LETTERS TO JMG

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

K Kahoński, 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. A wide spectrum of neo- plastic 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 have screened the role of colorectal polyposis and carcinomas in patients with confirmed BHD germine 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 TGFB2 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

Fifty-seven matched samples (from 37 patients), of which 10 were colon polyps with their matched carcinomas from the same patients, and 37 colorectal carcinomas, were obtained from the South Western Sydney Colorectal Tumour Bank (Liverpool Hospital, Australia). All tissue samples were collected prospectively with the informed consent of patients who underwent surgery in the South Western Sydney Area Health Service during the period 2000-2002. The lack of a family history of colorectal cancer or other familial cancer syndrome was ascertained by detailed questionnaire. This study was approved by the Institutional Review Board of the Van Andel Research Institute. Frozen sections (15 µm) were prepared from stored tumour specimens. The first, middle, and last slides (5 µm) were stained as reference slides. Manual micro-dissection was carried out on the unstained slides under low magnification.
power light microscopy (20-40x) by scraping of individual cell populations with a 28 gauge needle. DNA was isolated from microdissected tumour cells and specimens of normal colonic mucosa using the Qiagen DNeasy Mini system (Qiagen, Valencia, CA), according to the manufacturer’s instructions. DNA was extracted from peripheral blood leucocytes using the DNA isolation kit for mammalian blood (Roche Molecular Biochemicals).

Analysis of microsatellite instability (MSI) status

Paired colorectal carcinoma, polyp, and constitutional DNA samples (n=47) were analysed using a panel of 10 microsatellite markers comprising mononucleotide (BAT25, BAT26), dinucleotide (D2S123, D5S346, D18S34, D3S1611), and tetra- nucleotide (D17S740, D17S2196, D17S620). The relative distances (in cM) between each marker and their relationship to the BHD locus are indicated.

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

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. If the ratio was <0.67 or >1.3, the result was determined to be LOH. Initially, the two closest markers (D17S740 and D17S2196) were analysed for LOH. A designation of LOH was given when at least one of the matched 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). 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 DNAse free, RNAse free distilled water before storing at –20°C. The specific primers for methylated sequences were designed as follows: BHD-BISF-OF (5′-ATGTGGGATGAAAGTTAGTGGTTAATTT-3′) and BHD-BISF-IR (5′-ACAAAATCACCACCAAAAAACCC-3′) as the forward primer, and BHD-BISF-OR (5′-ACAAAATCACCACCAAAAAACCC-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 (Invitrogen), and 0.1 µmol/l of each primer. PCR conditions were 95°C for five minutes followed by 35 cycles of 94°C (30 seconds), 60°C (30 seconds), and 72°C (45 seconds). PCR was performed using 1 µl of the initial amplification reaction. The primers used were BHD-BISF-IF: 5′-GAAATGTTTCTTTTATTATTGAGTTG-3′ and BHD-BISF-IR: 5′-CCTAAAACAACCCAAACCCCAAAACC-3′, with conditions similar to those described for the preceding amplification, with the exception that 40 amplification cycles were carried out. The PCR products were purified using Microcon YM-100 columns (Millipore). After amplification, 20 µl of the 414 bp PCR product was incubated with 0.3 U of RsaI (New England BioLabs Inc, Beverly, MA) for two hours at 37°C. λDNA (0.3 µg) and distilled water were used respectively as positive and negative controls. Products of restriction digestion (20 µl) were electrophoresed on 2% agarose gels containing ethidium bromide, and visualised under UV illumination. The sizes of the RsaI digestion products were 160 and 254 bp.

RESULTS

Tumour MSI status

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

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

<table>
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P = polyp, – = absent; + = present; H = high level MSI; L = low level MSI; ND = not determined.

**Figure 2** Detection of mutations within the BHD gene in sporadic colorectal cancer. Two novel somatic mutations of the BHD gene were detected in two MSS colorectal carcinomas. Each of the mutations is not present in the matched normal tissues. (A) c.691C>G (S79W) in CRC-28 and (B) c.1788G>A (A445T) in CRC-54.
in 45 BHD affected and 38 BHD unaffected subjects, thus excluding any association between colonic neoplasia and BHD. Nevertheless, we recently reported six cases of colonic polyps and two cases of possible colon cancer in a BHD family with confirmed BHD germline mutations, 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 colorectal tissues, we can conclude that LOH at 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 the LOH pattern at the BHD region, which could represent two hits of the BHD gene in accordance with Knudson’s classical two hit theory. Apparent biallelic alteration of the BHD gene appears to be uncommon and the high frequency of LOH in the rest of the tumours without mutations suggests several possibilities. First, loss of a single allele may be the preferred mode of inactivation of the BHD gene and that haploinsufficiency contributes to tumorigenesis. Second, there may be mutations present in the regulatory region of the BHD gene which were not tested in this study. Finally there may be loss of other tumour suppressor genes in the vicinity of the BHD gene.

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

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

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Letter

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Branchio-otic syndrome (BOR, OMIM 113650) is an autosomal dominant disorder characterised by the association of hearing loss (HL), structural ear anomalies, branchial arch defects, and renal anomalies.\(^1\) The prevalence approximates 1:40 000 in the general population,\(^2\) and has been reported in about 2% of deaf children.\(^3\) The clinical expression of BOR exhibits wide intra- and inter-familial variability.\(^4\) In addition, reduced penetrance for BOR has been assumed.\(^5\) The major feature of BOR, which occurs in 93% of patients, is HL, which can be conductive, sensorineural, or mixed. Besides the classical ear, kidney, and branchial arch anomalies, different developmental manifestations of BOR in other organ systems have been described. Among these, dysfunction of the lacrimal duct system is a common feature.\(^6\) Thus, BOR represents a related disorder presenting with the same clinical features without renal anomalies.\(^7\)

Recessive mutations in the human homologue of the \(Drosophila\) eyes absent gene (EYA1) have been shown to cause BOR and BOS. A locus (BOS2) for autosomal dominant BOS has been localised to chromosome 1q31.\(^8\)

We performed a genome wide search for linkage in a large pedigree with BOS with more than 40 affected subjects and mapped a new gene locus (BOS3) to chromosome 14q21.3-q24.3. The highest multipoint lod score was \(Z_{\text{max}}=4.81\) \((q=4)\) for marker D14S980.\(^9\)

Identification of the gene causing branchio-otic syndrome type 3 will offer new insights into the development and molecular mechanisms of hearing.

