LETTERS TO JMG

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

K Kahnoski, S K Khoo, N T Nassif, J Chen, G P Lobo, E Segelov, B T Teh

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. 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. 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. 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.

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. A wide spectrum of neoplastic and non-neoplastic features has been described in BHD patients, including diverse types of kidney tumours and spontaneous pneumothorax. BHD has also been reported to be associated with colonic polyposis and colorectal neoplasia, although a large study of 223 patients from 33 BHD families could not establish such a relation. 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. The BHD gene has been mapped to chromosome subband 17p11.2 and recently identified to encode a novel protein named folliculin. 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), tract (nt 1733–1740) has been detected in BHD patients, and this repeat tract represents a BHD mutational hot spot. 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. 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 colon polyps 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 power magnification.
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, D18S534, D3S1611), and tetranucleotide (D15S18, D7S808, D2S432, D15S182) repeats. A microsatellite instability screen was performed on 6% sequencing gels and visualised by 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.15 If the ratio was <0.67 or >1.3, the result was determined to be LOH. Initially, the two closest markers (D17S740 and D17S196) 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).20,21 DNA was treated with sodium bisulphite, which converts all unmethylated cytosines to uracils, leaving methylated cytosines unchanged. Briefly, 2 µg 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 glycerol as a carrier, and resuspended in 20 µl DNAase free, RNAase free distilled water before storing at −20°C. The specific primers for methylated sequences were designed as follows: BHD-BISF-OF (5′-ATGTTGGATGAGGTTAGTTGTTAATTT-3′) as the forward primer, and BHD-BISF-OR (5′-ACAAGTTCAACACCCAAACCCCCC-3′) as the reverse primer. An aliquot of the bisulphite treated product (2 µl) was amplified in a 25 µl reaction containing 2 mmol/l MgCl2, 0.24 mmol/l each dNTP (Invitrogen), 0.02 U Taq DNA polymerase (Amersham-Pharmacia Biotech), 50 pmol/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. Radiosotope 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.22

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.13 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 µg/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.14 One µl of each PCR product was added to a cocktail containing 5 µl of DNAses and RNAses free, distilled water, 10 µl of Hi-Di formamide and 0.2 µl 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.15 If the ratio was <0.67 or >1.3, the result was determined to be LOH. Initially, the two closest markers (D17S740 and D17S196) 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.
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 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. However, one recent study 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.

---

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

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

P = polyp, – = absent; + = present; H = high level MSI; L = low level MSI; ND = not determined.
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 confirmed with BHD germine mutations, indicating that the BHD gene is involved in the tumorgenesis 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 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. 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 Thr 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. 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 a potential site of phosphorylation 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 is associated with 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. 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.

ACKNOWLEDGEMENTS

The first two authors contributed equally to this work. This study was supported by the Van Andel Foundation.

Authors' affiliations

K Kahnoski, S K Khoo, J Chen, B T Teh, Laboratory of Cancer Genetics, Van Andel Research Institute, Grand Rapids, MI-49503, USA
N T Nassif, G P Lobo, E Segelov, Cancer Research Laboratories, South West Sydney Clinical School, University of New South Wales, Liverpool Hospital, Liverpool, NSW 2170, Australia

Correspondence to: Dr N T Nassif, Department of Medicine, University of New South Wales, Level 6, Health Services Building, Cnr Goulburn & Campbell Streets, Liverpool, NSW 2170, Australia
n.nassif@unsw.edu.au or Dr B T Teh, Laboratory of Cancer Genetics, Van Andel Research Institute, Grand Rapids, MI-49301, USA; bin.teh@vai.org

REFERENCES


514 Letter

www.jmedgenet.com

Downloaded from http://jmg.bmj.com/ on June 17, 2017 - Published by group.bmj.com
A gene locus for branchio-otic syndrome maps to chromosome 14q21.3-q24.3


Branchio-otic-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. The prevalence approximates 1:40 000 in the general population, and has been reported in about 2% of deaf children. The clinical expression of BOR exhibits wide intra- and inter-familial variability. In addition, reduced penetrance for BOR has been assumed. A locus (1q31) to chromosome 14q21.3-q24.3 has been identified by linkage analysis. This new locus was initially described as a disorder distinct from BOR, featuring the same clinical symptoms as BOR with some additional renal anomalies. Genetic studies of this BOR locus in patients with and without an associated renal abnormality have been performed. The current data suggest that BOR and a related renal disorder may represent a clinical spectrum of the same genetic disorder.
the exception of renal anomalies. The large variety of clinical
phenotypes and the description of mutations in the EYA1
gene for BOR and BOS patients 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. 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 fea-
tures of BOR or BOS remains unsolved. We describe here a
geno-wide search for linkage in a large pedigree with BOS,
in which linkage to the EYA1 locus on chromosome 1q31 had
been excluded, resulting in a new locus (BOS3) on chromo-
some 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 sam-
ples were collected (14 from affected subjects, six from unaf-
fected 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 mod-
erate 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).

Table 1 Clinical data of affected subjects from the BOS kindred

<table>
<thead>
<tr>
<th>Subject</th>
<th>AO</th>
<th>R</th>
<th>L</th>
<th>R</th>
<th>L</th>
<th>EP</th>
<th>BC</th>
<th>LD</th>
</tr>
</thead>
<tbody>
<tr>
<td>II.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>II.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>II.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>II.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>II.6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>II.7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>II.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>II.9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>II.10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>III.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>III.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>III.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>III.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>III.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>III.6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>III.7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>III.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>III.9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>III.10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

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

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

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.
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, either directly from blood samples or after Epstein-Barr-virus transformation of peripheral blood lymphocytes. 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 map 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), D14S5748 (4.5 cM), D14S587 (4.1 cM), D14S980 (2.9 cM), D14S748 (4.5 cM), D14S587 (4.1 cM), D14S980 (2.9 cM), D14S53 (5.1 cM), D14S592 (8.0 cM), D14S588 (2.9 cM), D14S274 (3.9 cM), D14S1013 (3.2 Mb), 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, 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. Multitip analysis of the 11 markers resulted in a Zmax=4.81 at marker D14S980 at relative position 50.9 (fig 2). The 95% confidence interval at Zmax=1 extends over a 33.9 cM interval between the markers D10S1013 and D10553 within the set of 11 microsatellites. Marker D14S980 also showed the highest two point lod score value Zmax=4.11 (θ=0) (table 2).

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

<table>
<thead>
<tr>
<th>Marker</th>
<th>0</th>
<th>0.001</th>
<th>0.01</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>0.4</th>
<th>Zmax (θ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D14S599</td>
<td>-4.991</td>
<td>-3.645</td>
<td>-1.891</td>
<td>-0.659</td>
<td>-0.237</td>
<td>0.02</td>
<td>0.053</td>
<td>0.062</td>
<td>0.053 (0.3)</td>
</tr>
<tr>
<td>D14S306</td>
<td>-4.220</td>
<td>-2.777</td>
<td>-0.984</td>
<td>0.192</td>
<td>0.529</td>
<td>0.585</td>
<td>0.381</td>
<td>0.127</td>
<td>0.585 (0.2)</td>
</tr>
<tr>
<td>D14S1013</td>
<td>0.135</td>
<td>0.624</td>
<td>1.494</td>
<td>1.926</td>
<td>1.892</td>
<td>1.492</td>
<td>0.953</td>
<td>0.419</td>
<td>1.926 (0.05)</td>
</tr>
<tr>
<td>D14S5748</td>
<td>1.902</td>
<td>1.898</td>
<td>1.853</td>
<td>1.656</td>
<td>1.410</td>
<td>0.934</td>
<td>0.508</td>
<td>0.182</td>
<td>1.902 (0.0)</td>
</tr>
<tr>
<td>D14S587</td>
<td>3.268</td>
<td>3.261</td>
<td>3.202</td>
<td>2.933</td>
<td>2.590</td>
<td>1.881</td>
<td>1.170</td>
<td>0.518</td>
<td>3.268 (0.0)</td>
</tr>
<tr>
<td>D14S980</td>
<td>4.114</td>
<td>4.107</td>
<td>4.038</td>
<td>3.726</td>
<td>3.321</td>
<td>2.461</td>
<td>1.544</td>
<td>0.638</td>
<td>4.114 (0.0)</td>
</tr>
<tr>
<td>D14S274</td>
<td>2.163</td>
<td>2.157</td>
<td>2.097</td>
<td>1.830</td>
<td>1.492</td>
<td>0.831</td>
<td>0.286</td>
<td>0.005</td>
<td>2.163 (0.0)</td>
</tr>
<tr>
<td>D14S592</td>
<td>2.672</td>
<td>2.665</td>
<td>2.122</td>
<td>2.371</td>
<td>2.067</td>
<td>1.459</td>
<td>0.876</td>
<td>0.368</td>
<td>2.672 (0.0)</td>
</tr>
<tr>
<td>D14S588</td>
<td>1.962</td>
<td>1.958</td>
<td>1.922</td>
<td>1.756</td>
<td>1.544</td>
<td>1.108</td>
<td>0.679</td>
<td>0.298</td>
<td>1.962 (0.0)</td>
</tr>
<tr>
<td>D14S1002</td>
<td>3.359</td>
<td>3.353</td>
<td>3.295</td>
<td>3.033</td>
<td>2.696</td>
<td>1.995</td>
<td>1.275</td>
<td>0.583</td>
<td>3.359 (0.0)</td>
</tr>
<tr>
<td>D14S1025</td>
<td>2.254</td>
<td>2.249</td>
<td>2.201</td>
<td>1.988</td>
<td>1.717</td>
<td>1.166</td>
<td>0.634</td>
<td>0.214</td>
<td>2.254 (0.0)</td>
</tr>
<tr>
<td>D14S53</td>
<td>0.324</td>
<td>1.064</td>
<td>1.925</td>
<td>2.335</td>
<td>2.272</td>
<td>1.816</td>
<td>1.208</td>
<td>0.568</td>
<td>2.335 (0.05)</td>
</tr>
<tr>
<td>D14S606</td>
<td>-4.205</td>
<td>-2.769</td>
<td>-0.981</td>
<td>0.188</td>
<td>0.517</td>
<td>0.568</td>
<td>0.388</td>
<td>0.173</td>
<td>0.568 (0.2)</td>
</tr>
</tbody>
</table>

