.

Here, we describe a female with minor signs of the disease, who, however, is mosaic for a large deletion of the *NF1* gene region. She transmitted this deletion to her son, who developed a severe microdeletion syndrome. The deletion encompasses a segment of ~1.3 Mb and is the result of non-allelic recombination between the *KIAA0160* pseudogene on the proximal side and the functional *KIAA0160* gene located distally to the *NF1* gene (fig 2). This is the first published case of an NF1 microdeletion mediated by recombination within the *KIAA0160* gene. In 29 of 60 NF1 patients with constitutional microdeletions characterised so far with respect to the deletion boundaries, the breakpoints were identified in highly homologous segments of the *WI-12393* gene derived duplicons which flank the *NF1* gene region.¹²⁻¹⁴ The *KIAA0160* gene and its pseudogene are also duplicated segments, which are located in close proximity to the *WI-12393* gene derived low copy repeats (fig 2A). The *KIAA0160* gene, recently termed *JJAZ1* (Joined to Juxtaposed with Another Zinc Finger gene) has been shown to be disrupted by somatically acquired translocations t(7;17)(p15;q21) in endometrial stromal sarcomas. This type of translocation not only fuses the promoter and 5' end of the *JAZF1* gene on chromosome 7 to the coding portion of the *KIAA0160* (*JJAZ1*) gene on chromosome 17,²⁷ but may also reduce the physiological activity of the *KIAA0160* product as a result of hemizygosity at the *KIAA0160* locus. While it seems premature to construct a link between hemizygosity of the *KIAA0160* locus and tumour progression, it might be suspected that the triple event, loss of one *JAZF1* allele, creation of a new fusion transcript between *JAZF1* and *KIAA0160*, as well as the loss of one *KIAA0160* copy triggers the development of endometrial sarcomas as a whole. Similarly, haploinsufficiency of *KIAA0160* (*JJAZ*) may contribute to the outgrowth and progression of neurofibromas in microdeletion patients. Our speculation that *KIAA0160* (*JJAZF1*) serves some tumour suppressive function, which may also be disturbed in endometrial stromal tumours, is consistent with the high risk and early development of tumours observed in patients carrying constitutional microdeletions.

The present study shows that the *KIAA0160* gene in conjunction with its pseudogene represents a second recombinational pitfall in the *NF1* gene region, besides the *WI-12393* gene derived duplicons. Genotype analyses clearly indicate that the deletion occurred somatically in proband II.2 by an intrachromosomal (sister chromatid based) mechanism (figs 1 and 3).

According to previous studies, low copy repeat mediated de novo deletions are believed to be triggered by meiotic recombination during germ cell development.^{14, 28} This notion was based on PCR experiments that failed to identify the patient specific junction product in the blood of their healthy parents.¹⁴ López Correa *et al*²⁸ observed a strong maternal bias for 17q11.2 microdeletions and found that the 1.5 Mb spanning deletions occurred predominantly during maternal germ cell development. In five of the six informative families, interchromosomal recombination (non-sister chromatid exchange) was the underlying mechanism that caused the rearrangements.

Our study implies that somatic recombination between duplicated sequences at 17q11.2 during early embryonic development can lead to gonadosomal and tissue mosaicism with only mild signs of NF1, which do not meet the diagnostic criteria. Subsequent germline transmission of such a microdeletion, however, can generate a very severe form of NF1 in the offspring with developmental retardation and high tumour load. The risk of somatic mosaicism in patients with very mild or ambiguous signs of NF1 who do not fulfil the usual diagnostic criteria is widely ignored or underestimated, but can have dramatic consequences, as in our family.

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Unusual cognitive and behavioural profile in a Williams syndrome patient with atypical 7q11.23 deletion

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Williams syndrome (WS, MIM 194050) is a rare (frequency 1/20 000) multisystemic disorder¹ caused by haploinsufficiency of genes at 7q11.23.^{2–4} WS is associated with dysmorphic facial features, supravalvular aortic stenosis (SVAS) and other cardiovascular diseases, infantile hypercalcaemia, and growth deficiency. The full intelligence quotient (IQ) of WS subjects is usually in the 50s to 60s, with a unique cognitive profile, characterised by relatively good verbal abilities alongside a low level of spatial and constructive organisation.^{5–7} This different pattern of abilities has been named the “WS cognitive profile” (WSCP).⁸

More than 95% of clinically defined WS patients have a de novo deletion of about 1.5 Mb, with the breakpoints clustered within two highly homologous regions flanking the WS region.⁹ Several genes have been mapped within the deleted region,¹⁰ including syntaxin 1A (*STX1A*)¹¹ that codes for a component of the synaptic apparatus, and *RFC2*¹² that encodes a subunit of the replication factor C complex.

While *ELN* haploinsufficiency has been associated with the cardiovascular and possibly connective tissue abnormalities of WS,¹³ the role of other genes in the remaining clinical features of the disease is not known. In particular, it is not clear which gene(s) is responsible for the cognitive and personality profile characteristic of WS patients. It has been reported⁸ that patients with deletions of only *ELN* and *LIMK1* show the characteristic WSCP, generally without mental retardation, but analysis of additional patients harbouring small deletions involving *ELN* and *LIMK1*¹⁴ did not confirm these results. *Limk1* deficient mice exhibit significant abnormalities in spine morphology and synaptic function. They also show altered spatial learning and fear response.¹⁵ The *CYLN2* gene, coding for the cytoplasmic linker protein CLIP-115,¹⁶ localised in the dendritic lamellar bodies of neurones and cerebellar glia cells,¹⁷ has also been considered a good candidate. Very recently, targeted mutation of *Cyln2* has produced evidence that in the mouse haploinsufficiency of the gene produces brain abnormalities, hippocampal dysfunction, and particular deficits in motor coordination.¹⁸

We have identified a patient with many clinical features of WS and a peculiar cognitive profile, without specific spatial and constructive impairment, carrying a 7q11.23 deletion with an atypical telomeric breakpoint.

MATERIALS AND METHODS

Cytogenetic investigations

Chromosome analysis was performed on the proband's blood using standard high resolution techniques. Fluorescent in situ hybridisation (FISH) with the commercially available probe WSR (Vysis Inc, Downers Grove, IL) was performed on the proband's metaphase spreads. Other FISH experiments were performed with bacterial artificial chromosome (BAC) and prokaryotic artificial chromosome (PAC) clones labelled with biotin-dUTP (Vector Laboratories, Burlingame, CA) using nick translation; the labelled probes were visualised with FITC-avidin (Vector Laboratories) and the chromosomes were counterstained with DAPI (Sigma, Milano, Italy); hybridisations were analysed with a Zeiss Axioplan epifluorescence microscope and images captured with a Power Gene FISH System (PSI, Newcastle Upon Tyne, UK).

Key points

- We have identified a patient with a smaller deletion in the WS critical region and an atypical cognitive and behavioural profile.
- The patient had SVAS and vesicoureteric reflux with megaureter. He had normal development, with a mild delay in language acquisition.
- The subject's cognitive performance was compared to an age matched control group of nine WS subjects with a typical deletion (WSCG) and the results were significantly different for both general intelligence (borderline IQ v mild impairment) and for visuospatial and visuoconstructive abilities (relatively preserved v compromised). His cognitive profile did not show the usual WS cognitive and behavioural pattern. His development differs from both the WSCG and normally developing children.
- The patient's deletion ranges from the centromeric common breakpoint region to beyond marker D7S613 and includes elastin (*ELN*), *LIMK1*, and at least a portion of *CYLN2*.
- Our results suggest that deletion of *CYLN2* may cause cognitive impairment, but is not sufficient to produce the typical WSCP. The *GTF2I* gene located in the telomeric portion of the WS critical region could be responsible for some of the cognitive and behavioural features of the disease.

DNA analysis

Microsatellite analysis was conducted on peripheral blood DNA extracted by standard techniques following the protocol described in Perez-Jurado *et al.*⁹ The portion of intron 1 of the *CYLN2* gene containing the previously unreported 4 bp microsatellite polymorphism was amplified with primers *CYLN2* i1F (5'-CTCTTCCCTTTCGGTTGTAATGT-3', ABI-Fam labelled) and *CYLN2* i1R (5'-CGCCTCCACCTGCCTCTTCT-3') and the same PCR protocol used for the other polymorphisms. All primers were purchased from MWG Biotech (Ebersberg, Germany). The 473/477 bp fragments were visualised like all other polymorphisms on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Monza, Italy). The *CYLN2* i1 polymorphism was verified in 50 unrelated normal subjects: 36 were 473/473, 13 473/477, and one was 477/477. Allele frequencies were 0.85 for the 473 bp allele and 0.15 for the 477 bp allele. All sequencing reactions were performed with a Big Dye terminator cycle sequencing kit (Applied Biosystems). Sequences were run on an ABI Prism 310 Genetic Analyzer.

RESULTS

Clinical description

The proband is a 5½ year old boy, the second child of healthy, unrelated parents. Written informed consent for the neuropsychological examination and the genetic analysis was obtained from his parents. The pregnancy was uneventful,

Figure 1 Photograph of the subject aged 5½ years.

except for mild intrauterine growth retardation during the third trimester, shown by ultrasound examination. At birth the child's weight was 2450 g (25th centile), his length was 45 cm (3rd centile), and his head circumference was 34 cm (25th centile). His Apgar scores were 10/10. He had supraaortic stenosis (SVAS, surgically corrected at the age of 23 months) and vesicoureteric reflux with right megaureter (surgically corrected at the age of 17 months). The following facial dysmorphism was evident: mild coarsening of facial features, bitemporal narrowing, sparse eyebrows, downward slanting palpebral fissures, deep set eyes, prominent cheeks, bulbous triangular nasal tip, long and featureless philtrum, and macrostomia (fig 1).

Neurological evaluation was normal, except for slightly decreased muscle tone, with hypertonia of the tibiotarsal joints and increased deep tendon reflexes. Balance was adequate.

Neuropsychological testing

The patient's psychomotor milestones were mildly delayed; he sat at the age of 8 months, walked at 12 months, and spoke his first words at 18 months and first sentences at 36 months. His cognitive profile was assessed at the age of 5½ years. We used a general intelligence test, the Stanford Binet Development

Scale,¹⁹ and a neuropsychological battery, VMI block construction²⁰ and Rey Figure,²¹ in order to assess visual-spatial and visuoconstructive abilities; verbal and spatial working memory and recall memory for drawings were assessed respectively by Digit Span, Corsi Span,²² and Rey Figure Memory task (table 1). The subject showed borderline IQ (IQ=83), with some difficulties in vocabulary (mildly restricted), syntactic organisation (poor), and comprehension. His performance in visual-spatial and visuoconstructive tasks was borderline, like his IQ. Verbal and spatial working memory were mildly impaired, without significant differences between verbal and non-verbal domains. His social behaviour was friendly, and no anxiety trait or overfriendly manner could be detected.

The patient's performance was compared to a group of age matched subjects (six boys and three girls, mean age 5.12 years, SD 0.66) with Williams syndrome and the typical deletion (Williams syndrome Control Group, WSCG), who underwent the same test battery (table 1). The WSCG's performance was characterised by mild cognitive impairment (IQ=68.67, SD 16.29), major impairment in non-verbal abilities, mainly in visuoconstructive tasks, and a very low level of spatial organisation; verbal working memory was better than spatial. The proband's and WSCG's test performances are compared in the last column of table 1. The difference in IQ was statistically significant. The patient's performance was similar to the WSCG in language tasks, but significantly better in spatial and constructive organisation. In fact the nine children of the WSCG were not able to perform the Rey Figure task (copy and memory), which is sensitive to the ability to organise spatially and hierarchically a graphic performance by first copying from a model and later recalling it, while the proband performed at a level adequate to his mental age.

Similar data were obtained from the second copy and draw test, the VMI test. No significant differences with the WSCG were shown by the other tests (block construction, Digit and Corsi Span).