**Key points**

- Branchio-otic syndrome (BOR) is an autosomal dominant developmental disorder characterised by the association of hearing loss, branchial arch defects, and renal anomalies. Branchio-otic syndrome (BOS) represents a related disorder presenting with the same clinical features without renal anomalies.
- Recessive mutations in the human homologue of the \(Drosophila\) eyes absent gene (EYA1) have been shown to cause BOR and BOS. A locus (BOS2) for autosomal dominant BOS has been localised to chromosome 1q31.
- We performed a genome wide search for linkage in a large pedigree with BOS with more than 40 affected subjects and mapped a new gene locus (BOS3) to chromosome 14q21.3-q24.3. The highest multipoint lod score was \(Z_{\text{max}}=4.81\) \((q=4)\) for marker D14S980.
- Identification of the gene causing branchio-otic syndrome type 3 will offer new insights into the development and molecular mechanisms of hearing.

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**References**

10. Tatusova TA. Drosophila eyes absent gene \((EYA1)\) for autosomal \(\text{dominant BOS}\) has been localised on chromosome 8q13.\(^{11}\) Familial variability. In addition, reduced penetrance for BOR has been assumed.\(^{5}\) The major feature of BOR, which occurs in 93% of patients, is HL, which can be conductive, sensorineural, or mixed. Besides the classical ear, kidney, and branchial arch anomalies, different developmental manifestations of BOR in other organ systems have been described. Among these, dysfunction of the lacrimal duct system is a common association.\(^{12}\) Thus, BOR represents a clinically and genetically heterogeneous disease complex that manifests predominately during organogenesis. A gene locus for autosomal dominant BOR had been localised on chromosome 8q13.\(^{11}\) Subsequently, mutations in the human homologue of the \(Drosophila\) eyes absent gene \((EYA1)\) have been shown to cause autosomal \(\text{dominant BOS}\) (OMIM 601653).\(^{12}\) Branchio-otic syndrome \((\text{BOR})\) (OMIM 602588) was initially described as a disorder distinct from BOR, featuring the same clinical symptoms as BOR with high frequency.

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


**Letter**


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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 features of BOR or BOS remains unsolved. We describe here a genome wide search for linkage in a large pedigree with BOS, in which linkage to the EYA1 locus on chromosome 1q31 had been excluded, resulting in a new locus (BOS3) on chromosome 14q.

METHODS

Blood samples and clinical data for a large multigeneration family with over 40 affected subjects with BOS were obtained after informed consent was given by patients and unaffected relatives. The ethnic origin of the family was Anglo-Saxon Australian. Clinical examinations and renal ultrasound were performed in 32 affected family members. Twenty blood samples were collected (14 from affected subjects, six from unaffected relatives or partners) and DNA was extracted for molecular analysis. All 32 affected subjects had deafness (100%). In 17 affected family members, precise audiometric data were available. Diagnosis was sensorineural HL in 14 of them (82%) and mixed HL in three of them (18%). The affected frequencies varied from low to high frequencies as well as the presence of HL in all frequencies. Severity varied from mild to severe HL being still progressive in six cases. Among subjects IV.14, IV.17, and V.2 differences in the HL between the right and left ear were found. In IV.14 sensorineural HL was mild in the right ear whereas it was moderate to severe in the left ear. In IV.17, in addition to moderate to severe sensorineural HL on both sides, moderate to severe conductive HL was present only on the left side. In V.2 high frequency HL in the right ear differed from low frequency in the left ear. Age of onset was very variable with an average of 9.5 years, ranging from 3 weeks to 22 years. Eight subjects (25%) had branchial arch defects, three with branchial cysts, and six with ear pits as external ear manifestation (table 1).

### Table 1 Clinical data of affected subjects from the BOS kindred

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

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

Figure 1 Haplotypes on chromosome 14q12-2q3 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 centromere 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, and used. Order and sex averaged distances (in parentheses) between these markers from centromeric to q telomeric are as follows: D14S599 (2.9 cM), D14S306 (2.8 cM), D14S1013 (3.2 cM), D14S748 (4.5 cM), D14S587 (4.1 cM), D14S980 (2.9 cM), D14S274 (3.9 cM), D14S53 (5.1 cM), D14S592 (8.0 cM), D14S588 (2.9 cM), and D14S1013 (3.2 cM). D14S306 and D14S599 flanking the BOS3 region (fig 1) are used. Other subjects haplotypes were inferred if possible (fig 1). A total of 380 microsatellite markers from the Genethon final linkage map were available for haplotype analysis in 14 affected and six unaffected subjects for the genome wide search for linkage. By evaluating the results of the genome wide search, the locus for BOS on chromosome 1q31 was also excluded for this kindred (data not shown). From the total genome search for linkage, only for one locus was cosegregation of the haplotype pattern in all affected subjects found for markers D14S587, D14S592, and D14S588 on chromosome 14q21.3-q24.3, yielding a maximum two point lod score of Zmax=3.27 (θ=0) for marker D14S587 (table 1). Further fine mapping with an additional six markers confirmed the locus. Haplotype analysis showed clear evidence that the disease allele cosegregated with all affected subjects and was absent from unaffected subjects (fig 1). A recombination event in II.2 defined marker D14S1013 as proximally flanking, and a recombination in V.5 identified marker D14S53 as distally flanking the critical genetic region within a 37.7 cM interval on chromosome 14q21.3-q24.3. Multipoint analysis of the 11 markers resulted in a Zmax=4.81 at marker D14S980 at relative position 50.9 (fig 2). The 95% confidence interval at Zmax=1.0 extends over a 33.9 cM interval between the markers D10S1013 and D10S53 within the set of 11 microsatellites. Marker D14S5890 also showed the highest two point lod score value Zmax=4.11 (θ=0) (table 2).

DISCUSSION

Here we have reported a third gene locus for BOS, BOS3, which maps to chromosome 14q21.3-q24.3. According to the UCSC Genome Browser, the interval between markers D14S1013 and D14S53 spans a physical distance of approximately 33 Mb, relative marker positions are 41 383 995 and 74 328 130, respectively. A recombinatn 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 dominant model with 100% penetrance and a gene frequency for BOS of 0.0001. The “lodmax - 1 support interval” was defined as the genetic map positions intersecting the lod score curve at Zmax=1. For haplotyping and computation of multipoint lod scores, the program SIMWALK was used, assuming equal allele frequencies. Because of the reduced penetrance described in BOR/BOS, the calculations were performed on basis of an “affected only” strategy.

### Table 2

<table>
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<tr>
<th>Marker</th>
<th>Two point lod scores in the BOS kindred at various recombination fractions for markers at the BOS3 locus</th>
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</tr>
<tr>
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<tr>
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<tr>
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<tr>
<td>D14S606</td>
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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, severity, frequency, and the age of onset among the different family members and even between the ears of one patient, a characteristic feature of BOR and BOS. The low percentage of branchial arch defects compared to previously described families with BOS and BOR can be explained either by the known variable expressivity or by the genetic heterogeneity of BOS.

Genes encoding proteins involved in renal and otic histogenesis and organogenesis are good 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 392).