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

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. The two markers D14S1013 and D14S53 flanking the BOS3 region (fig 1) are underlined. cen, centromeric orientation; qter, q terminal orientation.
described by Kumar et al. 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, frequency, severity, 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 BOR and BOS can be explained either by the known variable expressivity or by the genetic heterogeneity of BOS.

Genes encoding proteins involved in renal and otic development may be ideal candidates. EYA-deficient mice have been shown to lack ears and kidneys and show abnormal apoptosis of organ primordia. Another member of the EYA gene family, EYA4, is responsible for late onset deafness. EYA2 and EYA3 are excluded from the BOS locus on chromosome 14, as they are localised on chromosomes 20 and 1, respectively. Gene loci for non-syndromic forms of autosomal dominant and autosomal recessive deafness 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.

ACKNOWLEDGEMENTS
We thank all the patients, their family members, and their physicians for their participation in this study. FH was supported by a grant from the German Research Foundation (SFB 592).

Authors’ affiliations
R G Ruf, M T F Wolf, E Otto, F Hildebrandt, Departments of Pediatrics and Human Genetics, University of Michigan, Ann Arbor, USA
J Berkman, M Gattas, J Kromberg, J Macmillan, Queensland Clinical Genetics Service, Royal Children’s Hospital, Brisbane, Queensland, Australia
P Nurnberg, G Nurnberg, Gene Mapping Centre and Department of Molecular Genetics, Max-Delbrueck Centre for Molecular Medicine, Berlin-Buch, Germany
P Nurnberg, Institute of Medical Genetics, Charité University Hospital, Humboldt University, Berlin, Germany
E-M Ruf, University Children’s Hospital, Freiburg, Germany
V Hyland, Molecular Genetics Laboratory, Queensland Health Pathology Service, Queensland, Australia
I Glass, Departments of Pediatrics and Medicine, University of Washington School of Medicine, Seattle, USA
Correspondence to: Dr F Hildebrandt, University of Michigan Health System, 8220C MSRB III, 1150 West Medical Center Drive, Ann Arbor, MI 48109, USA; fhilde@umich.edu

REFERENCES
25. Ansar M, Din MA, Arshad M, Sahal M, Faiyaz-Ul-Haque M, Haque S, Ahmad W, Leal SM. A novel autosomal recessive non-syndromic deafness locus (DFNB35) maps to 1q42.4-1.4q24.3 in large consanguineous kindred from Pakistan. Eur J Hum Genet 2003;11:77-80
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


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, phaeochromocytomas, 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. More than 70% of the germline mutations are protein truncating and are distributed throughout the coding region. 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 dysmorphia. This was recognised very early and confirmed by several studies. 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. 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. 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 leukocytes 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...
been characterised. Resource Center (www.chori.org/bacpac) and have previously clones used as FISH probes were purchased from the BAC/PAC obtained by a punch biopsy of the left axillary region. BAC standard methods. Skin fibroblasts of the mother were lymphocytes of her son (the index patient III.2) according to lymphocytes and skin fibroblasts of proband II.2 and blood Chromosome spreads were prepared from peripheral blood FISH analysis were diagnosed, which had to be surgically corrected several times. progressive dislocation of his hips and genu valgum 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. 10,11,15 PCR products used as FISH probes, DJ1686/1863, DJHJK10/11, 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 genomic DNA from peripheral blood leucocytes. The deleted interval of proband II.2 are present as single copies in the majority of peripheral blood leucocytes.

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 FAM 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′-CTGCACTAGGCGATTTGTAAT 3′) and DJ1910 (5′-AAACCTCTTGTGGCCTACTC 3′), DJ1561 (5′-GTCAATAG CAAGAAAAGAGCAA 3′) and DJ1562 (5′-CAAGAAAAGAGCAA 3′), DJ1948 (5′-GAGAAAATGAAAGGAGAGA 3′) and DJ1936 (5′-CAAGAAAAGAGCAA 3′) and DJ1936 (5′-CAAGAAAAGAGCAA 3′). PCR products were digested with the restriction enzyme MboI and analysed by agarose gel electrophoresis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>Amplified from BAC (Acc No)</th>
<th>Position on BAC product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DJ1576</td>
<td>GACTGAATGTTGATGATTTTCGCC</td>
<td>640N20 (AC090616)</td>
<td>67241</td>
</tr>
<tr>
<td>DJ1578</td>
<td>AATTACGGGGGGCTTGAGGAT</td>
<td>640N20 (AC090616)</td>
<td>79694</td>
</tr>
<tr>
<td>DJ1686</td>
<td>CAGGGTTATGGGAGGAGGAGGAGG</td>
<td>640N20 (AC090616)</td>
<td>98021</td>
</tr>
<tr>
<td>DJ1863</td>
<td>ACCAGGCGTATGAGGAGGAGG</td>
<td>640N20 (AC090616)</td>
<td>104095</td>
</tr>
<tr>
<td>DJHJK10</td>
<td>CAGGGCGGCTCAGAACCAGA</td>
<td>307A16 (AC030041)</td>
<td>4242</td>
</tr>
<tr>
<td>DJHJK11</td>
<td>GCCTGGGGAGGGAGAGGGAAGA</td>
<td>8373</td>
<td></td>
</tr>
</tbody>
</table>
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. 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 III.2 was also investigated by FISH. In her fibroblast cultures, 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 analysed (n=50). Upon this finding, the mother (patient II.2) of the index patient III.2 and 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).\(^{15,16}\) On metaphase chromosome spreads of the index patient III.2 and his mother, BACs 785C15, 3047G17, 499I20, 805L22, and 542B22 were 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. Using BACs 271K11 and 474K4 as FISH probes, which span the \( WI-12393 \) gene derived LCRs (fig 2A), 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 confirm the deletion 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 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.

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 (psex) 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 deletion breakpoints on the affected chromosome.

[B] Fine mapping of the deletion boundaries within the \( KIAA0160 \) sequences. The exons of the functional \( KIAA0160 \) 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 DJHK10/11 are indicated. The position of the functional \( NF1 \) gene region at 17q11.2 and the localisation of STS markers as well as FISH probes. The grey rectangles. (B) Fine mapping of the deletion boundaries within the \( KIAA0160 \) sequences. The exons of the functional \( KIAA0160 \) and \( WI-12393 \) genes and their pseudogenes are shown by vertical rectangles with numbers based on the functional genes. The position of the functional \( NF1 \) gene region at 17q11.2 and the localisation of STS markers as well as FISH probes. The horizontal bar represents chromosome 17. The position of the functional \( NF1 \) gene, the functional \( KIAA0160 \) gene, the functional \( WI-12393 \) gene, and their pseudogene exons (psex) 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 deletion breakpoints on the affected chromosome.

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).
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. 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 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 ($\sim 1 \times 10^4$/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). Somatic mosaicism is clearly implicated in the pathogenesis of segmental neurofibromatosis, but only documented at the cytogenetic level for one patient. With regard to point mutations, somatic mosaicism of the NF1 gene is difficult to

**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.
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 analysis7,13,14, and two were ascertained by marker analysis.1,15 Moreover, Tischendorf et al.16 described a patient with segmental NF1 who was 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.17,18 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 JJAZ1 gene on chromosome 7 to the coding portion of the KIAA0160 (JJAZ1) gene on chromosome 17,19 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 suggested that the triple event, loss of one JJAZ1 allele, creation of a new fusion transcript between JJAZ1 and KIAA0160, as well as the loss of one KIAA0160 copy, triggers the development of endometrial sarcomas as a whole. Similarly, haploinsufficiency of KIAA0160 (JJAZ1) may contribute to the outgrowth and progression of neurofibromas in microdeletion patients. Our speculation that KIAA0160 (JJAZ1) 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 recombination hotspot in the NF1 gene region, besides the WI-12393 gene derived duplicons. Genotype analysis clearly indicates that the deletion occurred somatically in proband II.2 by an intrachromosomal (sister chromatid based) mechanism (figs 1 and 3).

