SHORT REPORT

A new syndrome, congenital extraocular muscle fibrosis with ulnar hand anomalies, maps to chromosome 21qter


**Background:** Congenital fibrosis of the extraocular muscles (CFEOM) is a heterogeneous group of disorders that may be associated with other anomalies. The association of a CFEOM syndrome with ulnar hand abnormalities (CFEOM/U) has not been reported to date.

**Objective:** To describe a new autosomal recessive syndrome of CFEOM and ulnar hand abnormalities, and localise the disease causing gene.

**Methods:** Clinical evaluation of the affected members and positional mapping.

**Results:** Six affected patients with CFEOM/U (aged 2 to 29 years) from a large consanguineous Turkish family were studied. Ophthalmological involvement was characterised by congenital blepharoptosis and restricted eye movements.

**Conclusions:** CFEOM/U maps to a 1.5 Mb region at chromosome 21qter. Future identification of the disease causing gene may provide insights into the development of the extraocular muscles and brain stem motor neurones, as well as anteroposterior limb development.

**H**euk (1879) was the first to report the combination of congenital blepharoptosis and restricted eye movements.1 In 1950, Brown described this group of ocular abnormalities in detail and classified the syndromes into five distinct phenotypes: horizontal retraction syndromes; strabismus fixus; vertical retraction syndromes; superior oblique tendon sheath syndromes; and a general fibrosis syndrome.2 Currently, the horizontal retraction syndromes are referred to as Duane syndrome, the superior oblique tendon sheath syndromes as Brown syndrome, and the remaining syndromes as congenital fibrosis of the extraocular muscles (CFEOM).3 Recent neuropathological studies have shown that some of the fibrosis syndromes result from developmental defects of particular brain stem motor neurones and their corresponding axons.4

Duane syndrome, the most common of the CFEOM syndromes, is characterised by limited abduction, variably limited adduction, and globe retraction on attempted adduction. Most cases are sporadic and only about 10% are familial.5 Cytogenetic analyses of sporadic cases revealed deletions of 8q13. This locus was designated DURS1 (MIM 126800).45 Linkage analysis in autosomal dominant families mapped the familial disease to the DURS2 locus on 2q31 (MIM 604356).16 To date, neither the DURS1 nor the DURS2 gene has been identified.

Three other inherited CFEOM syndromes have been mapped to different genetic loci:

- **CFEOM1** (MIM 135700), an autosomal dominant disorder, was mapped to 12q21.17 and the disease causing gene was recently identified as KIF21A.6 Affected individuals have bilateral ptosis and restrictive ophthalmoplegia, and their eyes are fixed below the horizontally neutral position with or without secondary esotropia or exotropia. CFEOM1 is phenotypically variable, with some patients having a milder expression which resembles CFEOM3. However, these families with the milder phenotypes have been linked to the CFEOM1 locus,17 leading to their designation as CFEOM type 3A (MIM 607034).

- The CFEOM2 locus (MIM 602078), an autosomal recessive disorder, was mapped to chromosome 11q13.18 and subsequently mutations in the ARIX gene were described.19 Affected individuals with CFEOM2 have bilateral ptosis, with both eyes fixed in abduction.

- CFEOM3 (MIM 600638, formerly 604361), an autosomal dominant disorder, was mapped to chromosome 16q24.2.6 The phenotype of affected individuals in CFEOM3 families was variable and ranged from bilateral ptosis with fixed eyes in an infraverted and exotropis position to normally positioned eyes with minimal limitation of vertical gaze and unilateral or absent ptosis. To date, the gene causing CFEOM3 has not been identified.

Several CFEOM syndromes occur in association with other anomalies including the Duane radial ray syndrome (DRRS) (MIM 607323), the Wildervanck syndrome (MIM 314600), and familial horizontal gaze palsy with progressive scoliosis (MIM 607313).

DRRS is characterised by the Duane anomaly, radial ray abnormalities, and deafness. The DRRS syndrome—also known as Okhihiro syndrome7—is inherited as an autosomal dominant trait with variable expressivity. The DRRS locus was mapped to 20q13 and subsequently SALL4 was identified as the disease causing gene.8 9

The features of the Wildervanck syndrome include the Duane anomaly, the Klippel-Feil anomaly (fused cervical vertebrae), and congenital perceptive deafness. This disorder is mostly seen in females, suggesting that the syndrome is X-linked.