Data access. URL for data in this article are as follows: Genethon map: http://www.ncbi.nlm.nih.gov/Omim. UCSC Genome Browser: http://genome.ucsc.edu/.

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


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 dysmorphism. 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 leucocytes and skin fibroblasts. We determined the mechanism underlying the deletion and the size of the deleted interval at the cytogenetic and molecular level with respect to the duplicated sequences in the NF1 region.

MATERIAL AND METHODS

Patients

The 60 year old female investigated in this study (II.2, fig 1) has four café au lait spots on her thighs and forearms. Some minor freckling was found in the left axilla, but she did not have dermal neurofibromas or Lisch nodules. Her two sons, however, suffer from a severe form of NF1 (patients III.1 and III.2). In early childhood, they had multiple café au lait spots distributed all over the body and noticeably large hands and feet. In the younger brother, retarded psychomotor development was documented. Later in life, coarse facial features with hypertelorism were noticed, in addition to macrocephaly, axillary freckling, mild scoliosis, and multiple neurofibromas, which were too numerous to count. Multiple melanocytic naevi and low set nipples were additional features. The intelligence quotient of the sons was judged by their mother as normal, but was specifically tested only in her oldest son and was found to be in the normal range. Both attended regular school. Patient III.1 suffered from a malignant glandular schwannoma in the left gluteus which metastasised. At the age of 29 years, he died from intracranial bleeding before genetic investigations were started. His younger brother, patient III.2, who

Key points

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

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. PCR products used as FISH probes,
DJ1686/1863, DJHK10/11, and DJ1576/1578, were amplified
with primers listed in table 1 using the Expand Long Template
PCR System (Roche Molecular Biochemicals). The respective
markers in parentheses located within the deleted interval of proband II.2 are present as single copies in
the majority of peripheral blood leucocytes.

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

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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. Geno-
typing was performed with FAM labelled primers and capil-
ary 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 Lazarro et al.4 and analysed on an ALF-Express
Sequencer (Amersham Pharmacia). The single nucleotide
polymorphism SNP2 located in the KIAA1821 gene was ampli-
ﬁed with primers described by Eisenbarth et al.7 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-
polyomorphical 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’TGCCATAGGCAATGGTCTTTG 3’T), and DJ1910 (5’
AAACCCCTCTGTGCCCTACTC 3’T), DJ1561 (5’GTCAATAGG
CAAGAAAGAGACCA 3’T) and DJ1562 (5’TGGGGAAGAAA-
CATCAGTCAA 3’T) and analysed on an ALF-Express
Sequencer (Amersham Pharmacia). The single nucleotide
polyorphism SNP2 located in the KIAA1821 gene was ampli-
ﬁed with primers described by Eisenbarth et al.7 Resulting
PCR products were digested with the restriction enzyme MboI
and analysed by agarose gel electrophoresis.

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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 and comparison with the known sequences for 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 KIAAO160 pseudogene and the distal breakpoint to the functional KIAAO160 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 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 KIAAO160 pseudogene and the distal breakpoint to the functional KIAAO160 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. 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 (≈1 × 10⁶/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 gonado-somatic 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
Large deletions of the NF1 gene region have been reported in six 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 analysis and two were ascertained by marker analysis. Moreover, Tischler et al. described a 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. 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 JJAZF1 (Jointed to Juxtaposed with Another Zinc Finger gene) has been shown to be disrupted by somatically acquired translocations (7;17) (p15;q21) in endometrial stromal sarcomas. This type of translocation not only fuses the promoter and 5' end of the JJAZF1 gene on chromosome 7 to the coding portion of the KIAA0160 (JJAZ1) gene on chromosome 17, 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 JJAZF1 allele, creation of a new fusion transcript between JJAZF1 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 (JJAZF1) serves some tumour suppressive function, which may also be disturbed in endometrial stromal tumours, is consistent with the high risk and early development of tumours observed in patients carrying constitutional microdeletions.

The present study shows that the KIAA0160 gene in conjunction with its pseudogene represents a second recombinational pitfall in the NF1 gene region, besides the WI-12393 gene derived duplicons. Genotype analyses clearly indicate that the deletion occurred somatically in proband II.2 by an intrachromosomal (sister chromatid based) mechanism (figs 1 and 3).
According to previous studies, low copy repeat mediated de novo deletions are believed to be triggered by meiotic recombination during germ cell development.¹ This notion was based on PCR experiments that failed to identify the patient specific junction product in the blood of their healthy parents.² López Correa et al³ observed a strong maternal bias for 17q1 1.2 microdeletions and found that the 1.5 Mb spanning deletions occurred predominantly during maternal germ cell development. In five of the six informative families, interchromosomal recombination (non-sister chromatid exchange) was the underlying mechanism that caused the rearrangements.

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

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

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

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

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

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

MATERIALS AND METHODS

Cytogenetic investigations

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

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, DNA analysis

Microsatellite analysis was conducted on peripheral blood DNA extracted by standard techniques following the protocol described in Perez-Jurado et al.

DNA analysis

Microsatellite analysis was conducted on peripheral blood DNA extracted by standard techniques following the protocol described in Perez-Jurado et al. The portion of intron 1 of the CYLN2 gene containing the previously unreported 4 bp microsatellite polymorphism was amplified with primers CYLN2i1F (5' - CTCTCCCTTTCCGTTGTAATACTG-3'; ABI-Fam labelled) and CYLN2i1R (5' - CGCCTACCTGCCCCCTTCTC-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.
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 D7S613 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 intensity, which could be detected.

Table 1

<table>
<thead>
<tr>
<th>Tests</th>
<th>WS subjects with typical deletion</th>
<th>Proband score</th>
<th>p</th>
</tr>
</thead>
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<tr>
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<td>Average score</td>
<td>SD</td>
<td></td>
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<tr>
<td>Chronological age (y)</td>
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<tr>
<td>Mental age (y)</td>
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<td>4.82</td>
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<tr>
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<td>16.29</td>
<td>83.00</td>
</tr>
<tr>
<td>Corsi Span (score for years)</td>
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<td>Digit Span (score for years)</td>
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<tr>
<td>Rey Figure copy (score for years)</td>
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<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, D7S489A, 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 RP4-665P5 on the telomeric side (fig 4).

**DISCUSSION**

The subject described here meets the diagnostic criteria for WS (clinical features, SVAS, short stature, positive FISH analysis), but his clinical and neuropsychological profiles are unusual. Face features are typical for WS, but more mildly expressed; moreover, inspection of photographs taken at different periods through infancy and childhood showed a gradual reduction of WS facial features (data not shown). The cognitive profile displayed by the patient was different in a number of ways from the WSCG. He had borderline IQ, while the age-matched WSCG children were mildly impaired. He did not show the usual WS behavioural pattern. Moreover, his cognitive profile did not show the typical spatial and constructive impairment. However, the patient showed impairment in several competencies. In this sense, his development differs from both the WSCG and normally developing children.
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 WSR1 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, GTF2IPI. 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 Gtf2ird1 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.