Figure 5 The different origins of sporadic forms of NF1.
According to previous studies, low copy repeat mediated de novo deletions are believed to be triggered by meiotic recombination during germ cell development. This notion was based on PCR experiments that failed to identify the patient specific microdeletion in the blood of their healthy parents. López Correa et al. 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 gonadsomal 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.

ACKNOWLEDGEMENTS
We thank Helene Spöri and Antje Kollak for expert technical assistance. The Institute of Medical Biology and Human Genetics at the University of Graz is a member of the IBMS and was supported by the infrastructure programme UGP4 by the Austria Ministry of Education, Science and Culture. This research was supported by grants of the Oesterreichische Nationalbank (No 9522/EP), by the FOF (No S7403/PKM), and by the Deutsche Forschungsgemeinschaft (HA-1082 and KE-724 2-1/HK-S) and (KFO 113-1/DEJ).

Authors’ affiliations
E Petek, C Windpassinger, K Wagner, P M Kroisel, Institute of Medical Biology and Human Genetics, University of Graz, Austria
D E Jenne, W Lasinger, Max-Planck-Institute of Neurobiology, Department of Neuroimmunology, Martinsried, Germany
J Smolle, B Binder, Department of Dermatology, University of Graz, Austria
H Kehrer-Sawatzki, Department of Human Genetics, University of Ulm, Ulm, Germany
Correspondence to: Dr H Kehrer-Sawatzki, Department of Human Genetics, University of Ulm, Albert-Einstein-Allee 11, 89081 Ulm, Germany; hildegard.kehrer-sawatzki@medizin.uni-ulm.de

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

C Gagliardi, M C Bonaglia, A Selicorni, R Borgatti, R Giorda

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 megaloureter. 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 visuconstructive 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′-CTCTCCCTTTCGGTTGTAAATGT-3′, ABI-Fam labelled) and CYLN2 i1R (5′-CGCCCTCACCTGCGCTTCT-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 (Applera, 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 (Applera). 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 neurological examination and the genetic analysis was obtained from his parents. The pregnancy was uneventful,
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 supravalvular aortic 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 months) and vesicoureteric reflux with right megaureter (surgically corrected at the age of 17 months). The following months) and vesicoureteric reflux with right megaureter (surgically corrected at the age of 17 months). The following months) and vesicoureteric reflux with right megaureter (surgically corrected at the age of 17 months). The following months) and vesicoureteric reflux with right megaureter (surgically corrected at the age of 17 months). The following months) and vesicoureteric reflux with right megaureter (surgically corrected at the age of 17 months). The following months) and vesicoureteric reflux with right megaureter (surgically corrected at the age of 17 months).

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, and a neuropsychological battery, VMI block construction and Rey Figure, 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, 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 WSCG displayed the typical Williams syndrome cognitive profile. 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 D7S5613 loci. FISH with clone CTB-8H17, partially overlapping the centromeric cluster of repeated sequences (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. Additional FISH experiments with clones RP4-665P5 (fig 2C), RP11-815K3, and CTB-139P11 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.

<table>
<thead>
<tr>
<th>Tests</th>
<th>WS subjects with typical deletion</th>
<th>Proband score</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average score</td>
<td>SD</td>
<td></td>
</tr>
<tr>
<td>Chronological age (y)</td>
<td>5.12</td>
<td>0.66</td>
<td>5.60</td>
</tr>
<tr>
<td>Mental age (y)</td>
<td>3.39</td>
<td>0.63</td>
<td>4.82</td>
</tr>
<tr>
<td>IQ (score)</td>
<td>68.67</td>
<td>16.29</td>
<td>83.00</td>
</tr>
<tr>
<td>Corsi Span (score for years)</td>
<td>3.60</td>
<td>3.60</td>
<td>3.60</td>
</tr>
<tr>
<td>Digit Span (score for years)</td>
<td>1.80</td>
<td>2.14</td>
<td>3.00</td>
</tr>
<tr>
<td>VMI (score for years)</td>
<td>3.30</td>
<td>0.38</td>
<td>4.30</td>
</tr>
<tr>
<td>Rey Figure copy (score for years)</td>
<td>Unmeasurable</td>
<td>4.60</td>
<td></td>
</tr>
<tr>
<td>Rey Figure memory (score for years)</td>
<td>Unmeasurable</td>
<td>4.60</td>
<td></td>
</tr>
<tr>
<td>Blocks - WISC-R (score)</td>
<td>1.40</td>
<td>1.20</td>
<td>2.00</td>
</tr>
</tbody>
</table>
intensity on both chromosome 7 homologues, indicating absence of deletion.

Microsatellite analysis 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, D7S489B, and D7S669 were also heterozygous. All other 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 and CYLN2 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 RP4-665P05 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.
Very few subjects with deletions that do not span the entire WS region have been reported so far. Two patients carrying a deletion spanning from ELN to marker D7S1870 still had the full WS phenotype. Tassabehji et al. and Karmiloff-Smith et al. 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. (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. 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, 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. The GTF2IRD1 gene, coding for a putative transcription factor with ubiquitous expression, 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. 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. 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 Gif2IRD1 gene 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. 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.

ACKNOWLEDGEMENTS

The authors wish to thank the family of the patient for their cooperation, Lucy Osborne and Stephen Scherer for their gift of clones CTB-139P11 and RP11-815K3, and Uberto Pozzoli, Maria Teresa Bassi, and Manuela Sironi for their helpful suggestions. This study was supported in part by grant ICS 030.10/RP97.37 from the Italian Ministry of Health.
O bstructive pulmonary disease is an important health problem in all populations, and bronchiectasis of unknown aetiology (idiopathic bronchiectasis, IB) contributes significantly to the disease. 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 (Tn) in intron 8 (IVS8) have been observed in patients with IB. In our study, we observed a high frequency of T9 and CFTR mutations in children with idiopathic bronchiectasis.

**Authors' affiliations**

C Gogliardi, M C Bonaglia, R Borgatti, R Giorda, IRCCS “E Medea”, Basioso Parini, (LC), Italy; rgiorda@bp.lnf.it

Correspondence to: Dr R Giorda, IRCCS “E Medea”, Via Don Luigi Monza 20, 23842 Basioso Parini (LC), Italy; rgiorda@bp.lnf.it

REFERENCES


31 Osborne LR, Campbell T, Daradich A, Scherer SW, Tsui LC. Identification of a putative transcription factor gene (WBSSCR1) that is commonly deleted in Williams-Beuren syndrome. Genomics 1999; 57:279-84.


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

V N Ninis, M O Kýlyncz, M Kandemir, E Daşlý, A Tolun


www.jmedgenet.com

Downloaded from http://jmg.bmj.com/ on June 17, 2017 - Published by group.bmj.com
**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 74 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 47OM, 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. 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, obstructive azoospermia, allergic bronchopulmonary aspergillosis, and idiopathic chronic pancreatitis.

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. 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, α1-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) 68.3% (SD 24.1).

**Mutation analysis**

The methods have been described in detail by Kılıçoglu et al. 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). 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>T4 and 1811+1.6kbA>G, deletion mutations CFTRdele1,2,3 and CFTRdele19, and −33A>G in the minimal promoter region. 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. The three alleles (T5, T7, and T9) of Tn were amplified by allele specific PCR. 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. The alleles for the (TG)n tract upstream of Tn 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. The alleles for the biallelic GATT repeat in intron 6 and the three multiallelic microsatellites (IVS5CA in intron 8 and IVS17bTA and IVS17bCA in intron 17b) were resolved on polyacrylamide gels. Haplotypes were constructed by segregation analysis using the available DNA samples from parents and sibs.

**Statistical analysis**

χ2 test was applied to contingency tables to detect statistically significant differences in allele frequencies; p values of less
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 CFTPdele19, and two for K68E. Another patient was a compound heterozygote for −33G>A and CFTRdele2,3. 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 comparisons of T9 and T7, and T5 in the normal chromosomes to be 28, 162, and 10, respectively, in a total of 200. \( \text{Chi}^2 \) tests were used to compare the allelic frequencies at three polymorphic 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 total of 34 with mutations and 53 among the total of 112 without mutations.

In contrast, T5 IB chromosomes were mostly on backgrounds of bronchiectasis. Certain alleles of T9, T7, and T5 in the IB chromosomes in which no mutation was identified (no mutation chromosomes) to be T7, 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).

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 T5 IB chromosomes also showed great variation as compared to normals. There were 10 T5 chromosomes among the total of 34 with mutations and 53 among the total of 112 without mutations.

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 T913 and 470V.

Haplotypes
Haplotypes could be identified in 71 of the unrelated patients with respect to the alleles at the polymorphic loci (GATT), T9, 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 GATT-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 GATT-TG11-470M (five of 12) and GATT-TG11-470V (four of 12). The remaining three haplotypes were observed once: GATT-TG11-470M, GATT-TG11-470V-1001+1T-2694T, and GATT-TG13-470V-2692V-2694T. When the three microsatellites were also taken into account, no two of the T5 IB chromosomes had the same haplotype.