Genetic analysis

FISH analysis using the commercially available probe WSR (Vysis) showed hemizygosity at the *ELN*, *LIMK1*, and D7S613 loci. FISH with clone CTB-8H17, partially overlapping the centromeric cluster of repeated sequences²³ (BAC 1008H17), detected a partial deletion showing a small signal on one chromosome 7 compared to its homologue (fig 2A). The result was confirmed by performing a FISH analysis on a subject with typical WS deletion; the probe shows the same pattern as observed in our patient (fig 2B), as previously described.²³ Additional FISH experiments with clones RP4-665P5²⁴ (fig 2C), RP11-815K3, and CTB-139P11²⁵ gave signals of equal

Table 1 Neuropsychological test scores obtained from the proband, compared to the results obtained from a control group of nine WS subjects with typical deletion. The p value (normal distribution) is shown in the last column; significant p values (p<0.05) are highlighted in bold

Tests	WS subjects with typical deletion		Proband score	p
	Average score	SD		
Chronological age (y)	5.12	0.66	5.60	0.4654
Mental age (y)	3.39	0.63	4.82	0.0484
IQ (score)	68.67	16.29	83.00	0.0166
Corsi Span (score for years)	3.60		3.60	
Digit Span (score for years)	1.80	2.14	3.00	0.1595
VMI (score for years)	3.30	0.38	4.30	0.0353
Rey Figure copy (score for years)	Unmeasurable		4.60	
Rey Figure memory (score for years)	Unmeasurable		4.60	
Blocks - WISC-R (score)	1.40	1.20	2.00	0.2934

Figure 2 FISH analysis of metaphase chromosomes from the patient (A, C) and from a control subject with a typical WS deletion (B). In the patient (A), FISH with BAC clone CTB-8H17 generates a small signal on one chromosome 7 (arrowhead) compared to the other (arrow), indicating a partial deletion. The same result is obtained analysing a subject with a typical WS deletion (B). The clone CTB-8H17, partially overlapping the centromeric cluster of repeated sequences, gives a partial deletion pattern in subjects with the typical WS deletion, as described previously.²³ FISH with PAC probe RP5-665P05 (C) shows signals of equal intensity on both chromosomes 7 (arrows).

intensity on both chromosome 7 homologues, indicating absence of deletion.

Microsatellite analysis⁹ on peripheral blood DNA from the patient, his brother, and his parents confirmed hemizygosity for marker D7S613 and the paternal origin of the deletion, but showed dizygosity for D7S1870. Markers D7S653, D7S1816, D7S489A, and D7S669 were also heterozygous. All other

Figure 3 Molecular genotyping of the proband and his family with microsatellite probes in the WS region. The numbers indicate allele size in base pairs. Alleles belonging to the same haplotype have been vertically aligned and shaded. In the proband, markers D7S489B and D7S2476 have been drawn as deleted based on the FISH results with probe CTB-8H17; marker D7S2472 has been drawn as non-deleted because of FISH results with probe RP5-665P05; marker CYLN2 i1 has been considered deleted for the reasons outlined in the results section.

markers were uninformative (fig 3). The proband and his brother inherited different chromosome 7 haplotypes from their mother and the same haplotype from their father. In the proband the deletion was not associated with a recombination.

Typing of a previously undescribed 4 bp (TTCA) insertion/deletion polymorphism in intron 1 of the *CYLN2* gene (1066 bp downstream of exon 1; position 11736822 on sequence NT_007758.8) showed that the proband's deletion includes at least the 5' end of the gene. In fact, the proband and his brother inherited the same paternal haplotype carrying the 477 bp *CYLN2* i1 allele, but the proband only has the 473 bp allele (fig 3). He could have inherited his father's 473 bp allele only in the unlikely event of a double crossover. Sequence analysis of all *RFC2*¹² and *CYLN2*¹⁶ exons and of several intronic single nucleotide polymorphisms (SNPs) in the region (not shown) did not show any other informative polymorphism.

These results map the extent of the deletion from the typical WS breakpoint on the centromeric side to between intron 1 of *CYLN2* and PAC clone RP4-665P5 on the telomeric side (fig 4).

DISCUSSION

The subject described here meets the diagnostic criteria for WS (clinical features, SVAS, short stature, positive FISH analysis), but his clinical and neuropsychological profiles are unusual. Face features are typical for WS, but more mildly expressed; moreover, inspection of photographs taken at different periods through infancy and childhood showed a gradual reduction of WS facial features (data not shown). The cognitive profile displayed by the patient was different in a number of ways from the WSCG. He had borderline IQ, while the age matched WSCG children were mildly impaired. He did not show the usual WS behavioural pattern. Moreover, his cognitive profile did not show the typical spatial and constructive impairment. However, the patient showed impairment in several competencies. In this sense, his development differs from both the WSCG and normally developing children.

Figure 4 Physical map of the WS region at 7q11.23 (not drawn to scale) showing the relative location of the probes used for FISH analysis (thick horizontal lines), informative polymorphic markers (vertical lines), and selected genes (arrows).

Very few subjects with deletions that do not span the entire WS region have been reported so far.^{8 14 23 26–29} Two patients carrying a deletion spanning from *ELN* to marker D7S1870 still had the full WS phenotype.²⁶ Tassabehji *et al*¹⁴ and Karmiloff-Smith *et al*²⁸ accurately described a SVAS patient carrying a large deletion encompassing all genes in the WS region with the exception of *CYLN2*, *GTF2IRD1*, and *GTF2I*, but no clinical and cognitive WS phenotype. They used the British Abilities Scale II (BAS) scale for the assessment of cognitive abilities, and showed “an above average, even cognitive profile, with no indication of spatial impairment.” Since there is no standardisation of the BAS scale for the Italian population, we used a different cognitive assessment, but explored the same functional areas.

Our patient’s profile is different from that of the subject they described because of his borderline cognitive abilities but similar in the absence of the typical spatial constructive impairment. In the subject described by Korenberg *et al*²³ (subject RM1199), all genes between *FZD9* and *WSCR1* were deleted, while *RFC2*, *CYLN2*, *GTF2IRD1*, and *GTF2I* were not. This 8 year old girl had SVAS, some minor dysmorphisms, and mild mental retardation. No information on her cognitive or behavioural profile was available. Del Campo *et al*²⁹ reported a family with SVAS, borderline mental functioning, gregarious personality, minor facial WBS, and absence of visual/spatial deficits. Molecular analysis showed a 700 kb deletion including all genes from *ELN* to *GTF2IRD1*.

The *GTF2I* gene, located in the telomeric copy of the WS typical breakpoint region and deleted in all WS subjects with a typical deletion,³⁰ is not deleted in our patient. This gene encodes BAP-135, a protein phosphorylated by Bruton’s tyrosine kinase, as well as the transcription factor TFII-I. The centromeric copy of the WS breakpoint region contains a highly similar (99.9% throughout the coding region) transcribed pseudogene, *GTF2IP1*.³⁰ The *GTF2IRD1* gene, coding for a putative transcription factor with ubiquitous expression,^{31 32} may also be preserved. All the other genes in the WS region are deleted.

We cannot rule out that in our patient *CYLN2* may be expressed from an alternative promoter located downstream from the known exon 1, since the transcription pattern of human *CYLN2* has not been fully analysed and its protein

coding region starts in exon 2.¹⁶ On the other hand, a deletion involving at least exon 1 and the entire upstream regulatory portion of the gene is likely to have major effects on its expression.

Tassabehji *et al*¹⁴ suggested that all determinants of the WS phenotype, apart from SVAS, lie telomeric to *RFC2*. Our observations indicate that, while the WS phenotype is the result of the haploinsufficiency of a number of genes, the deletion of the *GTF2IRD1* and/or *GTF2I* genes located on the telomeric side of the WS region is necessary for the syndrome’s unique cognitive profile. Transgenic mice carrying a disrupted *Gtf2ird1* gene³³ did not show any obvious impairment, although it must be pointed out that they were not tested in detail for subtle cognitive and behavioural defects. It is also interesting to note that Osborne *et al*²⁴ described a subject with WS facies, developmental delay, and WS-like behavioural profile (subject 12503) carrying an inversion of the WS region, and hypothesised that *GTF2I* may have been affected by the rearrangement. The *GTF2I* gene may then be critical for the WSCP.

Our data also show that hemizygosity for *LIMK1* and *CYLN2*, while not sufficient to generate the WSCP, may cause alterations in the cognitive profile. Very likely, deletion of *GTF2I*, *GTF2IRD1*, and *CYLN2* (and perhaps *LIMK1*) is necessary to cause the typical WSCP. When, as in the case of our patient, one or more of these genes is preserved, the result is a milder phenotype, with some cognitive impairment (borderline IQ) and a variable loss of visual-spatial and constructive abilities.

Identification of additional subjects with atypical deletions, careful comparison of their genetic, clinical, and neuropsychological profiles, and the development of methods for the molecular analysis of *GTF2I* will be needed in order to assess the contribution of each gene to the WS phenotype.

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High frequency of T9 and CFTR mutations in children with idiopathic bronchiectasis

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Obstructive pulmonary disease is an important health problem in all populations, and bronchiectasis of unknown aetiology (idiopathic bronchiectasis, IB) contributes significantly to the disease. The gene responsible for cystic fibrosis (CF), the cystic fibrosis transmembrane regulator (*CFTR*), was shown to have a role in the manifestation of IB, as

gene mutations and a significantly high proportion of allele T5 of the polythymidine tract (T_n) in intron 8 (IVS8) have been observed in patients.¹⁻³ However, the complex genetic basis of the phenotype expression of IB remains largely unknown. *CFTR* mutations alone cannot be held responsible for the disease, as obligate *CFTR* mutation heterozygotes were shown not to have

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Key points

- We performed genetic analysis at the *CFTR* locus in 73 unrelated Turkish families affected with idiopathic bronchiectasis. Twenty-eight of the unrelated affected children were found to carry mutations, six of them on both *CFTR* chromosomes.
- We detected a total of nine different mutations in 34 of the 146 alleles (23.3%). The most frequent mutation was K68E, which we had previously identified as a rare novel mutation in a CF patient. The spectrum of mutations was very different from those observed in our CF patients. Also, the spectrum of polymorphic alleles was different from both the Turkish CF patients and the normal population.
- Frequencies of alleles T5 and T9 were highly significant compared to the normal population. T9 had not been reported to be frequent in IB patient groups from other populations and not reported to be associated with any disease. Association of 470M, but not a specific (TG)_n allele, with T9 was also highly significant.
- Genotypic homozygosity at the locus was very low, in spite of the high parental consanguinity. Also, all four IB sib pairs and six of the IB healthy sib pairs shared genotypes.
- We suggest that either a modifier gene works in concert with *CFTR* mutations and polymorphisms to manifest the IB phenotype or T9 works as an attenuator for CF.

an increased risk for IB.⁶ The *CFTR* gene seems to act in a multifactorial context, as both the mutations and polymorphic alleles exert their effects in an incompletely penetrant fashion. Therefore, environmental factors and/or other genes are believed to contribute to the disease. IB is only one of the several single organ diseases to which the *CFTR* gene contributes. Some other such diseases are asthma,⁷ obstructive azoospermia,^{8–11} allergic bronchopulmonary aspergillosis,¹² and idiopathic chronic pancreatitis.^{13,14}

Recently we conducted an extensive molecular genetic investigation at the *CFTR* locus in CF patients and showed that the Turkish population had the highest genetic heterogeneity among those studied so far. We also found that CF was quite common, with a carrier frequency of about 1 in 50.¹⁵ We now report the genetic analysis of the *CFTR* gene in Turkish children diagnosed with idiopathic bronchiectasis. What set our study apart from the previous studies are the large number of IB patients, the very high proportion of families with parental consanguinity, and the high consanguinity in the population. High consanguinity highlights the contribution of genetic factors.

MATERIALS AND METHODS

Patients and families

In total, 77 children with IB from 73 unrelated families were included in the study. Eight of the patients were sib pairs. Forty-five of the patients were girls and 32 were boys. DNA samples were available from the great majority of patients' parents and 51 healthy sibs. Thirty-eight patients had no sibs available for study, while 23 had one healthy sib each, nine had two, two had three, and one had four. There were no twins in the study group. Appropriate informed consent was obtained from the families.

The status of parental consanguinity was known in 52 of the families: 27 declared consanguinity (19 were first cousin marriages) and 25 denied it. Two parent pairs in the latter group had originated from the same village, thus some degree of consanguinity could not be excluded.

Clinical findings

Bronchiectasis was diagnosed by computed tomography scan or bronchography. Primary ciliary dyskinesia, α_1 -antitrypsin deficiency, and immunodeficiency were excluded as the cause of the disease. Patients had no other clinical findings such as malabsorption or sinus disease and had normal to borderline sweat chloride values (<60 mEq/l). Eleven of the patients were diagnosed as borderline CF late in the study. Broad clinical information was available for 46 of the patients, 30 girls and 16 boys. These patients had a mean age of 10.3 (SD 3.9) years at the time of the last clinical examination, and the disease manifested at 1 month to 12 years (mean 31.7, SD 44 months). In 12 of them the disease was disseminated. In the rest, it was localised most commonly in the lower left lobe (15), followed by lower right (9), and both lower lobes (8). Bronchiectasis was not localised in an upper lobe in any patient. Seven of the 46 patients had undergone lobectomy, while three others exhibiting a severe clinical course had been assessed in need of but unsuitable for operation. Eleven of the patients had chronic persistent cough and in 25 patients coughing was productive. Ten patients complained only of sputum production. One patient had haemoptysis, five had chest deformity, and 13 had clubbing. Pulmonary function tests were performed in 33 patients above the age of 6 years. The average forced vital capacity (FVC) was 72.6% (SD 23.8) and the forced expiratory volume in one second (FEV1) 68.5% (SD 24.1).