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

V N Ninis, M O Kýlync, M Kandemir, E Daýlý, A Tolun

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. The 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. However, the complex genetic basis of the phenotype expression of IB remains largely unknown. CFTR mutations alone cannot be held responsible for the disease, as obligate CFTR mutation heterozygotes were shown not to have
chronic pancreatitis.

common, with a carrier frequency of about 1 in 50.

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report the genetic analysis of the

The status of parental consanguinity was known in 52 of

an increased risk for IB. The CFTR gene seems to act in a mul-

tifactorial context, as both the mutations and polymorphic alle-

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 sev-

eral 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 investi-

ation 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 contribu-

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 clini-

cal 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 dis-

ease 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 Kylänc 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 CFTRdel42,30 and CFTRdelev9, and −33A>G in the minimal promoter region. Marker DNA samples for the latter region were kindly provided by Dr M Claustrat. 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) 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 asayed by either DGGE or restriction enzyme digestion. The alleles for the biallelic GATT repeat in intron 10 and the three multiallelic microsatellites (IVS8CA 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 CFTRdel19, and two for K68E. Another patient was a compound heterozygote for −33A>G and CFTRdel2c. 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 computing association with others, have been implicated in the aetiology of bronchiectasis. 1, 14 We investigated whether any alleles were associated with IB in our patients. We had found the frequencies of T9, T7, and T5 in the normal chromosomes to be 28, 162, and 10, respectively, in a total of 200. 15 We found the frequencies of T9, T7, and T5 in the IB chromosomes in which no mutation was identified (no mutation chromosomes) to be 7, 30, and 11, respectively, in 112. The frequency of T5 with respect to T7 in the IB no mutation chromosomes was found to be highly significant compared to the normal population (11/41 versus 10/172). The frequency of T9 with respect to T7 in no mutation IB chromosomes also was significantly higher than in the normals (71/101 versus 28/190). Regarding the IB chromosomes harbouring mutations, we found the frequencies of T9, T7, and T5 to be 20, 13, and 1, respectively, in 34. Thus, the frequency of T9 with respect to T7 was highly significant (20 versus 13) as compared to the CF mutant chromosomes (17 versus 102). 15

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

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

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

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). 13 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 CFTRdel19 and CFTRdel2c were observed in four and two chromosomes, respectively. One patient had W1282X and another F1052V, while the last one had the novel mutation E1228G, which resulted from an A to G transition at nucleotide 3815 (sequence data available on request). An uncharged polar amino acid was substituted for an acidic one at residue 1228 in exon 19 in the second nucleotide binding domain of the protein. The residue has been conserved in human, bovine, Xenopus, and dogfish. 14, 15 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. 14, 15

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, 14 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. 14 The numbers in CF patients were as follows: one K68E, four N1303K, one CFTRdel2c, and five W1282X. Three of the remaining IB mutations (3272-26A>G, CFTRdel19, F1052V) were identified in other studies in CF patients, 14, 16, 17, one (−33G>A) in a CBVAD patient, 21 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. 1, 14 We investigated whether any alleles were associated with IB in our patients. We had found the frequencies of T9, T7, and T5 in the normal chromosomes to be 28, 162, and 10, respectively, in a total of 200. 15 We found the frequencies of T9, T7, and T5 in the IB chromosomes in which no mutation was identified (no mutation chromosomes) to be 7, 30, and 11, respectively, in 112. The frequency of T5 with respect to T7 in the IB no mutation chromosomes was found to be highly significant compared to the normal population (11/41 versus 10/172). The frequency of T9 with respect to T7 in no mutation IB chromosomes also was significantly higher than in the normals (71/101 versus 28/190). Regarding the IB chromosomes harbouring mutations, we found the frequencies of T9, T7, and T5 to be 20, 13, and 1, respectively, in 34. Thus, the frequency of T9 with respect to T7 was highly significant (20 versus 13) as compared to the CF mutant chromosomes (17 versus 102). 15

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

Haplotypes

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

The T5 IB chromosomes also showed great variation as compared to normals. There were 10 T5 chromosomes among the 200 normals, and they were all on GATT7-TG11-470V background. Moreover, they had in total only three different haplotypes with respect to the three microsatellite polymorphisms IVS8CA, IVS7b1TA, and IVS17bCA: 14-30-13, 17-30-13, and 17-33-13 with frequencies of 3, 3, and 4, respectively. In contrast, T5 IB chromosomes were mostly on backgrounds GATT7-TG11-470M (five of 12) and GATT6-TG11-470V (four of 12). The remaining three haplotypes were observed once: GATT6-TG11-470M, GATT7-TG11-470V-1901 +11TP2694T, and GATT7-TG13-470V-F1052V-2694T. When the three microsatellites were also taken into account, no two of the T5 IB chromosomes had the same haplotype.

Table 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>CFTRdel19</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>CFTRdel2c</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>Try to Stop at 1282</td>
</tr>
<tr>
<td>Total alleles</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>
ian disseminated bronchiectasis patients, mutant alleles among the total of 19 detected in the adult Italian patients. 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 had claimed parental consanguinity (including 18 first cousin others had denied it. Patients from 26 other families who 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**

**Comparison of the genotypes of the sibs**

We compared the CFTR haplotypes of the patients to their sibs (Table 2). All of the four affected sib pairs shared both of their haplotypes, indicating that both of the CFTR chromosomes contributed to the phenotype. The genotypes were T9-N1303K homozygous, T9/T9-N1303K, T9/T9-3272-26A>G, and T7/T5. However, six affected-healthy sib pairs also shared both of the haplotypes, excluding the gene as the sole locus responsible for the disease phenotype. The genotype of one pair was T7/T7-CFTRdel19, three were T9/T9, and two were T9/T7.