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

<table>
<thead>
<tr>
<th>Mutation</th>
<th>No of alleles</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>K68E</td>
<td>9</td>
<td>Lys to Glu at 68</td>
</tr>
<tr>
<td>−33G&gt;A</td>
<td>7</td>
<td>Promoter mutation</td>
</tr>
<tr>
<td>N1303K</td>
<td>6</td>
<td>Arg to Lys at 1303</td>
</tr>
<tr>
<td>CFTPdele19</td>
<td>4</td>
<td>Deletion of exon 19</td>
</tr>
<tr>
<td>3272-26A&gt;G</td>
<td>3</td>
<td>mRNA splicing defect</td>
</tr>
<tr>
<td>CFTPdele2,3</td>
<td>2</td>
<td>Deletion of exons 2 and 3</td>
</tr>
<tr>
<td>F1052V</td>
<td>1</td>
<td>Phe to Val at 1052</td>
</tr>
<tr>
<td>E1228G</td>
<td>1</td>
<td>Glu to Gly at 1228</td>
</tr>
<tr>
<td>W1282X</td>
<td>1</td>
<td>Trip to Stop at 1282</td>
</tr>
<tr>
<td>Total alleles</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

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

**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. 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 CFTPdele19 and CFTPdele2,3 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* leavis, and dogfish. 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.

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, 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. The numbers in CF patients were as follows: one K68E, four N1303K, one CFTPdele2,3, and five W1282X. Three of the remaining IB mutations (3272-26A>G, CFTPdele19, F1052V) were identified in other studies in CF patients,\(^\text{21,23}\) one (−33G>A) in a CBVAD patient,\(^\text{22}\) and the last one (E1228G) in this study.

**Significance of the allelic frequencies at three polymorphic loci**
Certain alleles of T9 and (TG)m and allele 470M, alone or in association with others, have been implicated in the aetiology of bronchiectasis.\(^\text{11,12}\) 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.\(^\text{15}\) We found the frequencies of T9, T7, and T5 in the IB chromosomes in which no mutation was identified (no mutation chromosomes) to be T7, 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).

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.
ian disseminated bronchiectasis patients, mutant alleles among the total of 19 detected in the adult Ital-
our population was assessed as 1/50. This frequency is very high, as the carrier frequency in
chromosomes in our 73 unrelated idiopathic bronchiectasis
marriages) were not homozygous. Patients from 26 other families who
ily of one of them had declared parental consanguinity and of
four others had denied it. Patients from 26 other families who
with mutations and six with no mutation detected. The fam-
The haplotypes were evaluated to assess identity by descent. In

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. 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. This mutation comprised three of the mutations in our IB patients (3272-26A>G, *CFTR*dele19, and −33A>G) were not carried by any of our CF patients (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.17 It has been reported in only one other person, a CF patient in north eastern Italy.18 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.21 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.22 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.11 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.11,14 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

<table>
<thead>
<tr>
<th>IVS8 (TG)n</th>
<th>IVS8 (T)n</th>
<th>Polymorphism</th>
<th>Mutation</th>
<th>No of chroms</th>
<th>No of heterozyg patients</th>
<th>No of homozyg patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 5</td>
<td>470M</td>
<td>–</td>
<td>6</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>13 5</td>
<td>470V</td>
<td>–</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>470M</td>
<td>–</td>
<td>10</td>
<td>8</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>470V</td>
<td>–</td>
<td>10</td>
<td>8</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>470M</td>
<td>–</td>
<td>40</td>
<td>26</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>11 9</td>
<td>470M</td>
<td>–</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>470V</td>
<td>–</td>
<td>17</td>
<td>11</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>11 9</td>
<td>470V</td>
<td>–</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>11 7/9</td>
<td>470M/V</td>
<td>–</td>
<td>2</td>
<td>0</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td>11 5</td>
<td>470V, 2694T</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1001+11T, 4002G</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>470V, 2694T</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>875+40G</td>
<td>470V, 2694T</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>470V, 2694T</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>470V, 2694T</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>470M, 1001+11T</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>470M, 875+40G</td>
<td>–</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>470V</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>13 5</td>
<td>470V, 2694T</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>470V</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>470V</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>470V</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>470V</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>470V</td>
<td>–</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>470V</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>470M</td>
<td>–</td>
<td>9</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>470V</td>
<td>–</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>470V</td>
<td>–</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>470V</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>470M</td>
<td>–</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

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 haplotypes 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 haplotype of one pair was T7/T7-*CFTR*dele19, three were T9/T9, and two were T9/T7.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Polymorphism</th>
<th>Mutation</th>
<th>No of chroms</th>
<th>No of heterozyg patients</th>
<th>No of homozyg patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 9</td>
<td>470V</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>9 9</td>
<td>470M</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>9 9</td>
<td>470M</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>9 9</td>
<td>470M</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>9 9</td>
<td>470V</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>9 9</td>
<td>470M</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>9 9</td>
<td>470M</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>9 9</td>
<td>470M</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>9 9</td>
<td>470M</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

www.jmedgenet.com
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)), but not in two French and one Greek mostly adult patient group.1,10 In addition, the T5 chromosomes in our IB patients were mostly on backgrounds TGI1-470W 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. The 470V allele leads to a higher protein activity, 470M protein having 1.7-fold increased mRNA level and 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.11 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, the report highlights T9, an allele that has 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 common allele in the normal population worldwide, 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.10 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 no CFTR deletions (33G>A/CFTRD dele2,3). 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.12 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.

ACKNOWLEDGEMENTS
The work was supported by the Scientific and Technical Research Council of Turkey (SBAG-191T070), Boğaziçi University Research Fund (98B105), and the Turkish Academy of Sciences.

Authors' affiliations
V N Ninis, M O Kylync,1 A Tulun,2 Department of Molecular Biology and Genetics, Boğaziçi University, Istanbul, Turkey
M Kendemir, E Do˘blı,3 Department of Paediatrics, Marmara University Hospital, Istanbul, Turkey
Correspondence to: Dr A Tulun, Department of Molecular Biology and Genetics, Boğaziçi University, Bebek, Istanbul 34342, Turkey; tulon@boun.edu.tr
*Present address: School of Medicine, University of Louisville, Kentucky, USA.

REFERENCES


A significant correlation has been found between FMRP mosaicism and methylation mosaicism. Very recently, a highly significant correlation was reported in three male sibs with a varying degree of methylation mosaicism. The family was ascertained through a son of a fragile X family known to the Department of Clinical Genetics, Rotterdam. The three brothers who are the subjects of this report are from a fragile X family (60th centile). He had a normal face, except for a broad forehead, normal ears (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 normal/4 ml). He had hyperextensible finger joints and relatively normal teeth (fig 1). His testes were mildly enlarged for his age (4 ml/4 ml). He had hyperextensible finger joints and relatively normal teeth (fig 1).

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.

**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.

**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 isolated from blood leucocytes digested with HindIII and the methylation sensitive enzyme EagI and hybridised with probe pP2 according to standard protocols.

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. Further immunohistochemical incubations were performed according to procedures described previously (http://www.eur.nl/FGG/CH1/frax/). 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 1.10 m (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.
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.

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.
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 sub-classes 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.12-15 However, high functioning males with a size mosaic pattern have been described16-18 and Tassone et al19 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.20-22 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 ≥50% of lymphocytes.23-26 This situation is reminiscent of the situation in females with a full mutation where cognitive function is related to the X inactivation pattern.23-26

FMRP expression studies in blood smears of methylation mosaic males have been reported. Smects et al20 reported normal protein expression in cell lines of two normal functioning adults with an unmethylated full mutation. De Vries et al20 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 al21 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 ≥50% of lymphocytes.22 The findings of normal FMRP expression in partially unmethylated full mutations were in contrast with the report of Feng et al,23 who found markedly diminished FMRP production in fibroblasts 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 al24 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.

ACKNOWLEDGEMENTS

We thank the patients and their family for their kind support.

Authors’ affiliations
B B A de Vries, A Jacobs, Department of Human Genetics, University Medical Centre Nijmegen, The Netherlands
L A Severijnen, R Olmer, D J J Holley, B A Oosta, R Willemsen, Department of Clinical Genetics, Erasmus University Rotterdam, The Netherlands

Correspondence to: Dr B B A de Vries, Department of Clinical Genetics,
REFERENCES


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

G F Leal, E Roberts, E O Silva, S M R Costa, D J Hampshire, C G Woods


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 autozygos- ity 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.
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 markers selected from the ABI Linkage Mapping Panel Version I (Applied Biosystems), the Todd Panel,2 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

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.
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. Two point analysis was performed using the LINKAGE analysis programs 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 neurons and may have a role in glial cell growth and differentiation during development. 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. 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.

**ACKNOWLEDGEMENTS**

We express our gratitude to the members of the family studied. This work has been funded by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), FACEPE (Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco), the Wellcome Trust, and the West Riding Medical Research Trust. We thank the Research Center Aggeu Magalhães (CpqAM) for permitting us to use their equipment for DNA extraction and the staff of HGMF for computational assistance.