Mutation analysis

The methods have been described in detail by Kylnc *et al.*¹⁵ Briefly, all 27 exons of the *CFTR* gene and the flanking intronic sequences were amplified by polymerase chain reaction (PCR) and analysed by denaturing gradient gel electrophoresis (DGGE).^{16,17} Any pattern variation was investigated by comparing it to known DNA variant marker patterns, and, when necessary, by subsequent DNA sequence analysis. Amplification primers were kindly supplied by Professor M Goossens on behalf of the European Concerted Action for Coordination of Cystic Fibrosis Research and Therapy (ECACF). In addition, patients were screened for five mutations not detectable by the DGGE analysis described above. They were intronic mutations 3849+10kbc>T¹⁸ and 1811+1.6kba>G,¹⁹ deletion mutations *CFTR*dele2,3²⁰ and *CFTR*dele19²¹, and -33A>G in the minimal promoter region.²² Marker DNA samples for the latter region were kindly provided by Dr M Claustres. E1228G was identified by sequence analysis in an ABI 310.

Haplotype analysis

Patients and family members were assayed for a total of six intragenic DNA polymorphisms, five intronic and one exonic, as described previously.¹⁵ The three alleles (T5, T7, and T9) of T_n were amplified by allele specific PCR.³ Reliability of the technique was ascertained by verification by another method that involved nested PCR amplification, cleavage with a restriction enzyme at the created site, and size determination on 8% polyacrylamide gels.⁹ The alleles for the (TG)_n tract upstream of T_n that were associated with alleles T5 and T9 were determined by allele specific PCR amplification and size determination on 8% polyacrylamide gels. Polymorphism 470M/V (A/G variation at nucleotide 1540 in exon 10) was assayed by either DGGE or restriction enzyme digestion.²³ The alleles for the biallelic GATT repeat in intron 6 and the three multiallelic microsatellites (IVS8CA in intron 8 and IVS17bTA and IVS17bCA in intron 17b) were resolved on polyacrylamide gels.^{24–26} Haplotypes were constructed by segregation analysis using the available DNA samples from parents and sibs.

Statistical analysis

χ^2 test was applied to contingency tables to detect statistically significant differences in allele frequencies²⁷; *p* values of less

Table 1 The *CFTR* mutations identified in 73 unrelated IB patients

Mutation	No of alleles	Consequence
K68E	9	Lys to Glu at 68
-33G>A	7	Promoter mutation
N1303K	6	Asn to Lys at 1303
<i>CFTR</i> dele19	4	Deletion of exon 19
		Frameshift
3272-26A>G	3	mRNA splicing defect
<i>CFTR</i> dele2,3	2	Deletion of exons 2 and 3
		Frameshift
F1052V	1	Phe to Val at 1052
E1228G	1	Glu to Gly at 1228
W1282X	1	Trp to Stop at 1282
Total alleles	34	

than 0.05 were considered to indicate significance and less than 0.01 high significance.

RESULTS

In total, 69 unrelated children and four sib pairs with the IB phenotype were screened for mutations in the *CFTR* gene. Mutations were identified in 28 of the unrelated patients. Nine different mutations were identified in 34 (23.3%) of the 146 *CFTR* chromosomes analysed. Five patients were homozygous for *CFTR* mutations, one each for 3272-26A>G, N1303K, and *CFTR*dele19, and two for K68E. Another patient was a compound heterozygote for -33A>G and *CFTR*dele2,3. Twenty-two patients carried one mutation each. The chromosomal backgrounds were determined, and *CFTR* haplotypes were constructed to assess identity by descent. The frequencies of T9 and T5 as well as whether any alleles of 470M/V and (TG)_m were associated significantly were investigated by comparing our CF patient group and the normal control group reported previously.¹⁵

Spectrum of mutations

The mutations detected at the *CFTR* locus in the IB patients and their frequencies are shown in table 1. The most frequent mutation was K68E (nine of the total 34 mutant chromosomes), a mutation we recently identified in a Turkish CF patient.¹⁵ The next most frequent mutations were -33G>A and N1303K, observed on seven and six chromosomes, respectively, and 3272-26A>G was found on three chromosomes. Large deletion mutations *CFTR*dele19 and *CFTR*dele2,3 were observed in four and two chromosomes, respectively. One patient had W1282X and another F1052V, while the last one had the novel mutation E1228G, which resulted from an A to G transition at nucleotide 3815 (sequence data available on request). An uncharged polar amino acid was substituted for an acidic one at residue 1228 in exon 19 in the second nucleotide binding domain of the protein. The residue has been conserved in human, bovine, *Xenopus*, and dogfish.²⁸ No other mutant allele was identified upon screening for the two intronic mutations 3849+10kbC>T and 1811+1.6kbA>G, which were reported to be quite common in southern Mediterranean populations.^{18, 19}

The spectrum of mutations and their frequencies differed from those in our CF patient group. F508del, 1677-1678delTA, 2183AA>G, and G542X, the most common four mutations in our CF patients comprising 52% (64/125) of all mutant chromosomes,¹⁵ were not observed at all in the IB group. Only four of the total of nine different mutations we found in the IB group were also found in the 166 CF chromosomes we had analysed.¹⁵ The numbers in CF patients were as follows: one K68E, four N1303K, one *CFTR*dele2,3, and five W1282X. Three of the remaining IB mutations (3272-26A>G, *CFTR*dele19,

F1052V) were identified in other studies in CF patients,^{16, 21, 29} one (-33G>A) in a CBVAD patient,²² and the last one (E1228G) in this study.

Significance of the allelic frequencies at three polymorphic loci

Certain alleles of T_n and (TG)_m and allele 470M, alone or in association with others, have been implicated in the aetiology of bronchiectasis.^{1, 3, 4} We investigated whether any alleles were associated with IB in our patients. We had found the frequencies of T9, T7, and T5 in the normal chromosomes to be 28, 162, and 10, respectively, in a total of 200.¹⁵ We found the frequencies of T9, T7, and T5 in the IB chromosomes in which no mutation was identified (no mutation chromosomes) to be 71, 30, and 11, respectively, in 112. The frequency of T5 with respect to T7 in the IB no mutation chromosomes was found to be highly significant compared to the normal population (11/41 versus 10/172). The frequency of T9 with respect to T7 in no mutation IB chromosomes also was significantly higher than in the normals (71/101 versus 28/190). Regarding the IB chromosomes harbouring mutations, we found the frequencies of T9, T7, and T5 to be 20, 13, and 1, respectively, in 34. Thus, the frequency of T9 with respect to T7 was highly significant (20 versus 13) as compared to the CF mutant chromosomes (17 versus 102).¹⁵

The frequency of 470M was similar in the normal T9 chromosomes (13/28) and the normal T7 (66/157). Similarly, the difference in the association of 470M between the T7 IB no mutation chromosomes (16/29) and the T7 normals (66/157) did not reach significance. However, T9 no mutation IB chromosomes had a highly significant association with 470M in comparison to the T9 normals (44/70 versus 13/28). In addition, while all of the 10 normal T5 alleles were associated with 470M, five of 11 IB T5 no mutation chromosomes were on 470V background.

The frequencies of the (TG)_m alleles were similar in T9 IB no mutation chromosomes (58 TG9 and 13 TG11) and normal T9 chromosomes (24 TG9 and four TG11). In contrast, the association with the T5 chromosomes was different: seven of the T5 IB no mutation chromosomes were on TG11 background and four on TG13, while all of the 10 normals were on TG11.

In summary, T9 showed a highly significant association with the disease, and 470M was highly significant in the T9 no mutation IB chromosomes. Allele T5 also showed a highly significant association with the disease and increased association with both TG13 and 470V.

Haplotypes

Haplotypes could be determined in 71 of the unrelated patients with respect to the alleles at the polymorphic loci (GATT)_n, T_n, and 470M/V, rare polymorphisms detected in the course of mutation screening, and mutations. Also, the (TG)_m alleles associated with the T5 and T9 alleles were determined. We later refined the haplotypes by analysing three microsatellite loci. A large number of different haplotypes were observed: 22 among the total of 34 with mutations and 53 among the total of 112 without mutations.

The T5 IB chromosomes also showed great variation as compared to normals. There were 10 T5 chromosomes among the 200 normals, and they were all on GATT7-TG11-470V background. Moreover, they had in total only three different haplotypes with respect to the three microsatellite polymorphisms IVS8CA, IVS17bTA, and IVS17bCA: 14-30-13, 17-30-13, and 17-33-13 with frequencies of 3, 3, and 4, respectively. In contrast, T5 IB chromosomes were mostly on backgrounds GATT7-TG11-470M (five of 12) and GATT6-TG13-470V (four of 12). The remaining three haplotypes were observed once: GATT6-TG11-470M, GATT7-TG11-470V-1001+11T-2694T-4002G, and GATT7-TG13-470V-F1052V-2694T. When the three microsatellites were also taken into account, no two of the T5 IB chromosomes had the same haplotype.

Table 2 The IB haplotypes at *CFTR* with respect to (TG)_m, T_n, 470M/V, rare polymorphisms, and mutations

IVS8 (TG) _m	IVS8 (T) _n	Polymorphism	Mutation	No of chroms	No of heterozyg patients	No of homozyg patients
11	5	470M	–	6	4	1
13	5	470V	–	4	0	2
	7	470M	–	10	8	1
	7	470V	–	10	8	1
9	9	470M	–	40	26	7
11	9	470M	–	4	4	0
9	9	470V	–	17	11	3
11	9	470V	–	8	8	0
11	7/9	470M/V	–	2	0	(1)
11	5	470V, 2694T	–	1	1	0
	7	1001+11T, 4002G	–	1	1	0
	7	470V, 2694T	–	1	1	0
	7	875+40G	–	1	1	0
	7	470V, 2694T	–	1	1	0
	7	470V, 2694T	–	1	1	0
	7	470M, 1001+11T	–	1	1	0
	7	470M, 875+40G	–	5	3	1
9	9	470V, 2694T	–	1	1	0
13	5	470V, 2694T	F1052V	1	1	0
	7	470M	CFTRdele19	2	2	0
	7	470V	CFTRdele19	2	0	1
	7	470M	W1282X	1	1	0
	7	470V	CFTRdele2,3	2	2	0
	7	470M	E1228G	1	1	0
	7	470M	–33G>A	2	2	0
	7	470V	–33G>A	3	3	0
9	9	470V	–33G>A	2	2	0
9	9	470M	K68E	9	5	2
9	9	470V	N1303K	4	4	0
9	9	470M	N1303K	2	0	1
9	9	470V	3272-26A>G	1	1	0
9	9	470M	3272-26A>G	2	0	1

Comparison of the genotypes of the sibs

We compared the *CFTR* haplotypes of the patients to their sibs (table 2). All of the four affected sib pairs shared both of their haplotypes, indicating that both of the *CFTR* chromosomes contributed to the phenotype. The genotypes were T9-N1303K homozygous, T9/T9-N1303K, T9/T9-3272-26A>G, and T7/T5. However, six affected-healthy sib pairs also shared both of the haplotypes, excluding the gene as the sole locus responsible for the disease phenotype. The genotype of one pair was T7/T7-*CFTR*dele19, three were T9/T9, and two were T9/T7.

Identity by descent

The haplotypes were evaluated to assess identity by descent. In total, nine patients exhibited haplotype homozygosity, three with mutations and six with no mutation detected. The family of one of them had declared parental consanguinity and of four others had denied it. Patients from 26 other families who had claimed parental consanguinity (including 18 first cousin marriages) were not homozygous.

DISCUSSION

CFTR mutations were identified in 23.3% (34/146) of the *CFTR* chromosomes in our 73 unrelated idiopathic bronchiectasis patients. This frequency is very high, as the carrier frequency in our population was assessed as 1/50.¹⁵ It was intriguing that F508del was not observed in our IB patients, although it is the most common mutation (23.5% of the mutant chromosomes) in our CF patients.¹⁵ This mutation comprised three of the mutant alleles among the total of 19 detected in the adult Italian disseminated bronchiectasis patients,³⁰ three of 16 in the French patients,² and two of seven in the Greek mostly adult patients.⁵ Conversely, three of the mutations in our IB patients

(3272-26A>G, *CFTR*dele19, and –33A>G) were not carried by any of our CF patients¹⁵ (this study). Also, none of the mutations detected in our IB patients was found in the French, Italian, or Greek IB patients. This was surprising since N1303K and 3272-26A>G are both common CF mutations in most Mediterranean countries. K68E, the most common mutation in our IB group, was observed only once in the 166 CF chromosomes we had analysed. It manifested a very mild phenotype.¹⁵ It has been reported in only one other person, a CF patient in north eastern Italy.³¹ The next most common mutation/gene variation, –33A>G, had been identified on a CBAVD chromosome, out of 159 CBAVD, 376 CF, and 238 normal chromosomes, and was proposed to be either a rare polymorphism or a mutation that affected the regulation of the gene.²² We detected it in seven of the 146 IB chromosomes, but not in any of the 41 CF no mutation chromosomes or 44 control samples. Therefore, we propose that it is a pathogenic mutation, but perhaps associated with CBAVD, IB, or mild CF. It was associated *in trans* with either T5 (two patients), T7 (four patients), or T7-*CFTR*dele2,3 (one patient), but in no case with T9. The next most common mutations were N1303K and *CFTR*dele19, both of which manifest severe CF phenotypes.^{21–32} These five mutations make up 85.3% (29/34) of all mutant IB chromosomes, and 20 of these 29 chromosomes were on TG9-T9 background. It is worth mentioning here that in our population F508del is mostly on T7 background: 38 of the total 39 F508del chromosomes we had analysed were on this background.¹⁵ Also interesting was that all of the three 3272-26A>G mutant alleles in our IB patients were on T9 background in contrast to the European alleles which were all on T7 background.^{33–34} All of these observations point to a role of T9 in the aetiology of IB.