**Identity by descent**

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

<table>
<thead>
<tr>
<th>IVS8 (TG)m</th>
<th>IVS8 (T)n</th>
<th>Polymorphism</th>
<th>Mutation</th>
<th>No of chroms</th>
<th>No of homozyg patients</th>
<th>No of heterozyg patients</th>
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<tbody>
<tr>
<td>11</td>
<td>5</td>
<td>470M</td>
<td>–</td>
<td>6</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>470V</td>
<td>–</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>470M</td>
<td>–</td>
<td>10</td>
<td>8</td>
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</tr>
<tr>
<td>7</td>
<td>7</td>
<td>470V</td>
<td>–</td>
<td>10</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>470M</td>
<td>–</td>
<td>40</td>
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<td>7</td>
</tr>
<tr>
<td>11</td>
<td>9</td>
<td>470M</td>
<td>–</td>
<td>4</td>
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</tr>
<tr>
<td>9</td>
<td>9</td>
<td>470V</td>
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<td>17</td>
<td>11</td>
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<tr>
<td>11</td>
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</tr>
<tr>
<td>11</td>
<td>7/9</td>
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<tr>
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<td>5</td>
<td>470V, 2694T</td>
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</tr>
<tr>
<td>1001+11T, 4002G</td>
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<tr>
<td>875+40G</td>
<td>470V, 2694T</td>
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</tr>
<tr>
<td>7</td>
<td>7</td>
<td>470V, 2694T</td>
<td>–</td>
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<td>1</td>
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</tr>
<tr>
<td>7</td>
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<td>470M, 1001+11T</td>
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<tr>
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<td>470V, 2694T</td>
<td>–</td>
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<tr>
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<td>5</td>
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<tr>
<td>7</td>
<td>7</td>
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<td>CFTRdel19</td>
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<td>7</td>
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<td>W1282X</td>
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<td>7</td>
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<td>CFTRdel2,3</td>
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<td>E1228G</td>
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<td>7</td>
<td>470M</td>
<td>-33G&gt;A</td>
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<td>K68E</td>
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<td>9</td>
<td>9</td>
<td>470M</td>
<td>3272-26A&gt;G</td>
<td>2</td>
<td>0</td>
<td>1</td>
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</tbody>
</table>

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 with either T5 (two patients), T7 (four patients), or T7-CFTRdel2,3 (one patient), but in no case with T9. The next most common mutations were N1303K and CFTRdel19, both of which manifest severe CF phenotypes. These five mutations make up 85.3% (29/34) of all mutant IB chromosomes, and 20 of these 29 chromosomes were on T9 background. It is worth mentioning here that in our population 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 39 F508del chromosomes we had analysed on this background. 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. 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
such as disseminated bronchiectasis and obstructive 
manifest a milder form of CF. This fits in well with the model 
that mild 
Noone less the proportion of the mRNA lacking exon 9 sequences 
homozygous for haplotype T5-TG12-470V. She had defective 
increased frequencies of 
transfected cells, and the lower the (TG)m repeat number, the 
In addition, the T5 
chromosomes in our IB patients were mostly on backgrounds 
T9 IB chromosomes. T5 was shown to be high also in the Italian 
may be associated with defects in an as yet unidentified 
genotypes. We also observed a sig-
ificant association of 470M with the T9 no mutation IB 
Molecular studies are necessary to elucidate the 
Two opposing hypotheses could be proposed to explain the role of T9 in the aetiology of IB. T9 could be a predisposing 
Alternatively, T9 could be an attenuator for CF instead of 
a mutation with a role in the aetiopathogenesis of IB. A higher 
A modifier gene has already 
and 63 mEq/l, respectively), and he had developed CF-like 
and 63 mEq/l, respectively), and he had developed CF-like 
In addition to confirming the previous findings on increased frequencies of CFTR mutations and T5 in IB 
patients, this report highlights T9, an allele that had not 
been reported previously in association with any disease. 
Despite the fact that this allele results in normal transcripts 
(not lacking exon 9), it is not the most common allele in the 
normal population world wide, thus is not the most common 
allele. The high frequency of T9 chromosomes in our patients 
cannot be attributed to a possible association with an as yet 
unidentified common mutation, because the allele was on a 
large number of different haplotypes. We also observed a sig-
ificant association of 470M with the T9 no mutation IB 
mutations. 
identification of the gene would also 
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 
TB 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 groups. In addition, the T5 
chromosomes in our IB patients were mostly on backgrounds 
TG11-470V and TG13-470M, whereas the normal T5 chromo-
somes 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 470M allele leads to a higher protein activity, 470M protein having 1.7-fold 
invasive chloride channel activity compared to that of 470V in 
transfected cells, and the lower the (TG)m repeat number, the 
the (TG)m repeat number, the 
In addition, 470M was strongly associated with the 
470M allele was 
dissociated from the 470V allele in our IB patients 
IB when in association with defects in an as yet unidentified 
genotype. As for the no mutation patients, T9 would be 
expected to compensate for defects in the hypothetical 
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, simi-
lar 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 TB chromosomes (33G>ΔCFTRΔele2,3). 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. The four IB sib pairs and their six unaf-
fected sibs would give sufficient genetic information in a 
genoascane scan studying at the identification of the locus for 
the hypothetical gene. Identification of the gene would also 
increase our knowledge on the pathogenesis of CFTR defects 
and shed light on other diseases associated with CFTR.

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FMRF expression studies in blood and hair roots in a fragile X family with methylthionomaic

B B A de Vries, L-A Severijnen, A Jacobs, R Olmer, D J Halley, B A Oostra, R Willemsen

The fragile X syndrome is a common cause of familial mental retardation with an estimated prevalence of 1/4000–1/6000 for males in western countries. This X linked disorder is characterised by mental retardation with additional features like a long face with large protruding ears, macro-orchidism, and eye gaze avoidance. The causative mutation is an expansion of a CGG repeat in the 5' UTR of the FMR1 gene. Normal people have between 55 and 200, and affected subjects have more than 200 CGG repeats in their FMR1 gene, the so-called full mutation. The latter expansion is accompanied by hypermethylation of the repeat and its upstream region resulting in a shutdown of transcription and absence of the FMRP.

In fragile X patients, two special subclasses of mosaicism can be distinguished. These are the CGG repeat in the 5' UTR of the FMR1 gene. Normal people have between 55 and 200, and affected subjects have more than 200 CGG repeats in their FMR1 gene, the so-called full mutation. The latter expansion is accompanied by hypermethylation of the repeat and its upstream region resulting in a shutdown of transcription and absence of the FMRP.

In fragile X patients, two special subclasses of mosaicism can be distinguished:

1. **Subjects with a premutation in a proportion of their cells in addition to a full mutation**
   - In this group, the mutation is observed in a proportion of the cells, typically less than 50%
   - The premutation is associated with a smaller degree of mental retardation compared to the full mutation
   - Parents of affected individuals are often carriers of the premutation

2. **Subjects with full mutations in all cells**
   - This is the most severe form of the disease
   - Affected individuals have the full mutation in all their cells
   - The full mutation is associated with severe mental retardation

Both subclasses of mosaicism have implications for genetic counseling and the management of affected individuals. It is important to identify these subclasses to provide accurate genetic counseling and to告知 possible outcomes for future generations.