---

**REFERENCES**


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

<table>
<thead>
<tr>
<th>Marker</th>
<th>Lod score at $\theta = 0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D13S175</td>
<td>~</td>
</tr>
<tr>
<td>D13S275</td>
<td>5.90</td>
</tr>
<tr>
<td>D13S221</td>
<td>3.87</td>
</tr>
<tr>
<td>D13S1304</td>
<td>2.75</td>
</tr>
<tr>
<td>D13S1254</td>
<td>4.29</td>
</tr>
<tr>
<td>D13S1244</td>
<td>2.31</td>
</tr>
</tbody>
</table>

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

A Mubaidin, E Roberts, D Hampshire, M Dehyyat, A Shurbaji, M Mubaidien, A Jamil, A Al-Din, A Kurdi, C G Woods

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. Using polymorphic microsatellite markers we have shown that this family is not linked to the pantothenate kinase gene (PANK2) on chromosome 20. 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.

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 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. Dyssmetria, past pointing, dysdiadochokinesia, and intentional tremor were bilateral and symmetrical. The gait was wide based, swayed in all directions, and had a spastic

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.
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.

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.

MOLECULAR GENETIC STUDIES

We performed linkage analysis between the family's disease and the PANK2 gene that causes PKAN. 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, 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 5q23 (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. 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 syndrome) or atypical features with acanthocytosis, hypoprebetalipoproteinaemia and/or orofacial dyskinesia (known as HARP syndrome) 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. The family we report appears to have a demonstrably different condition from these two phenotypes because of the
<table>
<thead>
<tr>
<th>Disease title</th>
<th>Inheritance pattern</th>
<th>Age of presentation</th>
<th>Clinical features*</th>
<th>Radiological features†</th>
<th>Gene§</th>
<th>Chromosomal location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aceruloplasminaemia</td>
<td>Recessive</td>
<td>&gt;30 y</td>
<td>Dementia, ataxia, chorea, spasticity, rigidity</td>
<td>Normalness</td>
<td>CP</td>
<td>3q23.4</td>
</tr>
<tr>
<td>Neuroferritinopathy</td>
<td>Recessive</td>
<td>5–20 y</td>
<td>Rigidity, choreoathetosis, dystonia</td>
<td>Often declines</td>
<td>MIM 606159</td>
<td></td>
</tr>
<tr>
<td>PKAN/PANK2</td>
<td>Atypical and HARPM</td>
<td>&gt;15 y</td>
<td>Diverse extrapyramidal symptoms</td>
<td>Often declines</td>
<td>20p13</td>
<td></td>
</tr>
<tr>
<td>Karak syndrome</td>
<td>Recessive or X linked</td>
<td>6 y</td>
<td>Ataxia, chorea, dystonia</td>
<td>Declines “Eye of the tiger” and substantia nigra</td>
<td>Yes, atrophy</td>
<td></td>
</tr>
</tbody>
</table>

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

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

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.20 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. 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 aceruloplasminaemia.3 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.

ACKNOWLEDGEMENTS

We express our gratitude to the members of the family studied. The Wellcome Trust and the West Riding Medical Research Trust have funded this work.


Authors’ affiliations

A Mubaidin, M Dehyat, A Shurbaji, A Kurdi, Neurology Department, King Hussein Medical Centre, PO Box 926442, Amman, Jordan 2

E Roberts, D Hampshire, C G Woods, Molecular Medicine Unit, University of Leeds, Clinical Sciences Building, St James’s University Hospital, Beckett Street, Leeds, UK

M Mubaidien, Ophthalmology Department, King Hussein Medical Centre, Amman, Jordan

A Jamil, Radiology Department, King Hussein Medical Centre, Amman, Jordan

http://jmg.bmj.com/ on June 17, 2017 - Published by group.bmj.com
Myhre syndrome: new reports, review, and differential diagnosis

L Burglen, D Héron, A Moerman, A Dieux-Coeslier, J-P Bourguignon, A Bachy, J-C Carel, V Cormier-Daire, S Manouvrier, A Verloes

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, brachyphalangy and brachymetacarpalia, large epiphyses (notably the femoral heads and proximal phalanges of fingers 2 and 3), and mild platyspondylic appearance of the vertebral column). 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’ 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 thickened, 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

www.jmedgenet.com
**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 vertebral bodies with large pedicles. Moore-Federman syndrome, acromicro 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.

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 mU/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 oseotendinous 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
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, 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 brachydaulyy 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 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

<table>
<thead>
<tr>
<th></th>
<th>Myhre et al&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Saljak et al&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Garcia-Cruz et al&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Whiteford et al&lt;sup&gt;4&lt;/sup&gt;</th>
<th>Titomanlio et al&lt;sup&gt;5&lt;/sup&gt;</th>
<th>This report</th>
<th>Total or mean&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Paternal age at birth</strong></td>
<td>Patient 1</td>
<td>37</td>
<td>38</td>
<td>42</td>
<td>32</td>
<td>23</td>
<td>32</td>
</tr>
<tr>
<td>Sex</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td><strong>General</strong></td>
<td>Birth weight (g)</td>
<td>2100</td>
<td>2900</td>
<td>3200</td>
<td>2200</td>
<td>2100</td>
<td>2870</td>
</tr>
<tr>
<td></td>
<td>Height in SD</td>
<td>-5.5</td>
<td>-4.5</td>
<td>-4.5</td>
<td>-6</td>
<td>-3</td>
<td>-2</td>
</tr>
<tr>
<td></td>
<td>Adult height</td>
<td>(140 cm)</td>
<td>(146 cm)</td>
<td>(146 cm)</td>
<td>(139 cm)</td>
<td>(154 cm)</td>
<td>(140 cm)</td>
</tr>
<tr>
<td></td>
<td>Age at last observation</td>
<td>24</td>
<td>18</td>
<td>16</td>
<td>18</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td><strong>Craniofacial features</strong></td>
<td>OFC (SD)</td>
<td>-1</td>
<td>-1.5</td>
<td>?</td>
<td>-1.5</td>
<td>M</td>
<td>+1.5</td>
</tr>
<tr>
<td></td>
<td>Strabismus/fissures</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Midface hypoplasia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Short philtrum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Narrow mouth</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Thin upper lip</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Prognathism</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Class lip</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Small ears</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Skeletal anomalies</strong></td>
<td>Brachydactyly</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Thick calvaria</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Joint limitations</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Broad ribs</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Narrow pelvis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Thick femoral necks</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Mild platyspondaly</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Large pedicles</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Neuromuscular</strong></td>
<td>IQ</td>
<td>45</td>
<td>35–50</td>
<td>75</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td>Vary thick skin</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Deafness</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Ophthalmological anomalies</td>
<td>Hyperm</td>
<td>Hyperm</td>
<td>Hyperm</td>
<td>Hyperm</td>
<td>Hyperm</td>
<td>Hyperm</td>
</tr>
<tr>
<td></td>
<td>Heart malformation</td>
<td>+</td>
<td>ASD</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Kidney malformation</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Hypertension</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Inguinal hernia</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Hypospadias</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Cryptorchidism</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

ASD: atrial septal defect. PPS: peripheral pulmonary stenoses. PDA: patent ductus arteriosus. ?: not determined.
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 reported two unrelated males with mental retardation, facial dysmorphism (short palpebral fissures, maxillary hypoplasia, prognathism, short phaltrum, 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 vertebral with large pedicles. Five further cases were reported. 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 phaltrum, 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 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.5 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 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 hypergonadotropic hypogonadism. Although these phenotypic manifestations are discordant, they may point to a disturbance in the hypothalamic-hypophyso-gonalad axis that should be investigated in further cases.

Several syndromes were transiently considered in the differential diagnosis of our patients. OSMED syndrome shows megaeiphyses, irregular vertebral bodies, and deafness, but can be distinguished by a different facial dysmorphism (micrognathia), metaphyseal involvement, and progressive carpal fusion. Phenotypic similarities were noted between Myhre syndrome and GOMBO syndrome, but the latter has been shown to result from a cryptic translocation between chromosomes 3p and 22q. 14 Telomeric FISH analyses performed in two of our patients detected no cryptic rearrangement. Moore-Federman syndrome, acromicric dysplasia, and LAPS syndrome 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. 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.5 7 16 There is now convincing evidence to reject lumping with the recessively inherited geleophysic dysplasia as previously suggested.14 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 dysplasia 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. Myhre syndrome and geleophysic dysplasia share some features but hepatomegaly and

### Table 2 Differential diagnosis of Myhre syndrome

<table>
<thead>
<tr>
<th>Feature</th>
<th>Myhre</th>
<th>LAPS</th>
<th>Geleophysic dysplasia</th>
<th>Acromicric dysplasia</th>
<th>Moore-Federman</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short stature</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Brachydactyly</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Joint limitation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Muscular hypertrophy</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mental retardation</td>
<td>Freq</td>
<td>Learn difficulties</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Deafness</td>
<td>+/ 9</td>
<td>+ 3/5</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Abnormal skin</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Laryngotracheal stenosis</td>
<td>too young?</td>
<td>severe adult onset</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cardiac disease</td>
<td>Congenital malformations</td>
<td>Pericarditis</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Transmission</td>
<td>AD? X linked</td>
<td>1M/0F</td>
<td>AD? 1M/4F</td>
<td>Progressive cardiac valve disease</td>
<td>+/–</td>
</tr>
</tbody>
</table>

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 dysplasia 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. 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. Figueura suggested that the patient reported by Rosser et al as having geleophysic dysplasia had Myhre syndrome. Nevertheless, as pointed out by McGaughran and Donnai, geleophysic dysplasia seems a more convincing diagnosis for this patient who had hepatomegaly, thickened mitral valve, and “geleophysic dysplasia facial dysmorphism”.