In assessing the significance of T9 and T5, we compared their frequencies to those of T7, since it is the most common allele

world wide and has not been implicated in any kind of pathogenesis. Alleles T5 and T9 both showed a highly significant association with IB, and 470M was similarly associated with the T9 IB chromosomes. T5 was shown to be high also in the Italian patients (mean age 53 (SD 15.8)),⁴ but not in two French and one Greek mostly adult patient group.^{2,5,35} In addition, the T5 chromosomes in our IB patients were mostly on backgrounds TG11-470V and TG13-470M, whereas the normal T5 chromosomes were all on TG11-470M. Thus, the IB chromosomes all deviated from TG11-470M. This was not surprising, since the association of both 470V and a low number of TG repeats have been shown to lead to lower gene activity.³⁶ The 470M allele leads to a higher protein activity, 470M protein having 1.7-fold intrinsic chloride channel activity compared to that of 470V in transfected cells, and the lower the (TG)_m repeat number, the less the proportion of the mRNA lacking exon 9 sequences transcribed from T7 *CFTR* chromosomes in transfected cells. Noone *et al*³⁷ studied a patient who had CF type lung disease with normal to borderline sweat chloride values and was homozygous for haplotype T5-TG12-470V. She had defective *CFTR* mediated chloride conductance in epithelia.

In addition to confirming the previous findings on increased frequencies of *CFTR* mutations and T5 in IB patients,¹⁻⁵ this report highlights T9, an allele that had not been reported previously in association with any disease. Despite the fact that this allele results in normal transcripts (not lacking exon 9), it is not the most common allele in the normal population world wide, thus is not the most common allele. The high frequency of T9 chromosomes in our patients cannot be attributed to a possible association with an as yet unidentified common mutation, because the allele was on a large number of different haplotypes. We also observed a significant association of 470M with the T9 no mutation IB chromosomes. Molecular studies are necessary to elucidate the basis of the pathogenesis.

Two opposing hypotheses could be proposed to explain the role of T9 in the aetiology of IB. T9 could be a predisposing genotype that does not lead to disease on its own, but leads to IB when in association with defects in an as yet unidentified gene. Alternatively, T9 could be an attenuator for CF instead of a mutation with a role in the aetiopathogenesis of IB. A higher *CFTR* activity conferred by T9 (more so in association *in cis* with 470M) could dampen the effect of an *in cis* mutation and manifest a milder form of CF. This fits in well with the model that mild *CFTR* mutations lead to milder forms of disease, such as disseminated bronchiectasis and obstructive azoospermia.³⁸ As for the no mutation patients, T9 would be expected to compensate for defects in the hypothetical gene. The hypothetical gene would be expected to exert its effect in an autosomal recessive fashion, and the frequency of its defective form in the population would be lower than CF mutations. IB associated with this gene would thereby be noticeable only in populations with high consanguinity, similar to the Turkish population.

The attenuator hypothesis would explain why 12 of the patients developed a more CF-like disease several years after IB diagnosis. One such patient had mutations on both *CFTR* chromosomes (-33G>A/*CFTR*Δle2,3). He had the lower left lobe removed at the age of 4 and was referred to our clinic at the age of 5 with borderline sweat test values of 52 and 50 mEq/l. Eighteen and 20 months later the values were high (64 and 63 mEq/l, respectively), and he had developed CF-like gastrointestinal problems. The remaining five patients with two mutations had normal sweat test values (<40 mEq/l) and no gastrointestinal complaints at the ages of 7, 11, 13, 13, and 14. It will be interesting to follow whether these patients also develop CF-like clinical findings in the future.

Identification of an IB modifier gene would be of much clinical value, as no gene other than *CFTR* has been implicated in the aetiology of this common disease. A modifier gene has already been identified for idiopathic chronic pancreatitis associated

with *CFTR* mutations.³⁹ The four IB sib pairs and their six unaffected sibs would give sufficient genetic information in a genome scan study aiming at the identification of the locus for the hypothetical gene. Identification of the gene would also increase our knowledge on the pathogenesis of *CFTR* defects and shed light on other diseases associated with *CFTR*.

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FMRP expression studies in blood and hair roots in a fragile X family with methylation mosaics

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The fragile X syndrome is a common cause of familial mental retardation with an estimated prevalence of 1/4000-1/6000 for males in western countries.¹⁻³ This X linked disorder is characterised by mental retardation with additional features like a long face with large protruding ears, macro-orchidism, and eye gaze avoidance.⁴⁻⁶ The causative mutation is an amplification of a trinucleotide (CGG) repeat in the 5' UTR of the *FMR1* gene. Normal people have between six and 54 CGG repeats, carriers of the premutation have between

55 and 200, and affected subjects have more than 200 CGG repeats in their *FMR1* gene, the so called full mutation.^{7,8} The latter expansion is accompanied by hypermethylation of the repeat and its upstream region resulting in a shutdown of transcription and absence of the FMRP.⁹⁻¹¹

In fragile X patients, two special subclasses of mosaicism can be distinguished on the basis of size and methylation pattern: (1) subjects with a premutation in a proportion of their cells in addition to a full mutation, often referred to as "size

Key points

- In fragile X patients two special subclasses of mosaicism can be distinguished on the basis of size and/or methylation pattern: patients with full mutation and premutation, called "size mosaics", and patients with intercellular variations of the methylation status, called "methylation mosaics".
- Within a known fragile X family, three brothers with methylation mosaic patterns were studied using the FMRP antibody test on both blood smears and hair roots. The index patient aged 10 years (case 1) was diagnosed at the age of 5 years; he was mildly retarded and had some clinical fragile X features. DNA analysis showed a full mutation (200-250 repeats) with 86% unmethylated mutations. His 5 year old brother (case 2) had a mutation of 177 repeats that was unmethylated in 67% of cells. His (early) development was considered normal and he lacked additional fragile X features. The third brother (case 3) had a mutation of 183-187 repeats that was unmethylated in 86% of cells. His development was normal at the age of 3 years and he did not have any significant fragile X features.
- In cases 1, 2, and 3, FMRP expression in blood (in duplicate) was 20% and 10%, 22% and 10%, and 7% and 2%, respectively, and 67%, 85% and 88% of their hair roots expressed FMRP.
- These results suggest that FMRP expression in hair roots gives a better reflection of the mental development than FMRP expression in leucocytes, which is consistent with the common embryonic origin of hair roots and neuronal cells.

mosaics"; this pattern can be observed in 20-40% of male patients^{12,13}; (2) subjects with intercellular variations in the methylation status of a full mutation, "methylation mosaics".¹⁴ In a large multicentre study, "methylation mosaicism" was observed in 3% of the males with a full mutation.¹²

In 1995, Willemsen *et al*¹⁵ developed an FMRP antibody test for detecting the presence or absence of FMRP in lymphocytes and later in hair roots.¹⁶ This test allowed for screening for the fragile X syndrome among mentally retarded males¹⁷ and in addition made quantification of the number of FMRP expressing cells in patients possible. Using this technique in blood smears, Tassone *et al*¹⁸ were able to find correlations between FMRP expression and IQ in males with size mosaicism and methylation mosaicism. Very recently, a highly significant correlation has been found between FMRP expression in hair roots and cognitive functioning in females carrying a full mutation (R Willemsen, in press).

Here, a study of FMRP expression in blood and hair roots is reported in three male sibs with a varying degree of methylation mosaicism.

PATIENTS AND METHODS

The three brothers who are the subjects of this report are from a fragile X family known to the Department of Clinical Genetics, Rotterdam. The family was ascertained through a son of the mother's sister who had the classical clinical presentation of the fragile X syndrome confirmed by a fully methylated full mutation of the *FMR1* gene.

DNA analysis

Genomic DNA was isolated¹⁹ from blood leucocytes digested with *HindIII* and the methylation sensitive enzyme *EagI* and hybridised with probe pP2 according to standard protocols.²⁰

Sizing of the Southern blot and densitometry were done using a Kodak Electrophoresis Documentation and Analysis System 120.

Protein analysis

Blood smears were made from one drop of blood within two hours after collection. Slides were air dried. Hairs were plucked from different locations on the scalp and analysed within 24 hours. The FMRP was visualised by using monoclonal antibodies 1A1 against FMRP.²¹ Further immunoincubations were performed according to procedures described previously^{15,22} (<http://www.eur.nl/FGG/CH1/fragx/>). A total of 100 leucocytes were analysed per patient and scoring for FMRP expression was performed by two people independently.

Determination of IQ levels

The Wechsler Intelligence Scale for Children-Revised (WISC-R), the McCarthy Scales of Children's Abilities (MSCA), the Wechsler Preschool and Primary Scale of Intelligence (WPPSI-R), and the Peabody Picture Vocabulary Test-Revised (PPVT-R) were used to test the intellectual abilities by one examiner (AJ) who was not informed about the genetic status of the children tested. The WISC-R is suitable for children from the age of 6 years whereas the MSCA, the WPPSI-R, and PPVT-R should be used for younger children. The verbal, performance, and full scale IQ scores were calculated.

RESULTS

Case reports

Case 1

This boy was born after a normal pregnancy and delivery with a birth weight of 3250 g. In his first year of life he had frequent ENT related problems which disappeared after tonsillectomy and the insertion of grommets. His early development was somewhat slow but within the normal range: he sat at 8 months, walked unaided at 18 months, and spoke his first words at 1 year. At the age of 2 years hyperactive behaviour was noted which disappeared at 4 years of age. However, at that age he appeared to be unable to attend normal school. For further evaluation he was referred to our centre and because of the family history (the mother's sister had two sons with the fragile X syndrome) DNA analysis of the *FMR1* gene was performed.

At the age of 5 years he had normal physical measurements, height 1.17 m (70th centile) and head circumference 52.5 cm (75th centile). He had a long, narrow face with a normal chin, normally shaped and sized ears, periorbital fullness, and normal teeth (fig 1). His testes were mildly enlarged for his age (4 ml/4 ml). He had hyperextensible finger joints and relatively broad and short halluces. His behaviour was normal with normal eye contact.

Psychological testing at the age of 10 years, using the WISC-R, showed a full scale IQ score of 55 with a verbal IQ score of 56 and a performance IQ score of 61.

Case 2

This boy is the 4 year younger brother of case 1. He was also born after an uneventful pregnancy and delivery with a normal birth weight. His early development was normal: he sat at 9 months, stood at 10 months, and walked unaided at 14 months. Because of the diagnosis in his older brother, he was tested for the fragile X syndrome at the age of 1 year 3 months.

At the age of 5 years he had normal physical measurements, height 110 cm (25th centile) and head circumference 51.8 cm (60th centile). He had a normal face, except for a broad forehead which was observed in the father as well, some periorbital fullness, and normal ears (fig 1). His genitals were normal. His behaviour was normal with normal eye contact.

Figure 1 (A) Case 1 at the age of 5 years, (B) case 2 at the age of 5 years, and (C) case 3 at the age of 3 years.

Psychological testing at the age of 5 years showed a full scale IQ score of 81 with the MSCA and 75 with the WIPPSI-R.

Case 3

This boy is the 6 years younger brother of case 1. He was born after a normal pregnancy and delivery with a birth weight of 3750 g. His development was normal: he walked at 14 months and spoke normal sentences at 3 years.

At the age of 3 years he had normal physical measurements, height 1.00 m (50th centile) and head circumference 51.5 cm (70th centile). He had no dysmorphic facial features, except for a broad forehead which was observed in the father as well; he had normal sized and shaped ears. His genitals were normal. He had some hyperextensibility of MCP V. His behaviour was normal with normal eye contact. Psychological testing at the age of 3 years 8 months showed a full scale IQ score of 91 with the MSCA and 97 with the PPVT-R.

Molecular findings

In case 1, a full mutation was found in his leucocytes using Southern blot analysis: a 14% methylated 560 bp larger than

normal band (~217 repeats) and a 86% unmethylated 670 bp larger than normal band (~253 repeats) (fig 2).