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**References**

mosaics"; this pattern can be observed in 20-40% of male patients: (2) subjects with intercellular variations in the methylation status of a full mutation, "methylation mosaics." In a large multicentre study, "methylation mosaicism" was observed in 3% of the males with a full mutation. In 1995, Willemsen et al. developed an FMRP antibody test for detecting the presence or absence of FMRP in lymphocytes and later in hair roots. This test allowed for screening for the fragile X syndrome among mentally retarded males and in addition made quantification of the number of FMRP expressing cells in patients possible. Using this technique in blood smears, Tassone et al. were able to find correlations between FMRP expression and IQ in males with size mosaicism and methylation mosaicism. Very recently, a highly significant correlation has been found between FMRP expression in hair roots and cognitive functioning in females carrying a full mutation (R Willemsen, in press).

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

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

DNA analysis
Genomic DNA was 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 IA1 against FMRP. Further immunoincubations were performed according to procedures described previously (http://www.eur.nl/FGG/CHI/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 hallucs. 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 WPPSI-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.
DISCUSSION
According to the definition of mosaicism, all fragile X males are mosaic as they have different amplified repeat sequences in the FMR1 gene in different cells. However, two special subclasses can be distinguished; 20–40% of fragile X males have a premutation in a proportion of cells and the full mutation in the remaining (majority) of cells. In general, the proportion of cells with a premutation is lower than <30% and various studies have shown that the cognitive functioning of these fragile X patients is not significantly better than the males with a full mutation, suggesting that the number of FMRP expressing cells with a premutation is insufficient. However, high functioning males with a size mosaic pattern have been described and Tassone et al. detected 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. The findings of normal FMRP expression in partially unmethylated full mutations were in contrast with the report of Feng et al. who found markedly diminished FMRP production in fibroblast clones from transcripts with more than 200 repeats. These conflicting findings raise the question of whether unmethylated full mutations have normal or diminished FMRP expression, what is the relation to cognitive functioning, and what is the correct tissue to study. Interestingly, Tassone et al. 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 unmethylated alleles are all in the full mutation range whereas the other two brothers have unmethylated 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 unmethylated full mutations need to be tested to assess the validity of the relationship between FMRP expression in hair roots and mental functioning.

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

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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

Key points

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

Microcephaly is the clinical finding of a head circumference measurement greater than three standard deviations (SD) below the population mean for age and sex. It is usually accompanied by mental retardation and there are many diagnoses with both environmental and genetic aetiologies.1 Autosomal recessive primary microcephaly (MCPH) (MIM 251200) is a disorder in which affected subjects are born with a small head circumference, explained by a cerebral cortex of reduced size, and are mentally retarded. The brain is structurally normal and, apart from the intellectual impairment, there are no other significant neurological problems, dysmorphic features, or malformations.2,3 In a study carried out in The Netherlands,4 the incidence of MCPH was approximately 1/250,000 but it is probably greater in populations with a high rate of consanguineous marriages. MCPH has been shown to be genetically heterogeneous with the identification of five loci: MCPH1 on 8p23,5 MCPH2 on 19q13,6 MCPH3 on 9q34,7 MCPH4 on 15q15-q21,8 and MCPH5 on 1q31,9,10 MCPH1, 2, and 3 were mapped in northern Pakistani families, MCPH4 in a Moroccan family, and MCPH5 in northern Pakistani and Turkish families. Here we report the identification by autozygosity mapping11 of a novel locus for primary microcephaly, MCPH6, in a north eastern Brazilian family.

MATERIALS AND METHODS

Subjects

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

Molecular genetics

Linkage to the five known MCPH loci was ruled out (data not shown). An autosomal chromosome screen for regions of shared homozygosity was performed on seven of the eight...
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, 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 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 were selected from the ABI Linkage Mapping Panel Version I (Applied Biosystems), the Todd Panel, 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 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.

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 θ = 0 for markers in the critical region and results are shown in Table 1 with the highest lod score at 6.25 for marker D13S1275.

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

DISCUSSION

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

Within the larger MCPH6 region of 9 cM there is the potential candidate gene, fibroblast growth factor 9 (FGF9). In the nervous system of mice, FGF9 is produced mainly by neurones and may have a role in glial cell growth and differentiation during development. 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 evolves and has become so predominant in humans, and the perinatal to offer diagnostic, prenatal, and carrier testing for families.

ACKNOWLEDGEMENTS

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

<table>
<thead>
<tr>
<th>Marker</th>
<th>Lod score at θ = 0</th>
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<td>D13S175</td>
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<td>D13S1254</td>
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</table>

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

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Karag 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. We used polymorphic microsatellite markers and showed 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, aceruloplasminemia, 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. Dysmetria, 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 PNAK2 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
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.

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.\(^\text{10–12}\) However, in a large study of PKAN two groups were differentiated; firstly, cases with typical clinical symptoms (of what would have been once called Hallervorden-Spatz syndrome\(^\text{11}\)) or atypical features with acanthocytosis, hypoprebetalipoproteinaemia and/or orofacial dyskinesia (known as HARP syndrome\(^\text{14–16}\)) but all with the “eye of the tiger” sign on MRI brains scan and \( PANK2 \) mutations; secondly, a group of clinically atypical cases which did not have \( PANK2 \) mutations nor the “eye of the tiger sign” but did have hypodensity in the globus pallidus on T2 weighted MRI brains scan, cerebellar atrophy, and often evidence of iron deposition in the red nucleus and dentate nucleus.\(^\text{7}\) The family we report appears to have a demonstrably different condition from these two phenotypes because of the

\[ \text{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.\(^\text{7}\) \]

**MOLECULAR GENETIC STUDIES**

We performed linkage analysis between the family’s disease and the \( PANK2 \) gene that causes PKAN.\(^\text{3}\) 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,\(^\text{4}\) 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.\(^\text{10–12}\) However, in a large study of PKAN two groups were differentiated; firstly, cases with typical clinical symptoms (of what would have been once called Hallervorden-Spatz syndrome\(^\text{11}\)) or atypical features with acanthocytosis, hypoprebetalipoproteinaemia and/or orofacial dyskinesia (known as HARP syndrome\(^\text{14–16}\)) but all with the “eye of the tiger” sign on MRI brains scan and \( PANK2 \) mutations; secondly, a group of clinically atypical cases which did not have \( PANK2 \) mutations nor the “eye of the tiger sign” but did have hypodensity in the globus pallidus on T2 weighted MRI brains scan, cerebellar atrophy, and often evidence of iron deposition in the red nucleus and dentate nucleus.\(^\text{7}\) The family we report appears to have a demonstrably different condition from these two phenotypes because of the
Cerebral diseases in which excess iron accumulation is found on MRI scan