Hopkin et al described three patients with a disorder characterised by short stature, joint limitation, and progressive adult onset laryngotracheal stenosis. Lindor et al 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 characterised by short stature, joint limitation, and progressive adult onset 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 characterises 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.

Authors’ affiliations
L Burglen, Unité de Génétique Médicale, Service de Neuropédriatrie, Hôpital Trousseau, AP-HP, Paris, France
D Héron, A Moereman, Département de Génétique Médicale, Hôpital de la Pitié-Salpêtrière, AP-HP, Paris, France
A Moereman, A DieuX-Coëslier, S Manouvrier, Département de Génétique Médicale, Hôpital Jeanne de Flandre, Lille, France
J-P Bourguignon, University Department of Paediatrics, Liége University, Liége, Belgium
A Bachy, Department of Paediatrics, Clinique Notre Dame, Charleroi, Belgium
J-C Carel, Service d’Endocrinologie Pédiatrique, Hôpital Saint Vincent de Paul, AP-HP, Paris, France
V Cormier-Daire, Département de Génétique Médicale, Hôpital Necker-Enfants Malades, AP-HP, Paris, France
A Verloes, Unité de Génétique Clinique, Hôpital Robert Debré, AP-HP & INSERM E9935, Paris, France

Correspondence to: Dr A Verloes, Unité de Génétique Clinique, Hôpital Robert Debré & INSERM E9935, Paris, France; alain.verloes@rd.ap-hop-paris.fr

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

V N Ninis, M O Kýlýnç, M Kandemir, E Daðlý and A Tolun

J Med Genet 2003 40: 530-535
doi: 10.1136/jmg.40.7.530

Updated information and services can be found at:
http://jmg.bmj.com/content/40/7/530

These include:

Supplementary Material
Supplementary material can be found at:
http://jmg.bmj.com/content/suppl/2003/09/02/40.7.530.DC1

References
This article cites 37 articles, 6 of which you can access for free at:
http://jmg.bmj.com/content/40/7/530#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Errata
An erratum has been published regarding this article. Please see next page or:
/content/41/11/813.full.pdf

Topic Collections
Articles on similar topics can be found in the following collections

- Molecular genetics (1254)
- Cystic fibrosis (39)
- Pancreas and biliary tract (110)
- Pancreatitis (13)
- Asthma (21)
- Immunology (including allergy) (604)
- Ethics (220)
- Reproductive medicine (519)
- TB and other respiratory infections (24)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/
ORIGINAL ARTICLE

TLR4 and TNF-α polymorphisms are associated with an increased risk for severe sepsis following burn injury

R C Barber, C C Aragaki, F A Rivera-Chavez, G F Purdue, J L Hunt, J W Horton

Context: Sepsis, organ failure, and shock remain common among patients with moderate to severe burn injuries. The inability of clinical factors to identify at-risk patients suggests that genetic variation may influence the risk for serious infection and the outcome from severe injury.

Objective: Resolution of genetic variants associated with severe sepsis following burn injury.

Patients: A total of 159 patients with burns ≥20% of their total body surface area or any smoke inhalation injury without significant non-burn related trauma (injury severity score (ISS)≥16), traumatic or anoxic brain injury, or spinal cord injury and who survived more than 48 h post-admission.

Methods: Candidate single nucleotide polymorphisms (SNPs) within bacterial recognition (TLR4 +896, CD14 −159) and inflammatory response (TNF-α −308, IL-1β −31, IL-6 −174) loci were evaluated for association with increased risk for severe sepsis (sepsis plus organ dysfunction or septic shock) and mortality.

Results: After adjustment for age, full-thickness burn size, ethnicity, and gender, carriage of the TLR4 +896 G-allele imparted at least a 1.8-fold increased risk of developing severe sepsis following a burn injury, relative to AA homozygotes (adjusted odds ratio (aOR) 6.4; 95% confidence interval (CI) 1.8 to 23.2). Carriage of the TNF-α −308 A-allele imparted a similarly increased risk, relative to GG homozygotes (aOR = 4.5; 95% CI 1.7 to 12.0). None of the SNPs examined were significantly associated with mortality.

Conclusions: The TLR4 +896 and TNF-α −308 polymorphisms were significantly associated with an increased risk for severe sepsis following burn trauma.

Burn trauma is an important cause of morbidity and mortality worldwide. Sepsis, organ failure, and shock remain common among patients with moderate to severe burn injuries, despite a greater understanding of post-burn resuscitation, organ system support, and burn wound treatment. Sepsis accompanied by organ failure carries a 30–50% case-fatality rate and those patients who do survive often require prolonged and costly medical care. Although we have gained an increased understanding of the pathophysiological processes that follow burn injury and a wealth of information concerning the clinical factors that predispose burn victims to severe sepsis and septic shock, we are still unable to predict accurately which patients will follow a complicated clinical course.

The inability of clinical factors to identify at-risk patients suggests that genetic variation may influence the risk for serious infection and the outcome from severe injury. Indeed, associations between specific genetic variants and outcome following serious injury and infection have been reported.

Burn trauma offers certain advantages for the study of such associations. Notable among these is the highly quantifiable nature of the injury, in terms of burn depth and extent (burn size). The simple expression of burn size as a percentage of total body surface area (TBSA) adjusts for individual variation in body mass, enabling the direct comparison of patients. In addition, associated smoke inhalation can be diagnosed accurately. Finally, the typical burn patient is relatively young and free from comorbidity prior to the burn injury.

Identification of genetic polymorphisms that predispose burn patients to the most severe manifestations of infection would allow early targeting of high risk individuals for aggressive or novel treatment, potentially improving their clinical outcome. Eventually, rapid genetic screening of patients may enable physicians to tailor treatment to the individual, rather than the injury.

Selection of candidate single nucleotide polymorphisms (SNPs) was based upon location within a biologically relevant locus and evidence of a functional effect. Candidate SNPs were located within genes responsible for microbial recognition (TLR4 and CD14), as well as cytokine loci that are known to mediate the inflammatory response (TNF-α, IL-1β, and IL-6). In addition, candidate SNPs had at least one report of an effect upon protein function or abundance or a previous report of an association with an inflammation or immune response related phenotype.

METHODS

Study design and data collection

Patients admitted to the burn intensive care unit (BICU) unit at Parkland Memorial Hospital (Dallas, TX) with any smoke inhalation injury or with ≥20% TBSA burns were prospectively enrolled between April 1999 and December 2003, under a protocol approved by the Institutional Review Boards of the University of Texas Southwestern Medical Center and Parkland Memorial Hospital that waived the requirement for informed consent. In order to remove confounding variables that were unrelated to burn injury, individuals were excluded if they presented with significant non-burn related trauma (injury severity score (ISS)≥16), traumatic or anoxic brain injury, spinal cord injury, or if they failed to survive more than 48 h post-admission.

Clinical data were recorded daily and stored in a computerised database, concurrent with admission to the BICU.

Abbreviations: aOR, adjusted odds ratio; BICU, burn intensive care unit; ISS, injury severity score; LPS, lipopolysaccharide; PCR, polymerase chain reaction; SNPs, single nucleotide polymorphisms; TBSA, total body surface area
BICU. These data were supplemented with information from the prospectively maintained burn registry and the BICU database. The ISS is an index of injury severity that ranges from 0 to 75, with a value equal to or greater than 16 indicating severe injury. 20

DNA isolation and genotyping
Venous blood samples were collected into EDTA and genomic DNA was isolated by standard protocols. 21 Fragments containing each of the SNPs were amplified from genomic DNA by polymerase chain reaction (PCR) using Taq DNA polymerase (Roche Diagnostics, Indianapolis, IN). All amplifications were carried out in a PTC 200 thermal cycler (MJResearch, Watertown, MA) using a thermal profile, reaction conditions, and primer sequences that were optimised for each SNP (table 1). All the genotypes examined in this study were determined by Pyrosequencing (Pyrosequencing AB, Westborough, MA). Pyrosequencing is a DNA sequencing technique that is based upon the detection of pyrophosphate, which is released in direct proportion to the number of incorporated nucleotides during DNA synthesis. The energy retained by pyrophosphate was utilised in an enzymatic cascade to cleave a targeted substrate and produce visible light. The quantity of light produced was measured and genotypes were resolved using PSQ 96 SNP Software, v 1.2 AQ. Each SNP was assayed with a specific primer sequence (table 1), which enabled the scoring of heterozygotes and alternate homozygotes with equal reliability. 22 In addition, a representative sample of genotypes at each SNP was confirmed by restriction fragment length polymorphism and/or sequence analysis (data not presented).