Protein analysis in blood smears (in duplicate) showed 10% and 20% of the leucocytes and 67% of the hair roots (10/15) expressing FMRP.

In case 2, a mutation that was smaller than in case 1 was found in leucocytes using Southern blot analysis: a 33% methylated 440 bp larger than normal band (~177 repeats) and a 67% unmethylated 440 bp larger than normal band (~177 repeats).

Protein analysis in blood smears (in duplicate) showed 10% and 22% of the leucocytes and 85% of the hair roots (17/20) expressing FMRP.

In case 3, a mutation similar in size to that of case 2 was also found in leucocytes using Southern blot analysis: a 14% methylated 470 bp larger than normal band (~187 repeats) and an 86% unmethylated 460 bp larger than normal band (~183 repeats) (fig 2).

Protein analysis in blood smears (in duplicate) showed 2% and 7% of the leucocytes and 88% of the hair roots (23/26) expressing FMRP.

Table 1 Summary test results in the three brothers

	IQ score	<i>FMR1</i> % unmethylated blood	<i>FMRP</i>	
			Lymphocytes (in duplicate)	Hair roots
Case 1	55	86%	10 and 20%	67%
Case 2	75 and 81	67%	10 and 22%	85%
Case 3	91 and 97	86%	2 and 7%	88%

The test results are summarised in table 1.

DISCUSSION

According to the definition of mosaicism, all fragile X males are mosaic as they have different amplified repeat sequences in the *FMR1* gene in different cells. However, two special subclasses can be distinguished; 20–40% of fragile X males have a premutation in a proportion of cells and the full mutation in the remaining (majority) of cells. In general, the proportion of cells with a premutation is lower than <30% and various studies have shown that the cognitive functioning of these fragile X patients is not significantly better than the males with a full mutation, suggesting that the number of *FMRP* expressing cells with a premutation is insufficient.^{12–23} However, high functioning males with a size mosaic pattern have been described^{24–26} and Tassone *et al*¹⁸ detected a correlation between *FMRP* expression in blood smears and IQ in mosaic males.

For the second group of mosaic patients, the so called “methylation mosaics” the situation is different. These patients have a full mutation in all cells but in a proportion of cells the full mutation is unmethylated. The cells with an unmethylated full mutation are able to produce *FMRP* and can therefore function normally. Depending on their proportion, they are able to compensate for the loss of functioning of the cells with a methylated full mutation. Various reports on patients with methylation mosaicism suggest that

a proportion of cells with an unmethylated full mutation of at least 40% of normal is likely to be required for normal cognitive functioning.^{24–27–36} This is supported by actual *FMRP* studies in blood smears of patients with methylation mosaicism that showed that all mosaic patients with a normal IQ had *FMRP* in $\geq 50\%$ of lymphocytes.^{18–32} This situation is reminiscent of the situation in females with a full mutation where cognitive function is related to the X inactivation pattern.^{12–14–37–39}

FMRP expression studies in blood smears of methylation mosaic males have been reported. Smeets *et al*²¹ reported normal protein expression in cell lines of two normal functioning adults with an unmethylated full mutation. De Vries *et al*³² reported three cousins with 75%, 40%, and 10% cells expressing *FMRP* who had an unmethylated full mutation in 90%, 35%, and 10% of the cells, respectively. The latter two were both retarded whereas the adult males with 75% *FMRP* expressing cells had a normal IQ. Tassone *et al*¹⁸ found a correlation between IQ and *FMRP* expression in blood smears in 13 males with a partially methylated full mutation. They also found three non-retarded mosaic males with expression of *FMRP* in $\geq 50\%$ of lymphocytes.¹⁸ The findings of normal *FMRP* expression in partially unmethylated full mutations were in contrast with the report of Feng *et al*,⁴⁰ who found markedly diminished *FMRP* production in fibroblast clones from transcripts with more than 200 repeats. These conflicting findings raise the question of whether unmethylated full mutations have normal or diminished *FMRP* expression, what is the relation to cognitive functioning, and what is the correct tissue to study. Interestingly, Tassone *et al*⁴¹ reported a six-fold increase of *FMR1* mRNA levels in methylation mosaic males suggesting the existence of a compensatory response to impeded *FMRP* production.

In the oldest of the three reported brothers, the size of the (un)methylated alleles are all in the full mutation range whereas the other two brothers have (un)methylated alleles in the high premutation range; thus the latter two do have partially methylated premutation sized alleles which is quite rare.

The proportion of cells expressing *FMRP* in a blood smear ascertained by the *FMRP* antibody test did not correspond very well with the proportion of unmethylated *FMR1* alleles (pre- or full mutation sized) as ascertained by DNA blotting analysis. It suggests that in leucocytes the translation might also be hampered in the large unmethylated premutation sized alleles. It also shows that accurate prediction of mental functioning in males with an intercellular variation of the methylation status through *FMRP* studies in blood smears is, like DNA analysis, less valid. However, *FMRP* expression in hair roots did reflect the cognitive functioning in the three brothers. Both brothers with normal IQs (81 and 91) had a high proportion of *FMRP* expressing hair roots (85% and 90%, respectively). This is consistent with the common embryonic origin, ectoderm, of hair roots and neuronal cells whereas blood is of mesodermal origin. Of course a larger number of males with (un)methylated full mutations need to be tested to assess the validity of the relationship between *FMRP* expression in hair roots and mental functioning.

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A novel locus for autosomal recessive primary microcephaly (MCPH6) maps to 13q12.2

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Key points

- Autosomal recessive primary microcephaly (MCPH) is a genetic disorder in which an affected subject is born with a head circumference >3 SD below the expected mean and is mentally retarded.
- We report a novel locus (MCPH6) mapped to chromosome 13q12.2 in a Brazilian family.
- The minimal critical region spans 6 Mb between markers AL139378GT17 and D13S1244 with a maximum two point lod score of 6.25.

Microcephaly is the clinical finding of a head circumference measurement greater than three standard deviations (SD) below the population mean for age and sex. It is usually accompanied by mental retardation and there are many diagnoses with both environmental and genetic aetiologies.¹ Autosomal recessive primary microcephaly (MCPH) (MIM 251200) is a disorder in which affected subjects are born with a small head circumference, explained by a cerebral cortex of reduced size, and are mentally retarded. The brain is structurally normal and, apart from the intellectual impairment, there are no other significant neurological problems, dysmorphic features, or malformations.^{2,3} In a study carried out in The Netherlands,⁴ the incidence of MCPH was approximately 1/250 000 but it is probably greater in populations with a high rate of consanguineous marriages. MCPH has been shown to be genetically heterogeneous with the identification of five loci: MCPH1 on 8p23,⁵ MCPH2 on 19q13,⁶ MCPH3 on 9q34,⁷ MCPH4 on 15q15-q21,⁸ and MCPH5 on 1q31.^{9,10} MCPH1, 2, and 3 were mapped in northern Pakistani families, MCPH4 in

a Moroccan family, and MCPH5 in northern Pakistani and Turkish families. Here we report the identification by autozygosity mapping¹¹ of a novel locus for primary microcephaly, MCPH6, in a north eastern Brazilian family.

MATERIALS AND METHODS

Subjects

The consanguineous family had eight affected subjects (five males and three females, DNA available from seven subjects), with ages varying between 4 and 27 years (fig 1), in four sibships (fig 2). The head circumference of all affected subjects was noted to be small at birth and between 7–10 SD below the expected mean when examined by us. All had mental retardation of moderate severity: the three adults and the adolescent affected were unable to read or write but could speak simple phrases and had basic self-care skills. With the exception of intellectual impairment, there were no other neurological problems (including fits) and motor development had been normal. All eight were in good health and had growth parameters within normal limits. They were not dysmorphic and no syndrome diagnosis could be made. No past medical history or environmental causes could be found to explain the finding of microcephaly. The parents had normal head circumference and intelligence. Ophthalmological examination, standard lymphocyte karyotype (400 bands), and electroencephalogram performed in four affected subjects were normal, and brain scans in two showed no cerebral malformations or neuronal ectopia.

Molecular genetics

Linkage to the five known MCPH loci was ruled out (data not shown). An autosomal chromosome screen for regions of shared homozygosity was performed on seven of the eight

Figure 1 Six of the eight affected subjects with ages between 4 and 27 years with a diagnosis of autosomal recessive primary microcephaly.

Figure 2 Genotypes for eight markers used in the study at 13q12.2 arranged centromere to qter. Unaffected sibs have been omitted for clarity. Marker order was taken from the Marshfield linkage map. The boxed region shows the shared region of homozygosity in affected subjects. The *FGF9* gene, indicated by an arrow, is flanked by markers AL139378GT21 and AL139378GT17 and hence is excluded as a candidate gene from the common homozygous region in affected subjects.

affected subjects and their parents with the CHLC/Weber Human Screening Set version 8 (Research Genetics), which contains 365 autosomal microsatellite repeat markers spaced at approximately 10 cM intervals. PCR amplification of all markers was performed according to the manufacturer's specifications using a Roboseq 4200 (MWG BioTech Ltd). Amplified markers were pooled and electrophoresed on an ABI Prism 377 gene sequencer (Applied Biosystems) on 4.2% polyacrylamide gels, at 3000 V and 52°C, for 2.5 hours. Fragment length analysis was undertaken using the ABI Prism Genescan and Genotyper 1.1.1 analysis packages.

RESULTS

A single region of homozygosity common to all seven microcephalic subjects was identified on chromosome 13q defined by markers D13S787 and D13S1304. Further refinement of the region was conducted using the following mark-

ers selected from the ABI Linkage Mapping Panel Version I (Applied Biosystems), the Todd Panel,¹² and the Marshfield Linkage Maps: cen - D13S175 - D13S1275 - D13S787 - D13S221 - D13S1304 - D13S1254 - D13S1244 - D13S217 - D13S120 - D13S171 - D13S1493 - tel. This defined a shared homozygous region on chromosome 13 at band q12.2 with meiotic crossovers between markers D13S175-D13S1275 and D13S1254-D13S1244, with the centromeric and telomeric boundaries of a 9 cM region being defined by D13S175 and D13S1244. Information regarding marker order and relative distances was obtained from the Marshfield Linkage Maps. The marker order obtained from the Marshfield Linkage Maps was in agreement with that derived from analysis of the current draft human genome data.

A fully penetrant autosomal recessive mode of inheritance and a disease gene frequency of 0.003 were assumed. Owing to the complexity of the family structure, equal allele frequencies

Table 1 Two point lod scores at $\theta=0$ for each marker defining the MCPH6 region at 13q12.2

Marker	Lod score at $\theta = 0$
D13S175	—
D13S1275	6.25
D13S787	5.90
D13S221	3.87
D13S1304	2.75
D13S1254	4.29
D13S1244	2.31

were assumed for each marker when calculating the lod scores and the maximum number of alleles was set at 4. Pedigree allele inconsistencies were identified using PedCheck.¹³ Two point analysis was performed using the LINKAGE analysis programs¹⁴ at $\theta=0$ for markers in the critical region and results are shown in table 1 with the highest lod score at 6.25 for marker D13S1275.

Novel microsatellite markers to refine the region further were designed using the Human Genome Browser and the Primer3 program, and designated [human BAC accession number][microsatellite repeat unit][number of unit repeats in the reference BAC], for example, AL356285TG25 (fig 2). These allowed us to redefine the centromeric boundary marker as AL139378GT17.

DISCUSSION

Haplotype and lod score analysis both suggest that the chromosome region 13q12.2, designated the MCPH6 locus, contains a gene which when mutated causes autosomal recessive primary microcephaly.

Within the larger MCPH6 region of 9 cM there is the potential candidate gene, fibroblast growth factor 9 (*FGF9*). In the nervous system of mice, *FGF9* is produced mainly by neurones and may have a role in glial cell growth and differentiation during development.¹⁵⁻¹⁶ The redefinition of the region to 6 cM using novel microsatellite markers flanking *FGF9* resulted in the exclusion of this gene (fig 2). We now therefore consider that the gene causing this form of autosomal recessive primary microcephaly must lie within this smaller region of approximately 6 Mb. To date, only the MCPH1 gene, microcephalin, and the MCPH5 gene, *ASPM*, have been identified.¹⁷⁻¹⁸ Future identification of the MCPH6 gene may be aided by an insight into how these proteins function and interact within the human brain, such as mitotic spindle activity in the case of *ASPM*. The discovery of MCPH genes will lead to a greater understanding of normal and abnormal human fetal cerebral cortex growth, giving potential insights into the question of how the mammalian cerebral cortex evolved and has become so predominant in humans, and the wherewithal to offer diagnostic, prenatal, and carrier testing for affected families.