**Abbreviations:** CFEOM, congenital fibrosis of extraocular muscles; CFEOM/U, congenital fibrosis of extraocular muscles with ulnar hand abnormalities; DRRS, Duane radial ray syndrome; SNP, single nucleotide polymorphism; OD, right eye; OS, left eye
CFEOM with ulnar anomalies maps to 21qter

Distantly related
cousins

METHODS

Subjects and medical evaluation

Six affected individuals from the consanguineous Turkish family (fig 1) were clinically evaluated at the division of medical genetics of the Child Health Institute and the orthoptics clinic of the department of ophthalmology, Istanbul Medical Faculty of Istanbul University, Turkey. The study was approved by the Institutional Review Board of the Child Health Institute of Istanbul University and informed consent was obtained from each participant. Five of the six affected individuals had complete ophthalmological examinations, skeletal x-rays, abdominal ultrasound, echocardiography, and cranial magnetic resonance imaging (MRI).

Ophthalmological studies

Visual acuity was measured using a Snellen letter chart projector transilluminated at approximately 100 cd/m² and line acuity performances at 6 m were recorded. Non-cycloplegic refractive data were obtained using a retinoscope or a Topcon KR-7000P autokeratorefractometer, and binocular status was evaluated with a Clement–Clarke synoptophore. Range of ocular movements was evaluated with Hess screen tests in patients with binocular vision potential. Direct and indirect papillary reactions were recorded, and photographic records of each patient were obtained. Duction

inherited as a sex linked dominant with lethality in affected males.

Familial horizontal gaze palsy with progressive scoliosis is an autosomal recessive disorder characterised by progressive external ophthalmoplegia and scoliosis. The disease causing gene has been mapped to chromosome 11q23–q25.20

Here, we describe a new autosomal recessive CFEOM syndrome with prominent ulnar hand abnormalities in a consanguineous Turkish family. The six affected individuals, aged 2 to 29 years, presented with right eye involvement and bilateral postaxial oligodactyly/oligosyndactyly of the hands, with the right more severely affected than the left. A genome scan of DNAs from family members mapped the disease locus with the right more severely affected than the left. A genome scan of DNAs from family members mapped the disease locus to 21q with a multipoint LOD score of 4.525 at microsatellite marker D21S1259. Further interrogation of the locus narrowed the critical region to ~1.5 Mb between D21S1897 and the telomere of the long arm.

Figure 1 Pedigree of the CFEOM/U family and 21qter haplotype data. The haplotypes of the most telomeric two markers, D21S1446 and rs881827, which segregated with the disease in the whole family are shown in black. The inferred haplotypes of I-4 are indicated in parentheses. CFEOM/U, congenital fibrosis of extraocular muscles with ulnar hand abnormalities. (See online supplemental table 1 for all marker data, obtainable from http://www.jmedgenet.com/supplemental/)

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Table 1

<table>
<thead>
<tr>
<th>Family member</th>
<th>II-9</th>
<th>II-14</th>
<th>III-10</th>
<th>III-11</th>
<th>IV-1</th>
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<td>Marked</td>
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<td>Chin elevation</td>
<td>Slight head tilt</td>
<td>Head turn</td>
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<tr>
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<td>Ab 5</td>
<td>Ab 5; small fifth finger bud</td>
<td>Ab 5; small fifth finger bud</td>
<td>Ab 5; small fifth finger bud</td>
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<td>Ab MP 4, 5</td>
<td>Ab MP 4, 5</td>
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</table>

Ab, absent; Cl, clinodactyly; hT, hypotropia; IO, inferior oblique muscle; IR, inferior rectus muscle; Lev, levator palpebrae superioris muscle; M, metacarpal bone; P, phalangeal bone; PD, prism diopters; SR, superior rectus muscle; Sy, syndactyly; XT, exotropia.