Quantification of sepsis severity
Sepsis was defined according to the American College of Chest Physicians/Society of Critical Care Medicine consensus definitions. Severe sepsis was defined as sepsis that was temporally accompanied by the need for intravenous vasopressor drug support (excluding dopamine at $\leq 5 \mu g/kg/min$) to maintain blood pressure (despite adequate fluid resuscitation) along with the presence of perfusion abnormalities, or metabolic acidosis (pH $\leq 7.30$) or the development of respiratory, renal, hepatic, or haematological failure. Specifically, a patient was considered to have sepsis related organ failure if a Marshall organ dysfunction score of $\geq 3$ (failure) was assigned in one or more organs concurrent with, or within 48 h of, the diagnosis of sepsis. 23 Mild sepsis was defined as sepsis without any evidence of organ dysfunction or the need for intravenous vasopressor drug support to maintain blood pressure. Inhalation injury was defined as a history of being burned in a closed, smoke filled space, with or without positive bronchoscopy and acute respiratory distress syndrome (bilateral fluffy infiltrates in the absence of congestive heart failure, P:F $< 200$, and the need for more than 96 h of mechanical ventilation, independent of airway protection).

Data analysis
Data analysis was performed using SPSS 10.1 (SPSS, Chicago, IL) and SAS 8.02 (SAS Institute, Cary, NC) statistical software. Descriptive statistics included counts and percentages for categorical variables and medians with associated 25th and 75th quartiles for continuous data. Categorical data were compared using the $\chi^2$ test, while continuous data were compared by Mann-Whitney U. Best sets multiple logistic regression was used to simultaneously evaluate the effects of multiple variables as risk factors for severe sepsis. Actual $p$ values are reported for all analyses. The adjusted odds ratios (aORs) obtained from the regression analysis are presented with their associated 95% confidence intervals. Due to the low allele frequencies, homozygous carriers of the TNF-$\alpha$ -308A-, TLR4 +896G-, and IL-6 -174C- alleles were grouped with heterozygotes for analysis. Alternate homozygotes at the more common IL-1$\beta$ -31 and CD14 -159 SNPs were analysed separately from heterozygotes. The IL-1$\beta$ and CD14 SNPs were analysed both ways and the results did not differ (data not presented).

RESULTS
Demographics and clinical outcomes
Between April 1, 1999 and December 31, 2003, 159 patients admitted to the BICU at Parkland Memorial Hospital, Dallas, TX with $\geq 20\%$ TBSA burns or any inhalation injury were enrolled. A subgroup of these patients was included in a previous report concerning the clinical features of sepsis and organ failure after burn trauma. 24 Demographic and clinical outcome data are summarised in table 2 and described briefly below. Of the 159 patients, 25 (16%) died. The major causes of injury were flame (63%) or scalding (21%).

<table>
<thead>
<tr>
<th>Locus</th>
<th>Amplimer length</th>
<th>Primer sequences</th>
<th>Cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-$\alpha$ -308</td>
<td>107 bp</td>
<td>PCR (For): AAGCCAATAGTTTGTAGGAGGCCAT&lt;br&gt;PCR (Rev): TCTCCCTCTGTCGATTCCG&lt;br&gt;Pyrosequencing (Rev): GGCCTAAGCCTGGTCC</td>
<td>One cycle: 95˚C - 4 min; 35 cycles: 95˚C - 3 s, 60˚C - 30 s, 74˚C - 30 s; one cycle: 74˚C - 6 min</td>
</tr>
<tr>
<td>IL-6 - 174</td>
<td>111 bp</td>
<td>PCR (For): CGCTAGGCTCAATGAGC&lt;br&gt;PCR (Rev): CAGGGTGGGCTGATTGGAA&lt;br&gt;Pyrosequencing (For): CCCTAGTTGTGTCTTGC&lt;br&gt;Pyrosequencing (Rev): GGCTGAACCGTCC</td>
<td>One cycle: 95˚C - 4 min; 35 cycles: 95˚C - 45 s, 63˚C - 30 s, 74˚C - 45 s; one cycle: 74˚C - 6 min</td>
</tr>
<tr>
<td>IL-1$\beta$ -31</td>
<td>222 bp</td>
<td>PCR (For): CCTTACTTCCTCGTCTGG&lt;br&gt;PCR (Rev): AAGAGAGAGAGAGAGAAATGC&lt;br&gt;Pyrosequencing (Rev): TCCCTCGTGTTTTA</td>
<td>One cycle: 95˚C - 4 min; 35 cycles: 95˚C - 30 s, 55˚C - 30 s, 74˚C - 30 s; one cycle: 74˚C - 6 min</td>
</tr>
<tr>
<td>CD14 -159</td>
<td>193 bp</td>
<td>PCR (For): ATACATCCTCCCCACACC&lt;br&gt;PCR (Rev): GCCCCCCCTCTCCTGG&lt;br&gt;Pyrosequencing (Rev): TACGAGGAGGGGG</td>
<td>One cycle: 95˚C - 4 min; 35 cycles: 95˚C, 30 s, 64˚C - 30 s, 74˚C - 30 s; one cycle: 74˚C - 6 min</td>
</tr>
<tr>
<td>TLR4 +896</td>
<td>193 bp</td>
<td>PCR (For): AGTCATCTGCTTGGGCTTG&lt;br&gt;PCR (Rev): CACACTACACCGGAAAAATG&lt;br&gt;Pyrosequencing (Rev): ACAATTAAAAAATGTCAATAA</td>
<td>One cycle: 95˚C - 4 min; 35 cycles: 95˚C - 60 s, 55˚C - 60 s, 74˚C - 60 s; one cycle: 74˚C - 6 min</td>
</tr>
</tbody>
</table>

Pyrosequencing primer sequences are also presented.
Clinical risk factors for and outcome from severe sepsis
Of the 159 patients, 36 (23%) developed severe sepsis, defined as sepsis complicated by organ dysfunction or septic shock. Of these 36 patients with severe sepsis, 79% had evidence of a clinically relevant Gram-negative, or a mixed Gram-negative/Gram-positive infection (table 3). One patient with severe sepsis had candidiasis and two patients had infections of unknown characteristics. In the majority (63%) of patients, pneumonia was the cause of severe sepsis. Patients had relatively few comorbid medical conditions, with alcohol abuse (n = 28, 18%) and hypertension (n = 23, 14%) being reported most commonly. Pre-existing liver, lung, and renal disease was uncommon (<1%). Age, burn size, and gender were associated with severe sepsis.

Association between SNPs and outcome of burn injury
Unadjusted analysis
Genotype frequencies for the candidate SNPs among patients with and without severe sepsis are shown in table 3. The TNF-α −308A allele was associated with an increased risk of severe sepsis by unadjusted analysis. Patients who were carriers of the A-allele at TNF-α −308 had a 41% risk (16/39) for severe sepsis versus a 17% risk (20/120) for patients homozygous for the wildtype G-allele (p = 0.002). The unadjusted relative risk for severe sepsis associated with carriage of the TNF-α −308 A-allele was 3.47 (95% CI 1.56 to 7.73). Similarly, carriers of the TLR4 +896G allele had a slight, but non-significant increased risk (unadjusted relative risk = 2.46, 95% CI 0.88 to 6.90) for severe sepsis by unadjusted analysis (p = 0.08). Seven (39%) of the 18 G-allele carriers developed severe sepsis, versus 29 (21%) of the 141 AA homozygotes. Data for the remaining SNPs (CD14, IL-1β, and IL-6), which were not significantly associated with outcome, are included in table 4.

Logistic regression
In order to evaluate the candidate SNPs in the context of other potential risk factors, we performed a best sets multivariate logistic regression. Adjusting for age, burn size, ethnicity, and gender, carriage of the TNF-α −308 A-allele and the TLR4 +896 G-allele was significantly associated with an increased risk for severe sepsis. The aORs were 4.47 (95% CI 1.67 to 11.96) and 6.41 (95% CI 1.77 to 23.17) for the TNF-α −308 A- and TLR4 +896 G-alleles, respectively (table 5). The SNPs within the CD14, IL-1β, and IL-6 loci were not associated with increased risk for severe sepsis. Ethnicity, pre-existing medical conditions, and the presence of an inhalation injury did not affect risk. Clinical risk factors for severe sepsis did not differ significantly between alternate genotypes at the candidate SNPs.
**DISCUSSION**

We hypothesised that the associations between the candidate SNPs and severe sepsis might have been influenced by the degree of clinical risk. Previous analysis of a large dataset determined that age $\geq 50$ years and full-thickness burn size $\geq 30\%$ TBSA identified patients at increased risk for death. Patients were categorised according to the presence or absence of these clinical risk factors (full-thickness burn size $\geq 30\%$ TBSA and/or age $\geq 50$ years). The risk for severe sepsis and death according to clinical risk stratification is presented in table 6. A total of 76 (48%) patients were younger than 50 years of age with full-thickness burns covering less than 30% TBSA; these patients constituted the low risk group. Some 74 (47%) patients were either $\geq 50$ years old or had $\geq 30\%$ TBSA full-thickness burns and nine (6%) patients were both $\geq 50$ years of age with $\geq 30\%$ TBSA full-thickness burns. Therefore, a total of 83 patients were categorised into the high risk group. The association between A-allele carriage at the TLR4 $-308$ SNP and severe sepsis was similar in both the high and low risk groups (Mantel-Haenszel $\chi^2 = 0.05$; $p = 0.821$). However, the increased risk for severe sepsis associated with the TLR4 $+896$ G-allele was limited to the low risk group (Mantel-Haenszel $\chi^2 = 8.04$; $p = 0.005$). The unadjusted relative risk for the TLR4 $+896$ G-allele among low and high risk patients was 7.44 and 0.99, respectively, versus 2.25 and 2.26 for the TNF-$\alpha -308$ A-allele (fig 1).