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Electronic Database Information. URLs for data in this article are as follows: Center for Medical Genetics, Marshfield Medical Research Foundation available at <http://research.marshfieldclinic.org/genetics/>

(for genetic linkage maps). Online Mendelian Inheritance in Man (OMIM): <http://www.ncbi.nlm.nih.gov/omim>. Human Gene Nomenclature Database, HUGO Gene Nomenclature Committee: <http://www.gene.ucl.ac.uk/nomenclature/>. Draft human genome browser: <http://genome.cse.ucsc.edu/>. For primer creation (for novel microsatellite markers): http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi.

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Karak syndrome: a novel degenerative disorder of the basal ganglia and cerebellum

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We report a Jordanian Arab family where two sibs developed the classical clinical and radiological features of pantothenate kinase associated neurodegeneration (PKAN, formerly known as Hallervorden-Spatz disease) but in addition had an early onset cerebellar ataxia.^{1,2} Using polymorphic microsatellite markers we have shown that this family is not linked to the pantothenate kinase gene (*PANK2*) on chromosome 20.³ We hypothesise that the disorder, Karak syndrome, is novel and a member of the growing family of neurological diseases involving excess cerebral iron accumulation, for example, PKAN, neuroferritinopathy, aceruloplasminaemia, and Friedreich's ataxia.^{3–6}

CLINICAL STUDIES

Both affected members (fig 1, IV.1 and IV.2) were the product of a normal pregnancy and birth and had normal developmental milestones and progress at school until disease onset at the age of 6 years. They developed an ataxic gait that was slowly progressive, and was associated with decreased school performance. At 8 years of age they developed inverted feet (calcaneovarus), which was associated with frequent falls. Around the age of 9 years, both started to have choreiform

Key points

- Two brothers are reported with early onset progressive cerebellar ataxia, dystonia, spasticity, and intellectual decline.
- Neuroradiology showed cerebellar atrophy and features compatible with iron deposition in the putamen (including the "eye of the tiger sign") and substantia nigra.
- Diagnosis was compatible with pantothenate kinase associated neuropathy resulting from pantothenate kinase 2 mutation (PKAN due to *PANK2*) but linkage to *PANK2* was eliminated suggesting Karak syndrome to be a novel disorder.
- The "eye of the tiger" sign has previously only been reported to occur in PKAN due to *PANK2*

movements of all four limbs, more marked in the upper limbs than in the lower limbs. By the age of 10 years the condition had progressed and they were unable to walk without assistance, and they left school soon after. Their mother found difficulty in feeding them because of swallowing problems after the age of 10 years and they were unable to dress, bathe, or feed themselves by their mid-teens. There were no visual or auditory symptoms or history of epilepsy. Both parents and four older sibs, two brothers and two sisters, were in good health. The parents were first cousins and came from an inbred family (fig 1). The family lived in Karak, a town in southern Jordan.

On examination at the ages of 12 and 14 years respectively, the patients were not dysmorphic and were well grown (height, weight, and head circumference between the 10th–50th centiles). No telangiectasia was found on skin or mucus membrane, nor were skeletal abnormalities detected. Pursuit eye movements showed bilateral limitation of upward gaze in both children; saccadic eye movements were abnormal with bilateral hypometric saccades. Visual acuity was normal, as were the pupillary responses, fundoscopy of the optic discs and retina, and slit lamp examination. Both affected children had dysarthric scanning speech with dystonic features. There was dystonic movement of the tongue and facial muscles and choreiform movement was present in both upper and lower limbs, being more marked in the lower limbs, along with dystonic posture of the distal feet. Significant bradykinesia was present in both upper and lower limbs. Muscle bulk was normal with no wasting or fasciculation. Generalised hypertonia was evident in both upper and lower limbs of a mixed spastic-rigid type. Motor examination showed normal strength testing throughout. Evaluation of the different sensory modalities showed no abnormalities. Deep tendon reflexes were +2 and symmetrical, with bilateral extensor plantar responses. Dysmetria, past pointing, dysidiadochokinesia, and intentional tremor were bilateral and symmetrical. The gait was wide based, swayed in all directions, and had a spastic

Figure 1 A simplified pedigree of the research family. Affected subjects with Karak syndrome are shown as shaded symbols. The closest link between family members is shown, although other more distant links also exist. The haplotype results for the polymorphic microsatellite markers tel-D20S906-D20S193-D20S116-D20S482-D20S895-cen are shown under each person who was genotyped. The *PANK2* gene lies between markers D20S193 and D20S116. The box highlights the allele results of the two affected males and an unaffected sib, all of whom have an identical heterozygous haplotype.

Figure 2 MRI T2 weighted images of the brain of the older affected sib. (A) A parasagittal view showing a moderate degree of pancerebellar atrophy. (B) Decreased signal intensity in the substantia nigra, indicated by a black arrow. (C) The “eye of the tiger” sign with decreased signal intensity in the globus pallidus, indicated by a black arrow, in the centre of which a small high signal spot was seen, indicated by a white arrow.

quality. Neurological examination showed evidence of impairment of cognitive functions and on the Wechsler Adult Intelligence Scale they both achieved less than 60 for verbal IQ.

The following investigations were normal in both children: blood count and film, haemoglobin electrophoresis, liver enzymes, lipid profile, urine testing, serum immunoglobulins, alpha-fetoprotein, serum B12, folate, copper, ceruloplasmin, creatinine phosphokinase, very long chain fatty acids, ferritin,

urine organic acids (by gas chromatography and mass spectroscopy), audiogram, ECG, cardiac echo, chest radiographs, electromyography, motor and sensory conduction studies including studies of the late responses, and visual and brainstem evoked potentials. The potential differential diagnoses of PKAN, neuroferritinopathy, and aceruloplasminemia were eliminated by these results. Muscle biopsy from the older affected child was normal with no evidence to suggest a mitochondrial myopathy. MRI studies of the brain of both affected sibs showed a moderate degree of pancerebellar atrophy (fig 2A). T2 weighted images showed markedly decreased signal intensity restricted to the substantia nigra (fig 2B) and the globus pallidus (fig 2C). In the centre of the globus pallidus a small high signal spot was seen (“eye of the tiger” sign) (fig 2C). There was no abnormal signal in the putamen, caudate nucleus, red nucleus, or dentate nucleus. T1 weighted inversion recovery images were not remarkable. The striking low attenuation on T2 weighted findings suggests an increased iron deposition in the globus pallidus, and is an obligatory radiological feature of PKAN caused by *PANK2* gene mutations.⁷

MOLECULAR GENETIC STUDIES

We performed linkage analysis between the family’s disease and the *PANK2* gene that causes PKAN.² DNA was extracted using standard techniques from blood samples obtained from nuclear family members. Polymorphic microsatellite markers were sought that flanked the *PANK2* gene by scrutiny of the Marshfield Linkage Maps, the DeCode genetic map,⁸ and the draft Human Genome Browser: D20S906-D20S193-*PANK2* gene-D20S116-D20S482-D20S895 (at the following distances from 20pter: 1.5 Mb-3.3 Mb-3.85 Mb- 4.05 Mb-4.5 Mb-5.05 Mb). PCR amplification of all markers was performed using a Roboseq 4200 (MWG BioTech Ltd). Amplified markers were pooled and electrophoresed on an ABI Prism 377 gene sequencer (Applied Biosystems) on 4.2% polyacrylamide gels, at 3000 V and 52°C, for 2.5 hours. Fragment length analysis was undertaken using the ABI Prism Genescan and Genotyper 1.1.1 analysis packages. Fig 1 shows the family genotyping results. The two affected subjects and one of the unaffected sibs all had the same heterozygous results for the informative markers surrounding the *PANK2* gene. This suggests that linkage of the disease to the *PANK2* gene is very unlikely. A further implication of the result is that the affected sibs do not have two separate conditions, one *PANK2* related and the other causing cerebellar ataxia. The results do not help determine if the condition is X linked or autosomal recessive. We also eliminated linkage to the mitochondrial ferritin gene on chromosome 5q23⁹ (data not shown).

DISCUSSION

The disorder documented here presented at 6 years of age with cerebellar ataxia. Later, extra pyramidal motor features typical of classical PKAN due to *PANK2* gene mutation developed and intellectual ability declined. There have been a few case reports in which ataxia is associated with PKAN, but none with ataxia as a presenting feature.^{10–12} However, in a large study of PKAN two groups were differentiated; firstly, cases with typical clinical symptoms (of what would have been once called Hallervorden-Spatz syndrome¹³) or atypical features with acanthocytosis, hypoprebetalipoproteinaemia and/or orofacial dyskinesia (known as HARP syndrome^{14–16}) but all with the “eye of the tiger” sign on MRI brains scan and *PANK2* mutations; secondly, a group of clinically atypical cases which did not have *PANK2* mutations nor the “eye of the tiger sign” but did have hypodensity in the globus pallidus on T2 weighted MRI brain scans, cerebellar atrophy, and often evidence of iron deposition in the red nucleus and dentate nucleus.⁷ The family we report appears to have a demonstrably different condition from these two phenotypes because of the

Table 1 Cerebral diseases in which excess iron accumulation is found on MRI scan

Disease title	Inheritance pattern	Age of presentation	Clinical features*	Intellect	Radiological features†	Cerebellar features‡	Gene§	Chromosomal location
Aceruloplasminemia MIM 604290	Recessive	>30 y	Dementia, ataxia, chorea, dystonia	Declines	Caudate dentate, putamen, red, thalamus, on T1 + T2	Yes, normal	CP	3q23-4
Neuroferritinopathy MIM 606159	Dominant	40–55 y	Choreoathetosis, dystonia, spasticity, rigidity	Normal	Caudate, dentate, globus pallidus, putamen, substantia nigra	Normal	FTL	19q13.3-4
PKAN/PANK2 MIM 234200	Recessive	5–20 y	Rigidity, choreoathetosis, dystonia,	Often declines	"Eye of the tiger" and substantia nigra	Normal	PANK2	20p13
PKAN/PANK2 Atypical and HARP MIM 607236	Recessive	>15 y	Diverse extrapyramidal symptoms	Often declines	"Eye of the tiger"	Normal	PANK2	20p13
PKAN/not PANK2 Karak syndrome	Probably recessive Recessive or X linked	>15 y 6 y	Diverse extrapyramidal symptoms Ataxia, chorea, dystonia	Often declines Declines	Dentate, globus pallidus, red "Eye of the tiger" and substantia nigra	Often, atrophy Yes, atrophy	? ?	? ?

Disease title and OMIM number, as described in McKusick's Online Mendelian Inheritance in Man (OMIM).

PKAN/PANK2 and PKAN/not PANK2 is panthothenate kinase-associated neuropathy with or without a panthothenate kinase 2 gene mutation.

*Clinical features, lists the presenting and major neurological features only.

†Radiological features lists the (consistent and major) abnormal findings reported on cerebral T2 MRI brain scans. Caudate, dentate, and red refer to the caudate nucleus, dentate nucleus, and red nucleus respectively. "Eye of the tiger" sign is of decreased signal intensity in T2 weighted images, compatible with iron deposits, and of a small area of hyper intensity in its internal segment (illustrated in reference 6).

‡Cerebellar features documents whether clinical findings have been reported followed by cerebellar MRI brain scan findings. Normal means that neither clinical cerebellar signs nor abnormal cerebellar scan findings are present.

§Gene abbreviations used. CP is ceruloplasmin, FTL is ferritin light chain, PANK2 is panthothenate kinase 2 and ? no identified gene.

Freidreich ataxia was not included in the table as there is no evidence on MRI brain scan of excess pathological iron deposition in the central nervous system.

clinical presentation and the neuroradiological findings. The "eye of the tiger" sign is present as is hypodensity in the substantia nigra, but with no abnormal signal in the putamen, caudate nucleus, red nucleus, and dentate nucleus. Exclusion of linkage to the *PANK2* gene provides further support that Karak syndrome is a distinct disorder. In the original paper mapping PKAN due to *PANK2*, family HS9 was the only unlinked family presented. However, insufficient clinical details are given to determine if this single Japanese male case was similar to the family we report.^{17 18}

The highest concentration of iron in the brain is found within the basal ganglia (substantia nigra, putamen, caudate nucleus, and globus pallidus), red nucleus, and dentate nucleus. Cerebral iron accumulates with age, particularly in the basal ganglia.¹⁹ While iron is essential for normal brain function, free iron ions impose an oxidative stress on cells, owing to the production of oxygen free radicals, leading to cell damage. Cerebral diseases in which excess iron accumulation is shown on MRI scan are summarised in table 1. All cause clinical features of basal ganglia disease and all exhibit excess iron deposition in some or all of the sites where iron concentrations are the highest in the disease free state. Iron is stored predominantly complexed with ferritin within brain cells, and dysfunction of the ferritin light chain causes neuroferritinopathy.³ Ceruloplasmin is a ferroxidase with roles including ferric iron uptake by transferrin (which transports iron in the ferric state in the circulation) and dysfunction leads to aceruloplasminemia.⁶ How mutations in the *PANK2* gene, a brain specific CoA biosynthesis regulatory enzyme, cause pathological iron accumulation is unclear, but may relate to an accumulation of cysteine, which can chelate iron. A potential common pathogenic mechanism for neurological disease involving excess cerebral iron accumulation would be the release of free iron ions, either in excess or in a non-physiological situation or both.²⁰ Because of this we sought, but did not find, linkage between the mitochondrial specific ferritin gene and Karak syndrome.