<table>
<thead>
<tr>
<th>Disease title</th>
<th>Inheritance pattern</th>
<th>Age of presentation</th>
<th>Clinical features*</th>
<th>Intellect</th>
<th>Radiological features†</th>
<th>Cerebellar features‡</th>
<th>Gene§</th>
<th>Chromosomal location</th>
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<tr>
<td>Aceruloplasminemia</td>
<td>Recessive</td>
<td>&gt;30 y</td>
<td>Dementia, ataxia, chorea, dystonia</td>
<td>Declines</td>
<td>Caudate, dentate, putamen, red, thalamus, on T1 + T2</td>
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<td>Caudate, dentate, globus pallidus, substantia nigra</td>
<td>Normal</td>
<td>FTL</td>
<td>19q13.3-4</td>
</tr>
<tr>
<td>PKAN/PANK2</td>
<td>Recessive</td>
<td>5–20 y</td>
<td>Diverse extrapyramidal symptoms</td>
<td>Often declines</td>
<td>“Eye of the tiger” and substantia nigra</td>
<td>Normal</td>
<td>PANK2</td>
<td>20p13</td>
</tr>
<tr>
<td>PKAN/PANK2</td>
<td>Recessive</td>
<td>&gt;15 y</td>
<td>Diverse extrapyramidal symptoms</td>
<td>Often declines</td>
<td>“Eye of the tiger”</td>
<td>Normal</td>
<td>PANK2</td>
<td>20p13</td>
</tr>
<tr>
<td>PKAN/PANK2</td>
<td>Probably recessive</td>
<td>&gt;15 y</td>
<td>Diverse extrapyramidal symptoms</td>
<td>Often declines</td>
<td>Caudate, dentate, globus pallidus, putamen, red</td>
<td>Often, atrophy</td>
<td>?</td>
<td>?</td>
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<tr>
<td>Karak syndrome</td>
<td>Recessive or X-linked</td>
<td>6 y</td>
<td>Ataxia, chorea, dystonia</td>
<td>Declines</td>
<td>“Eye of the tiger” and substantia nigra</td>
<td>Yes, atrophy</td>
<td>?</td>
<td>?</td>
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</tbody>
</table>

Note: *Clinical features list the presenting and major neurological features only.†Radiological features list the consistent and major abnormal findings reported on cerebral T2 MRI brain scans.‡Caudate, dentate, red refer to the caudate nucleus, dentate nucleus, and red nucleus respectively. “Eye of the tiger” sign is a decreased signal intensity in T2 weighted images, compatible with iron deposits, and of a small area of hyperintensity in its internal segment (illustrated in reference 6).§Gene abbreviations used; CP is ceruloplasmin, FTL is ferritin light chain, PANK2 is pantothenate kinase 2 and no identified gene.¶Freidrich ataxia was not included in the table as there is no evidence on MRI brain scans of excess pathological iron deposition in the central nervous system.

**Acknowledgements**

We express our gratitude to the members of the family studied. 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 within the 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 in pathological disease involving excess cerebral iron accumulation is unclear, but may relate to an accumulation of cysteine, which can chelate iron. Because of excess or physiological situation or both.

The highest concentration of iron in the brain is found within the basal ganglia. Cerebral diseases in which excess iron accumulation is found on MRI scan are summarised in Table 1. All cause neurological disease involving excess cerebral iron accumulation is unclear, but may relate to an accumulation of cysteine, which can chelate iron. Because of excess or physiological situation or both.


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- Molecular Medicine Unit, Faculty of Health Sciences, University of Jordan, Amman, Jordan

Electronic database information


Mental retardation of variable severity was present in all brae with large pedicles. Five further cases were reported. hypoplastic iliac wings, broad ribs, and large, flattened verte-decreased joint mobility, mixed hearing loss, and cleft lip and retardation, facial dysmorphism (short palpebral fissures,nosis of Myhre syndrome, expanding the behavioural profile GOMBO syndrome, and LAPS (Laryngotracheal stenosis, S

J-C Carel, V Cormier-Daire, S Manouvrier, A Verloes
diagnosis
Myhre syndrome: new reports, review, and differential

REFERENCES

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, acromic dysplasia, geleophysic dysplasia, GOMBO syndrome, and LAPS (Laryngotracheal stenosis, Arthrophytha, Prognathism and Short stature) syndrome. In 1980 Myhre et al reported two unrelated males with mental retardation, facial dysmorphism (short palpebral fissures, maxillary hypoplasia, prognathism, short philtrum, small mouth), short stature, brachydactyly, muscle hypertrophy, decreased joint mobility, mixed hearing loss, and eft lip and palate in one of them. X rays showed a thickened calvarium, hypoplastic iliac wings, broad ribs, and large, flattened vertebrae with large pedicles. Five further cases were reported. Mental retardation of variable severity was present in all patients. Three of the older patients had hypertension.

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

CASE REPORTS
Patient 1
Patient 1, a male, was the second child of healthy, non-consanguineous parents. The father was 181 cm tall and the mother was 165 cm tall. They were aged 40 and 34 years, respectively, at the 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 thick and, on the right side, fused with the oval window. This appearance was compatible with primary stapedial dysplasia or with other causes. The absence of evolution of the hearing loss was considered as an argument for the dysplasia hypothesis. He wore a hearing aid. He had hypermetropia.

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

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

![Figure 1](https://www.jmedgenet.com)

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 mUI/ml, normal <11), and normal LH level. This was compatible with primary (hypergonadotrophic) hypogonadism, possibly related to germinal cell aplasia (Sertoli cell only syndrome) or to another anomaly of spermatogenesis or anomalies of the FSH receptor.

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

**Patient 2**

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

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

**Patient 3**

This male patient was the second child of a 32 year old G2 P2 mother and a 38 year old father. The brother was healthy and a short neck. The hands were short with brachydactyly. He had difficult social relationships. He was 154 m tall and OFC was 56.5 cm (fig 1A). The facial dysmorphism included a square face, upward slanting eyebrows, short nose, very short philtrum, narrow mouth, and a large, prognathic chin. He had short hands with mild cutaneous syndactyly (fig 1B), and short feet with Y shaped syndactyly of toes 2 and 3. The skin was hard. There was generalised joint limitation, noted by the age of 10 and slowly worsening with time, and generalised muscular hypertrophy with extremely hard muscles. Neurological examination was normal, but osteotendinous reflexes were very weak. At the ages of 10 and 20, CK, EMG, and motor conduction velocity were normal. Muscle light microscopy at the age of 10 showed no abnormality. Hypertension (up to 170/100) was noted on different occasions during the last two years; no specific reason could be found. Echocardiography was normal. High resolution G banding and FISH with subtelomeric probes were normal.

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**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, ultrasoundography 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 46 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, deep set eyes, low set and small, round ears, a short philtrum, slightly convex profile of the nose, thin lips, and prognathism. The fingers were short with distal phalangeal hypoplasia. Extension of the elbows, knees, and ankles and pronosupination were limited. Achilles tendons were shortened but he had no spasticity or pyramidal signs. The skin was thick.