**Figure 1** The patient population was stratified on clinical risk for severe sepsis, using age $\geq 50$ years and/or full-thickness burn size $\geq 30\%$ TBSA as cut points. The unadjusted relative risk for severe sepsis following burn injury that was associated with TLR4/TNF-$\alpha$ genotype in the low versus high clinical risk groups was then analysed in a Mantel-Haenszel test. While the relative risk associated with carriage of the TLR4 $-308$ A-allele was similar in both groups, the relative risk for carriage of the TLR4 G-allele differed between risk groups. TLR4 genotype was significantly associated with increased risk for severe sepsis among low risk patients only.

**Table 5** Risk factors for the development of severe sepsis after burn injury following adjustment for multiple factors

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>OR</th>
<th>95% CI</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-$\alpha -308$ A-allele carriage</td>
<td>4.47</td>
<td>1.67 to 11.96</td>
<td>0.0008</td>
</tr>
<tr>
<td>TLR4 $+896$ G-allele carriage</td>
<td>6.41</td>
<td>1.77 to 23.17</td>
<td>0.0103</td>
</tr>
<tr>
<td>Age $\geq 50$ years</td>
<td>5.74</td>
<td>1.99 to 16.55</td>
<td>0.0001</td>
</tr>
<tr>
<td>Full-thickness burn $\geq 30%$TBSA</td>
<td>4.62</td>
<td>1.79 to 11.88</td>
<td>0.0002</td>
</tr>
<tr>
<td>Female gender</td>
<td>3.84</td>
<td>1.47 to 10.00</td>
<td>0.0055</td>
</tr>
</tbody>
</table>

Odds ratios were determined by multivariate logistic regression using a “best sub-sets” model. This analytical model selected the most parsimonious set of prognostic factors that best described the risk for severe sepsis.

**Table 6** The frequency of severe sepsis and mortality following burn injury in high versus low clinical risk groups

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Low clinical risk (n = 76)</th>
<th>High clinical risk (n = 83)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe sepsis</td>
<td>8 (11%)</td>
<td>28 (34%)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Death</td>
<td>3 (4%)</td>
<td>22 (27%)</td>
<td>$&lt;0.001$</td>
</tr>
</tbody>
</table>

The adverse outcomes of severe sepsis and death were more common among patients in the high clinical risk group. Clinical risk groups were stratified using age $\geq 50$ years of age and/or full-thickness burn size $\geq 30\%$ TBSA as cut points.

Associated with an increased risk for developing severe sepsis. The increase in risk associated with carriage of variant alleles at these SNPs was comparable to the risk associated with traditional clinical factors such as age and full thickness burn size.

While there has been recent controversy regarding the validity of genetic association data, confidence in the legitimacy of our results was strengthened by independent evidence that the TLR4 $+896$ and TNF-$\alpha -308$ SNPs have functional relevance. That is to say, alternate alleles at these SNPs seem to impact protein abundance or function. For example, recent studies have suggested that altered TLR4 signalling plays a role in bacterial resistance as well as the development of sepsis and septic shock. TLR4 is a transmembrane protein that initiates a signalling cascade that triggers an innate immune response to endotoxin. In mammals, TLR4 initiates a signal upon activation by CD14-bound lipopolysaccharide (LPS) that is transmitted through a series of adapter molecules and protein kinases resulting in nuclear translocation of NF-kB and a subsequent cascade of inflammatory cytokines and chemokines. The non-synonymous adenine to guanine (A$\rightarrow$G) transition at nucleotide $+896$ of the human TLR4 mRNA that occurs within the extracellular domain of the TLR4 protein results in the substitution of asparagine with glycine at amino acid residue 299 (Asp299Gly). Carriers of the G-allele at this SNP exhibit reduced LPS responsiveness as well as an increased risk for septic shock and susceptibility to Gram-negative sepsis.

Furthermore, transient transfection experiments in THP-1 cells indicated that the 299Gly allele was able to disrupt LPS signalling. Importantly, adenoviral transfection of a wild-type TLR4 construct was able to rescue LPS responsiveness in airway epithelial cells and alveolar macrophages derived from individuals with TLR4 mutations. Finally, associations between altered TLR4 signalling and a number of inflammatory disease states have been reported.

Similar evidence of a functional effect exists for the TNF-$\alpha -308$ SNP. Importantly, TNF-$\alpha$ has been demonstrated to play a major role in the pathogenesis of sepsis and its complications after burn trauma. Furthermore, there is evidence that the guanine to adenine substitution (G$\rightarrow$A) at nucleotide $-308$ within the TNF-$\alpha$ promoter affects transcriptional regulation of TNF-$\alpha$. Carriage of the less common A-allele at this position has been associated with unfavourable clinical outcomes and increased risk of acquiring several infectious and inflammatory diseases in a number of clinical settings.

It was of interest that carriage of variant alleles at TLR4 and TNF-$\alpha$ have a seemingly opposite impact upon the amplitude of the endotoxin response. While carriage of the TLR4 $+896$G allele appears to decrease the intensity of the LPS response signal, the TNF-$\alpha -308$A allele is associated with an increased rate of TNF-$\alpha$ transcription. In order to further investigate the effects of the TLR4 and TNF-$\alpha$ SNPs upon risk for severe sepsis, patients were stratified into low and high clinical risk groups.
and high clinical risk groups using two established clinical risk factors: full thickness burn size ≥30% TBSA and/or age ≥50 years. A Mantel-Haenszel test was used to evaluate interaction between clinical risk and carriage of variant alleles at the candidate SNPs. Interestingly, the test for homogeneity between high and low clinical risk groups was significant for the TLR4 +896 G-allele, indicating that the clinical risk status significantly impacted the effect of TLR4 +896 G-allele carriage. Carriage of the TLR4 G-allele was significantly associated with an increased risk for severe sepsis among low risk patients only. The biological implication of this observation may be that reduced toll pathway signalling by TLR4 +896 G-allele carriers fails to control local infection in cases of lower clinical risk and that these uncontrolled local infections proceed to become systemic. Alternatively, when the clinical risk is sufficiently severe, stimulation of the endotoxin response through TLR4 is strong enough to overcome a genetic predisposition for reduced signalling. In the case of the TNF-α SNP, the higher level of transcription that has been ascribed to the −308 A-allele appears to increase risk for severe sepsis in all cases. However, there is an important caveat to this reasoning: the Mantel-Haenszel results could have been statistically spurious. Stratification of our relatively small patient sample into clinical risk groups increased this possibility by effecting differential association. Nonetheless, these results were the result of subgroup analysis, which is well known to inflate the likelihood of type I (false positive) error.

In addition, although we adjusted for ethnicity in our logistic regression, unmeasured genetic differences in this diverse cohort may have contributed to the risk for severe sepsis in unappreciated ways. Allelic frequencies of many polymorphisms are known to vary across ethnic groups and this variation may either reduce statistical power by generating undetected heterogeneity within the genomic background or produce spurious results if clinically relevant traits are in linkage disequilibrium with one or more candidate SNPs by chance. Further investigation with a much larger, preferably geographically distinct, patient population and/or the development of sophisticated statistical models will be required to fully resolve these issues.

In conclusion, this study provides strong evidence regarding the association of the TNF-α −308 promoter and TLR4 +896 coding region polymorphisms with an increased risk for severe sepsis following burn trauma. Furthermore, the independent predictive value of A-allele carriage at the −308 position in the TNF-α promoter and G-allele carriage at TLR4 +896 appear to impart the same risk as traditional clinical factors.

ACKNOWLEDGEMENTS

We thank Ms Audrey Cusano and Mr Thomas Wheeler for their outstanding technical contributions to this project.

Authors’ affiliations

R C Barber, F A Rivera-Chavez, G F Purdue, J L Hunt, J W Horton, The Department of Surgery, The University of Texas Southwestern Medical Center, Dallas, TX, USA

C C Aragaki, Divisions of Epidemiology and Biostatistics, School of Public Health, Dallas Regional Campus, University of Texas Health Science Center at Houston, Dallas, TX, USA

This work was supported by NIGMS grant #5R01GM061681-38.

Conflict of interest: none declared.

REFERENCES


www.jmedgenet.com
TLR4 and TNF-α SNPs and severe sepsis risk


Retraction statement

The authors of the following manuscript Ninis VN, KyliΜnc MO, Kandemir M, Daёblй E, Tolun. High Frequency of 9T and CFTR Mutations in Children with Idiopathic Bronchiectasis. J Med Genet 2003;40:530–5, are retracting it because the polythyminide track genotype data are not correct. Recently the authors repeated the genotyping or 17 of the subjects to check whether the reported genotypes were correct and found out that they were not. At the time of submission of the manuscript, the authors were very confident of the data, since they had employed two independent methods for the genotyping of all subjects. However subsequently the authors were prompted to the recheck Vasiliki N Ninis results and have been unable to confirm them. The authors regret that we did not find out prior to publication.