The inheritance pattern of Karak syndrome may be autosomal recessive or X linked recessive. The lack of other affected males in the matrilineal line and parental consanguinity favour recessive inheritance but clarification awaits further reports of the condition. We hypothesise that the disorder is novel and a member of the growing family of neurological disease involving excess pathological cerebral iron accumulation. Potentially, the gene mutated in Karak syndrome could be involved in iron sequestration, transport, storage, ferrous/ferric status control, intercalation of iron into enzymes, and oxidative respiration.

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Electronic database information. For genetic linkage maps: Center for Medical Genetics, Marshfield Medical Research Foundation at <http://research.marshfieldclinic.org/genetics/> For data on polymorphic markers: Genome Database (GDB) at <http://gdbwww.gdb.org/> For physical contig information of the human genome: the draft Human Genome Browser at <http://genome.cse.ucsc.edu/> For genetic disease details: McKusick's Online Mendelian Inheritance in Man at <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>

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Myhre syndrome: new reports, review, and differential diagnosis

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Several conditions characterised by short fingers, reduced joint mobility, short stature, and muscular build with or without mental retardation have been delineated during the past 30 years: Moore-Federman syndrome, Myhre syndrome, acromicric dysplasia, geleophysic dysplasia, GOMBO syndrome, and LAPS (Laryngotracheal stenosis, Arthropathy, Prognathism and Short stature) syndrome. In 1980 Myhre *et al*¹ reported two unrelated males with mental retardation, facial dysmorphism (short palpebral fissures, maxillary hypoplasia, prognathism, short philtrum, small mouth), short stature, brachydactyly, muscle hypertrophy, decreased joint mobility, mixed hearing loss, and cleft lip and palate in one of them. *X* rays showed a thickened calvarium, hypoplastic iliac wings, broad ribs, and large, flattened vertebrae with large pedicles. Five further cases were reported. Mental retardation of variable severity was present in all patients. Three of the older patients had hypertension.

We report here four new unrelated patients who fit a diagnosis of Myhre syndrome, expanding the behavioural profile of the disorder, and discuss the differential diagnosis.

CASE REPORTS

Patient 1

Patient 1, a male, was the second child of healthy, non-consanguineous parents. The father was 181 cm tall and

the mother was 165 cm tall. They were aged 40 and 34 years, respectively, at time of delivery. Birth weight was 2130 g, length 44.5 cm, and OFC 36 cm at 36 weeks' of gestation based on LMP (40 weeks based on morphological score of maturity). Recurrent hypoglycaemia requiring glucose infusion was recorded during the first weeks. Psychomotor development was normal, but dysarthria required long term speech therapy. He had bilateral deafness, with loss of 40 dB in low frequencies to 20 dB on the right side, and from 60 dB to 35 dB on the left side. MRI and CT showed bilateral dysplasia of the external semicircular canal. The stapedial footplate was bilaterally thick and, on the right side, fused with the oval window. This appearance was compatible with primary stapedial dysplasia or with otosclerosis. The absence of evolution of the hearing loss was considered as an argument for the dysplasia hypothesis. He wore a hearing aid. He had hypermetropia.

Progressive growth catch up occurred during the first year of life, reaching the 25th centile curve, then he slowly came back to the 3rd centile curve. OFC stayed around the 25th centile. Repeated bone age studies always showed skeletal maturation concordant with chronological age. Longitudinal skeletal survey showed generalised brachydactyly with brachyphalangy and brachymetacarpalia, large epiphyses (notably the femoral heads and proximal phalanges of fingers 2 and 3), and mild platyspondylic appearance of the vertebral

Key points

- Myhre syndrome is characterised by facial dysmorphism (short palpebral fissures, maxillary hypoplasia, prognathism, short philtrum, small mouth), short stature, brachydactyly, muscle hypertrophy, decreased joint mobility, hearing loss, and mental retardation.
- Radiological findings are a thickened calvarium, hypoplastic iliac wings, broad ribs and large, flattened vertebrae with large pedicles. Moore-Federman syndrome, acromicric dysplasia, geleophysic dysplasia and LAPS syndrome are the main differential diagnoses of Myhre syndrome.
- We report here four new unrelated patients who fit a diagnosis of Myhre syndrome. Absence of mental retardation was noted in one patient. Abnormal behaviour (impairment in social interactions and/or repetitive/stereotypic behaviour) was noted in 2/4 patients as in one previous report.
- Mixed hearing loss and hypertension (4/5 patients over 16 years old) seem constant in older patients. Thick skin was noted in three of our patients as in 3/7 previously reported patients and could be a major feature of Myhre syndrome.
- Paternal age was increased in half of the reported cases, suggesting a new mutation of an autosomal dominant gene. X linked transmission cannot be excluded since all reported cases (11/11) were males.

Figure 1 Patient 1. (A) Muscular build. (B) Brachydactyly. (C) Thickened calvarium. (D) Large vertebral pedicles.

bodies with irregular endplate (fig 1D). The pelvis was narrow. The calvarium and ribs were not thickened (fig 1C).

Bilateral cryptorchidism was surgically corrected at the age of 3. Puberty began at 13 years, but at the age of 17, pubertal development scored only Tanner stage P4 G4 with a subnormal testicular volume of 10/15 ml, which prompted endocrinological studies. These showed normal testosterone levels (5.61 ng/ml), high FSH levels (16.0 mIU/ml, normal <11), and normal LH level. This was compatible with primary

(hypergonadotrophic) hypogonadism, possibly related to germinal cell aplasia (Sertoli cell only syndrome) or to another anomaly of spermatogenesis or anomalies of the FSH receptor.

When evaluated at the age of 20 he was a university student. He had difficult social relationships. He was 154 m tall and OFC was 56.5 cm (fig 1A). The facial dysmorphism included a square face, upward slanting eyebrows, short nose, very short philtrum, narrow mouth, and a large, prognathic chin. He had short hands with mild cutaneous syndactyly (fig 1B), and short feet with Y shaped syndactyly of toes 2 and 3. The skin was hard. There was generalised joint limitation, noted by the age of 10 and slowly worsening with time, and generalised muscular hypertrophy with extremely hard muscles. Neurological examination was normal, but osteotendinous reflexes were very weak. At the ages of 10 and 20, CK, EMG, and motor conduction velocity were normal. Muscle light microscopy at the age of 10 showed no abnormality. Hypertension (up to 170/100) was noted on different occasions during the last two years; no specific reason could be found. Echocardiography was normal. High resolution G banding and FISH with subtelomeric probes were normal.

Patient 2

This boy was the second child of healthy, non-consanguineous, young parents. Heights of the parents were 176 cm (father) and 160 cm (mother). The two sisters were healthy. At birth at 41 weeks' gestation, weight was 2130 g, length 42 cm, and OFC 32 cm. Neonatal examination showed left cryptorchidism, short hands, and slight facial dysmorphism. During the first year, growth was retarded and gluten allergy was suspected. A gluten free diet had no effect. Motor development was normal but later learning disabilities were noted at school. When he was 9 years old hearing loss was suspected and confirmed by audiogram. Hearing improved with bilateral hearing aids. He was admitted to an institution for deaf persons but poor school performances were noted. At the age of 9, height was 113 cm (less than the 3rd centile), regularly on this curve since birth. Endocrinological investigations showed normal GH, IGF1, T4, and TSH levels. Chromosomal analysis was normal, 46,XY. Bone age was 8 years. Echocardiography was normal. A diagnosis of acromicric dysplasia was suspected but facial dysmorphism was strikingly different. A few weeks later, growth velocity improved rapidly, and testis growth began. Endocrinological studies (testosterone and LH-RH test) confirmed central precocious puberty. Cerebral MRI was normal. Gonadotrophin releasing hormone agonist therapy was started and continued until the age of 14.

Clinical findings at 14 years showed short stature (136 cm, less than the 3rd centile), weight 44.5 kg (25th centile), and OFC 55.5 cm (50th centile). He had facial dysmorphism with small palpebral fissures, small round ears, a broad nasal bridge, short philtrum, small mouth, marked prognathism, and a short neck. The hands were short with brachydactyly. He had a striking muscular build. Moderate joint limitation was present in the elbows and the skin was very thick (fig 2). Ophthalmological examination showed hypermetropia and astigmatism. Longitudinal radiological findings were thick calvarium, platyspondyly with irregular endplate, very large vertebral pedicles, and large epiphyses (hands, femoral heads).

Patient 3

This male patient was the second child of a 32 year old G2 P2 mother and a 38 year old father. The brother was healthy and family history was negative. During the pregnancy, ultrasonography showed short femora at 31 weeks' gestation. Fetal blood sampling was performed and karyotype was normal. Birth weight at 39 weeks' gestation was 2350 g, birth length 44 cm, and OFC 32 cm. Psychomotor development was normal in

Figure 2 Patient 2. (A) Facial dysmorphism, short philtrum, thin lips. (B) Muscular build, joint limitation (elbows). (C) Brachydactyly.

the first months but language was delayed. At 2 years auditory evoked potentials showed hypoacusis. Hearing improved partially after transtympanic drains. Behaviour was abnormal. He had a severe impairment in social interaction (gaze and physical contact avoidance), abnormal communication (repetitive/stereotypic language), repetitive/stereotypic activities, difficulties with new situations, attention deficit, hyperactivity, and slight auto-aggressiveness. No standardised diagnosis of autism was available. These difficulties partially improved with time and psychotherapy. Growth was on the 20th centile in the first five years.

At 5 years, height was 1 m (5th centile), weight 19 kg (60th centile), and OFC 50 cm (25th centile). Clinical examination showed muscular hypertrophy and facial dysmorphism. He had brachycephaly, short palpebral fissures, deep set eyes, low set and small, round ears, a short philtrum, slightly convex profile of the nose, thin lips, and prognathism. The fingers were short with distal phalangeal hypoplasia. Extension of the elbows, knees, and ankles and pronosupination were limited. Achilles tendons were shortened but he had no spasticity or pyramidal signs. The skin was thick.

High resolution G band analyses, FISH with subtelomeric probes, cerebral CT scan, cardiac and renal ultrasound, and CK were normal. Ophthalmological examination showed hypermetropia, astigmatism, and strabismus. Skeletal x ray showed large vertebral pedicles and bone age was retarded (fig 3).

Patient 4

This male patient was born to healthy, non-consanguineous parents. He had a healthy older sister and three healthy half sibs from his mother and a healthy half sister from his father. The father was 168 cm tall and the mother was 153 cm tall. They were respectively aged 43 and 35 at the time of delivery. At birth weight was 2500 g, length was 47 cm, and OFC 32 cm at 38 weeks' of gestation. Global hypertonia and brachydactyly were noticed. Clinical findings at 5 years of age showed short stature (1 m, -2 SD) with normal OFC (53 cm, $+1$ SD), "muscular" habitus, and thick skin. A peculiar square face was

Figure 3 Patient 3. (A) Facial dysmorphism: short palpebral fissures, short philtrum, and thin lips. (B) Short neck and small ears. (C) Muscular build. (D) Brachydactyly.