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

**Patient 4**
This male patient was born to healthy, non-consanguineous parents. He had a healthy older sister and three healthy half sibs from his mother and a healthy half sister from his father. The father was 168 cm tall and the mother was 153 cm tall. They were respectively aged 43 and 35 at the time of delivery. At birth weight was 2500 g, length was 47 cm, and OFC 32 cm at 38 weeks of gestation. Global hypertonia and brachydactyly were noticed. Clinical findings at 5 years of age showed short stature (1 m, −2 SD) with normal OFC (53 cm, +1 SD), “muscular” habitus, and thick skin. A peculiar square face was 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>Soljak 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>
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<tbody>
<tr>
<td></td>
<td>Patient 1</td>
<td>Patient 2</td>
<td>Patient 1</td>
<td>Patient 1</td>
<td>Patient 2</td>
<td>Patient 1</td>
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<td>3200</td>
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</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 philtrum, small mouth), short stature, brachydactyly, muscle hypertrophy, decreased joint mobility, mixed hearing loss, and cleft lip and palate in one of them. X rays showed a thickened calvarium, hypoplastic iliac wings, broad ribs, shortened tubular bones, and large, flattened vertebrae with large pedicles. Five further cases were reported. 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, 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. 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 hypergonadotrophic hypogonadism. Although these phenotypic manifestations are discordant, they may point to a disturbance in the hypothalamo-hypophysy-gonadal axis that should be investigated in further cases.

Several syndromes were transiently considered in the differential diagnosis of our patients. OSMED syndrome shows megaepiphyses, irregular vertebral bodies, and deafness, but can be distinguished by a different facial dysmorphism (microglossia), 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. Telomeric FISH analyses performed in two of our patients detected no cryptic rearrangement. Moore-Federman syndrome, acromicric dysplasia, geleophysic 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 –2 SD noted after the age of 2 years, short and sturdy 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. There is now convincing evidence to reject lumping with the recessively inherited geleophysic dysplasia as previously suggested. 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>Frequent</td>
<td>Learning difficulties</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Deafness</td>
<td>+ 9/11</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>+ (childhood onset)</td>
<td>+ (childhood onset)</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>Progressive cardiac valve disease</td>
<td>AR</td>
<td>AD</td>
</tr>
</tbody>
</table>

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).

Several syndromes were transiently considered in the differential diagnosis of our patients. OSMED syndrome shows megaepiphyses, irregular vertebral bodies, and deafness, but can be distinguished by a different facial dysmorphism (microglossia), 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. Telomeric FISH analyses performed in two of our patients detected no cryptic rearrangement. Moore-Federman syndrome, acromicric dysplasia, geleophysic dysplasia, and LAPS syndrome are the main differential diagnoses of Myhre syndrome. Table 2 summarises the similarities and differences between these entities.

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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. Figuera suggested that the patient reported by Rosser et al. 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-hypophysogonadal 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 cleanly distinguishes LAPS syndrome from Myhre syndrome, but this could perhaps be explained by insufficient follow up, as the older Myhre patient was only aged 24 whereas onset of obstructive symptoms was between 17 and 30 in LAPS cases. Despite these similarities, some major differences exist. The impressive muscular hypertrophy, which is a cardinal feature of Myhre syndrome, was never reported in LAPS patients. Finally, the sex ratio is strikingly dissimilar, 11 M:0 F in Myhre versus 1 M:4 F in LAPS. These two latter arguments make lumping of LAPS and Myhre hazardous at this point. Further reports and longer follow up of Myhre cases would probably help to solve this nosological issue in the future.

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

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REFERENCES
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 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 <5 µg/kg/min) to maintain blood pressure (despite adequate fluid resuscitation) along with the presence of perfusion abnormalities, or metabolic acidosis (pH ≤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 ≥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 χ² 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-α −308A-, TLR4 +896G-, and IL-6 −174C-alleles were grouped with heterozygotes for analysis. Alternate homozygotes at the more common IL-1β −31 and CD14 −159 SNPs were analysed separately from heterozygotes. The IL-1β 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 ≥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-α −308</td>
<td>107 bp</td>
<td>PCR (For): AGGCAATAGGTTTGGGGCCAT PCR (Rev): TCCCTCCGTCGGATGCCC</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></td>
<td>111 bp</td>
<td>PCR (For): CGTCAAGCTCAATGAC PCR (Rev): CCGTGGGGGGCTGATGGGAA</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-1β −31</td>
<td>222 bp</td>
<td>PCR (For): CTCCTACCTTCTGGCTTGAAGCC PCR (Rev): AAGAGAAGAGAAGAGAGAATG</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): ATACATCATCCGCTCACCACC PCR (Rev): GCCCCCTTCTCTCTGTTGA</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): AGCTATGGTGGTGGTGCTGG PCR (Rev): AACATCACCCAGGGAAAAATG</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 for 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 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.
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-α −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 ≥50 years</td>
<td>5.74</td>
<td>1.99 to 16.55</td>
<td>0.0001</td>
</tr>
<tr>
<td>Full-thickness burn ≥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.

Discussions

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 ≥50 years and full-thickness burn size ≥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 ≥30% TBSA and/or age ≥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 ≥50 years old or had ≥50% TBSA full-thickness burns and nine (6%) patients were both ≥50 years of age with ≥50% 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-α −308 A-allele (Fig 1).

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 ≥50 years of age and/or full thickness burn size ≥30% TBSA as cut points.

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

<table>
<thead>
<tr>
<th></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 results provide the first evidence that genetic polymorphisms within innate immunity loci are associated with an increased risk for severe sepsis after burn injury. Carriage of the TNF-α −308 A- or the TLR4 +896 G-allele was 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 TNF-α −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→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 TLR4 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-α −308 SNP. Importantly, TNF-α 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→A) at nucleotide −308 within the TNF-α promoter affects transcriptional regulation of TNF-α. 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-α have a seemingly opposite impact upon the amplitude of the endotoxin response. While carriage of the TLR4 +896G allele appears to decrease the intensity of the LPS response signal, the TNF-α −308A allele is associated with an increased rate of TNF-α transcription. In order to further investigate the effects of the TLR4 and TNF-α SNPs upon risk for severe sepsis, patients were stratified into low
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 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 generate a 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 increases the 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 efficiency of statistical power. In other words, these results were the result of subgroup analysis, which is well known to inflate the likelihood of type 1 (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 TLR4 promoter and G-allele carriage at TLR4 +896 appear to impart the same risk as traditional clinical factors.

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TLR4 and TNF-α SNPs and severe sepsis risk


Retraction statement

The authors of the following manuscript Ninis VN, Kylicz MO, Kandemir M, Daely E, Tolun J. High Frequency of 9T and CFTR Mutations in Children with Idiopathic Bronchiectasis. J Med Genet 2003;40:530–5, are retracting it because the polythymidine tract 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.