Figure 4 Patient 4. (A) Facial dysmorphism: short palpebral fissures, short philtrum, thin lips, and prominent mandible. (B) Broad ribs. (C) Large vertebral pedicles. (D) Brachydactyly.

observed with short palpebral fissures, short and broad nose, very short and marked philtrum, narrow mouth with thin lips, large, prognathic chin, and small, dysplastic, prominent ears. He had short hands with brachydactyly and short feet with slight II-III syndactyly. Osteotendinous reflexes were very weak. Psychomotor development was delayed and behaviour was abnormal with sleeping difficulties, frustration intolerance, hyperactivity, encopresis, and aggressiveness towards himself and others. Ophthalmological examination showed bilateral subcapsular cataract, which was surgically removed at the age of 6 years. Abdominal ultrasonographic scan

Table 1 Clinical data of Myhre syndrome patients

	Myhre <i>et al</i> ¹		Soljak <i>et al</i> ²	Garcia-Cruz <i>et al</i> ³		Whiteford <i>et al</i> ⁴	Titomanlio <i>et al</i> ⁵	This report				Total or mean*
	Patient 1	Patient 2	Patient 1	Patient 1	Patient 2	Patient 1	Patient 1	Patient 1	Patient 2	Patient 3	Patient 4	
Paternal age at birth	37	38	42	32	23	32	33	40	28	38	43	35*
Sex	M	M	M	M	M	M	M	M	M	M	M	11 M
General												
Birth weight (g)	2100	2900	3200	2200	2100	2870	2110	2100	2130	2,350	2,500	2.4*
Birth height	?	?	?	?	?	?	?	44.5	42	44	47	44.4*
Height in SD (adult height)	-5.5 (140 cm)	-4.5 (146 cm)	-4.5 (146 cm)	-6 (139 cm)	-3	<-2	-4	-2 (154 cm)	-3.6	-2	-2	-2/-6
Age at last observation	24	18	16	18	6	13	14	20	14	5	10	14.3*
Craniofacial features												
OFC (SD)	-1	-1.5	?	-1.5	M	+1.5	+1	-1	+0.5	-1	0	-1/+1.5
Prosis	?	?	?	?	-	-	?	-	+	+	slight	3/6
Short palpebral fissures	+	+	+	+	+	+	+	+	+	+	+	11/11
Midface hypoplasia	+	+	+	-	+	+	?	+	+	+	+	9/10
Short philtrum	+	+	+	+	+	+	+	+	+	+	+	11/11
Narrow mouth	+	+	+	+	+	+	+	+	+	+	+	11/11
Thin upper lip	+	+	+	+	+	+	+	+	+	+	+	11/11
Prognathism	+	+	+	?	+	?	+	+	+	+	+	9/9
Cleft lip	+	-	-	-	-	-	-	-	-	-	-	1/11
Small ears	?	?	?	?	+	?	?	+	+	+	+	5/5
Skeletal anomalies												
Brachydactyly	+	+	+	+	+	+	+	+	+	+	+	11/11
Thick calvaria	+	+	+	+	+	+	+	-	+	?	+	9/11
Joint limitations	+	+	+	+	+	+	+	+	+	+	+	11/11
Broad ribs	+	+	+	+	+	+	?	-	+	?	+	8/9
Narrow pelvis	+	+	+	+	+	+	+	+	+	?	+	10/10
Thick femoral necks	-	-	+	+	-	+	?	-	?	?	+	4/8
Large epiphyses	?	?	?	?	?	?	?	+	+	?	+	3/3
Mild platyspondyly	+	+	-	+	+	+	?	+	+	+	+	9/10
Large pedicles	+	+	+	?	?	+	+	-	+	+	+	8/9
Neuromuscular												
IQ	45	35-50	75	Low	Low	Low	Low	Normal	Low	50-60	55-60	
Behaviour	,	?	,	?	?	?	autistic	Difficult relationships	?	Repetitive/ stereotypic behavior, Hyperactivity	Hyperactivity encopresis	
Muscular hypertrophy	+	+	+	+	+	+	+	+	+	+	+	11/11
Muscle histology	?	?	Normal	Minor changes	?	Normal	Minor changes	Normal	?	?	?	
EMG/muscle ultrasound	?	?	EMG myopathic	Normal	?	Myopathic US changes	EMG neurogenic	EMGnNormal	?	?	?	
Miscellaneous												
Very thick skin	?	?	+	+	?	?	+	+	+	+	+	7/7
Deafness	+	+	+	-100dB	-	+	Mild	+	+	+	-	9/11
Abnormal inner ear	?	?	?	?	?	Ossicular fixation	?	+	?	?	?	
Ophthalmological anomalies	Hypermetropia	Hypermetropia	Hypermetropia	?	?	Cataract	?	Hypermetropia	Hypermetropia	Hypermetropia	Cataract	8/8
Heart malformation	+	ASD	-	-	-	PDA, PPS	Left atrium mild dilatation	-	-	-	Aortic and pulmonary valve stenosis	5/11
Kidney malformation	-	-	-	-	-	-	-	-	-	-	Right kidney agenesis	1/11
Hypertension	+	-	+	+	-	?	-	+	-	-	-	4/10
Inguinal hernia	+	-	-	-	-	-	+	-	-	-	-	2/11
Puberty	?	?	Normal	Delayed	?	?	?	Delayed	Precocious	-	Precocious	
Hypospadias	+	-	-	-	-	-	-	-	-	-	-	1/11
Cryptorchidism	+	+	-	?	-	-	+	+	+	-	-	5/10

ASD: atrial septal defect. PPS: peripheral pulmonary stenoses. PDA: patent ductus arteriosus. ?: not determined.

Table 2 Differential diagnosis of Myhre syndrome

	Myhre	LAPS	Geleophysic dysplasia	Acromicric dysplasia	Moore-Federman
Short stature	+	+	+	+	+
Brachydactyly	+	+	+	+	+
Joint limitation	+	+	+	+	+
Muscular hypertrophy	+	—	—	+/-	—
Mental retardation	Frequent	Learning difficulties	—	—	—
Deafness	+ 9/11	+ 3/5	—	—	—
Abnormal skin	+	+	+	—	+
Laryngotracheal stenosis	— (too young ?)	+ (severe adult onset)	+ (childhood onset)	+ (childhood onset)	—
Hepatomegaly	—	—	+	—	+
Cardiac disease	Congenital malformations	Pericarditis	Progressive cardiac valve disease	—	+/-
Transmission	AD? X linked? (11M/0F)	AD? (1M/4F)	AR	AD	AD

showed right kidney agenesis and cardiac ultrasonographic scan showed moderate aortic stenosis. Hearing was normal. Karyotype was normal 46,XY. Subsequently growth followed the -2 SD curve, behavioural difficulties persisted, but sleeping improved. Generalised muscular hypertrophy and “pugilistic” posture were more prominent. Progressive joint limitation was observed with enlargement of the big joints and limited extension of the elbows, knees, ankles, fingers, and limited pronosupination. At 10 years of age precocious central puberty occurred with advanced bone age. Cerebral MRI was normal (fig 4).

DISCUSSION

Myhre *et al*¹ reported two unrelated males with mental retardation, facial dysmorphism (short palpebral fissures, maxillary hypoplasia, prognathism, short philtrum, small mouth), short stature, brachydactyly, muscle hypertrophy, decreased joint mobility, mixed hearing loss, and cleft lip and palate in one of them. X rays showed a thickened calvarium, hypoplastic iliac wings, broad ribs, shortened tubular bones, and large, flattened vertebrae with large pedicles. Five further cases were reported.²⁻⁵ The four patients reported here fit a diagnosis of Myhre syndrome.

Clinical data on Myhre syndrome patients are presented in table 1. The most important features are low birth weight (mean 2415 g), variable short stature, facial dysmorphism (midface hypoplasia, narrow palpebral fissures, short philtrum, and prognathism), short hands, muscular build, very peculiar thick skin, and joint limitation. Birth length was not previously reported but short stature was of prenatal onset in our four patients. Mental retardation is frequent but was absent in our patient 1. However, this patient had behavioural disturbances with difficult relationships. Abnormal behaviour was noted in the patient reported by Titomanlio *et al*⁶ who was diagnosed as autistic. No standardised diagnosis of autism was available for our patient 3 but he had at least an “autistic-like condition”. Further observations with a careful description of behaviour are needed in order to know if autistic or other behavioural disturbances are features of Myhre syndrome. Deafness is a frequently reported feature that seems constant in older patients, suggesting that hearing loss could be progressive. Hearing loss is mixed in most cases. One of our patients had cataracts, as did one previously reported patient.⁴ Agenesis of one kidney is reported for the first time and could be a rare feature like cleft lip that was reported in one case. Thickness of the skin is present in 6/11 patients and is a major feature of Myhre syndrome. In patient 3, thickness of the skin was noted as early as 3 years old. No skin biopsy was performed in our patients. Titomanlio *et al*⁶ reported dermis thickening with collagen infiltration in their patient. Four of 11 patients had hypertension. All were between 16 and 20 years old. This feature is possibly not present in younger children. Blood pressure had to be regularly measured in patients

with Myhre syndrome. One hypothesis could be abnormal arterial stiffness but this remains to be confirmed by specific studies. Another clinical feature of interest is the abnormal onset of puberty observed in three of our cases, two showing early onset (before the age of 9 years) and another one showing delayed puberty with hypergonadotrophic hypogonadism. Although these phenotypic manifestations are discordant, they may point to a disturbance in the hypothalamo-hypophyso-gonadal axis that should be investigated in further cases.

Several syndromes were transiently considered in the differential diagnosis of our patients. OSMED syndrome shows megaepiphyses, irregular vertebral bodies, and deafness, but can be distinguished by a different facial dysmorphism (micrognathia), metaphyseal involvement, and progressive carpal fusion.⁶ Phenotypic similarities were noted between Myhre syndrome and GOMBO syndrome,^{7,8} but the latter has been shown to result from a cryptic translocation between chromosomes 3p and 22q.⁹ Telomeric FISH analyses performed in two of our patients detected no cryptic rearrangement. Moore-Federman syndrome,¹⁰ acromicric dysplasia,^{11,12} geleophysic dysplasia,^{13,14} and LAPS syndrome^{15,16} are the main differential diagnoses of Myhre syndrome. Table 2 summarises the similarities and differences between these entities.

Acromicric dysplasia is characterised by short stature below 3 SD noted after the age of 2 years, short and stubby hands and feet, and mild dysmorphic signs, including narrow palpebral fissures, full cheeks, and a short and anteverted nose. Radiological anomalies include short metacarpals (especially 2-5) with an internal notch on the second one, short and broad proximal and medial phalanges, cone shaped epiphyses, internal notch on the femoral necks, and delayed bone maturation. Hoarse voice, generalised joint limitation, and muscular build have recently been added to the clinical picture of acromicric dysplasia.¹² Microscopically, there is disorganised cartilage, abnormal organisation of collagen, and accumulation of glycogen in most chondrocytes. The distinction between acromicric dysplasia and the Moore-Federman syndrome, which are both dominantly inherited, is still a disputed issue, with both lumping and splitting suggested.^{12,17} There is now convincing evidence to reject lumping with the recessively inherited geleophysic dysplasia as previously suggested.¹⁸ There are obvious similarities between acromicric dysplasia and Myhre syndrome. Nevertheless, Myhre syndrome can be distinguished on the basis of facial features, IUGR, mental retardation, and on the severity of muscular and cutaneous involvement. Geleophysic dysplasia¹³ is characterised by short stature with a “happy looking” facial appearance, short hands, joint contractures, thickened skin, hepatomegaly, and cardiac valve dysplasia. This is considered to be a storage disease with progressive worsening and a poor prognosis. Storage vacuoles are present in hepatocytes, chondrocytes, and fibroblasts.¹⁴ Myhre syndrome and geleophysic dysplasia share some features but hepatomegaly and

cardiac valve dysplasia have never been reported in patients with Myhre syndrome even in older subjects. Facial dysmorphism is different with a short philtrum, small mouth, and frequent prognathism in Myhre syndrome. Additionally, short stature is postnatal in geleophysic dysplasia but was of prenatal onset in all our Myhre patients. Figuera¹⁹ suggested that the patient reported by Rosser *et al* as having geleophysic dysplasia had Myhre syndrome. Nevertheless, as pointed out by McGaughran and Donnai²⁰ geleophysic dysplasia seems a more convincing diagnosis for this patient who had hepatomegaly, thickened mitral valve, and "geleophysic dysplasia facial dysmorphism".

Hopkin *et al*¹⁵ described three patients with a disorder characterised by short stature, joint limitation, and progressive adult onset laryngotracheal stenosis. Lindor *et al*¹⁶ reported two further patients and coined the acronym LAPS syndrome for Laryngotracheal stenosis, Arthropathy, Prognathism, and Short stature. Short stature, brachydactyly, joint limitation, prognathism, small, round ears, short palpebral fissures, abnormal skin, thick calvaria, and deafness are observed in Myhre and LAPS syndromes. A disturbance in the hypothalamo-hypophyso-gonadal axis may be another common feature. Abnormal onset of puberty was observed in three Myhre patients; among LAPS patients, one had precocious puberty, three had irregular menses, and one secondary amenorrhoea. Progressive laryngotracheal stenosis apparently clearly distinguishes LAPS syndrome from Myhre syndrome, but this could perhaps be explained by insufficient follow up, as the older Myhre patient was only aged 24 whereas onset of obstructive symptoms was between 17 and 30 in LAPS cases. Despite these similarities, some major differences exist. The impressive muscular hypertrophy, which is a cardinal feature of Myhre syndrome, was never reported in LAPS patients. Finally, the sex ratio is strikingly dissimilar, 11 M:0 F in Myhre versus 1 M:4 F in LAPS. These two latter arguments make lumping of LAPS and Myhre hazardous at this point. Further reports and longer follow up of Myhre cases would probably help to solve this nosological issue in the future.

The pattern of inheritance of Myhre syndrome remains unknown. All reported patients were sporadic and paternal age was increased in half of the cases (7/11) suggesting dominant new mutations. However all cases have been males (11/11) and X linked transmission cannot be excluded to date.

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