DNA extraction and genotyping

Genomic DNA was isolated from peripheral blood collected in EDTA from patients and family members using the DNA isolation kit for mammalian blood (Roche, Istanbul, Turkey). For the initial genome scan, DNAs were analysed using 422 autosomal microsatellite markers from the genome-wide human screening set (version 9) and single chromosome scan set (Invitrogen Life Technologies, Carlsbad, California, USA). As needed, additional microsatellite markers were obtained from public databases (NCBI, Marshfield Institute, deCode), or new microsatellite markers were designed using the tandem repeat finder program. All new markers were on the genomic contig NT_011515 and were named by their positions on the contig in kb (Human May 2004[hg17] assembly[NCBI Build 35] on the UCSC human genome browser). The new markers and their positions on chromosome 21 were as follows: 1305K (44 748 500 base pairs (bp)), 2044K (45 585 432 bp), 2849K (46 292 500 bp), 3086K (46 529 500 bp), 3258K (46 701 500 bp) (for primer information, see online supplemental table 2, obtainable from http://www.jmedgenet.com/supplemental/). The primers were designed using Primer3 software, and the fluorescent dye labelled forward primers were synthesised by Invitrogen Life Technologies. When no informative microsatellite markers were found in a particular region, single nucleotide polymorphisms (SNPs) were used for linkage analysis. SNP information was obtained from the UCSC Genome Browser and NCBI web sites, and primers were designed as above.

Genomic DNAs were polymerase chain reaction (PCR) amplified in 96-well microtitre plates in an oil-free system using a DNA Engine PTC-200 thermal cycler (MJ Research, Waltham, Massachusetts, USA). Reaction mixtures (10 μl) contained 10 ng of genomic DNA, 2 mM MgCl₂, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 nM of each primer, 0.2 mM dNTPs, and 0.5 U of Taq DNA polymerase (AmpliTaq Gold, Applied Biosystems, Foster City, California, USA). For PCR, the reaction mixtures were initially incubated at 95°C for 10 minutes, and amplified for 27 cycles with denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension step at
72°C for seven minutes. PCR products were analysed with either an ABI Prism 3100 genetic analyser or on an ABI Prism 377 DNA sequencer using GeneScan analysis software (version 3.1.2) and Genotyper software (version 2.5) (Perkin-Elmer-Cetus, Norwalk, Connecticut, USA).

PCRs for SNPs were undertaken in a final volume of 50 ml and at specific annealing temperatures for each fragment for 35 cycles. Amplicons were sequenced on an ABI Prism 3700 capillary array sequencer using the ABI Prism BigDye Terminator ready reaction mix (Perkin-Elmer-Cetus). Electropherograms were inspected using ABI Prism sequencing analysis software (version 3.4.1).

**Linkage analysis**

The multipoint LOD score calculations for each chromosome were individually carried out with the SimWalk2 program (version 2.86) under the assumption of autosomal recessive inheritance with full penetrance. The Mega2 program (version 2.5) was used to create input files for the SimWalk2 program. As data on the population incidence of this unique disorder were unavailable, we used 0.001 as the disease allele frequency. Loci with suggestive LOD scores were genotyped with a denser marker set. The highest LOD score obtained with SimWalk2 was confirmed with the Linkmap program (Fastlink package version 4.1).

**RESULTS**

**Clinical evaluations**

Figure 1 shows the pedigree of the consanguineous Turkish family with six affected members in three sibships, of which four (II-9, II-10, II-14, and IV-1) were offspring of first cousin marriages, suggesting autosomal recessive inheritance. Of note, two affected siblings (III-10 and III-11) were the offspring of an affected father (II-9) and a healthy mother (II-8). Although consanguinity between the parents could not clearly be documented, both originated from the same small village and thought they were distantly related. The physical and neurological examinations of all affected members were normal with the exception of the ophthalmological and hand abnormalities. The feet were normal and there were no abdominal ultrasound or echocardiographic abnormalities in the affected individuals. The relevant findings in each patient are described below and summarised in table 1.

**Patient 1 (II-9)**

This was a 29 year old man who had a right divergent strabismus with hypotropia and 20/20 visual acuity bilaterally, with no refractive error. He had 14 DD exotropia and 25 DD hypotropia OD, with no torsional component and a slight “chin up” anomalous head posture. He had a markedly restricted elevation OD, as well as secondary overaction of the inferior oblique muscle OS. Hess screen test results showed restriction of the entire right superior gaze field. Cranial MRI showed volume loss of the right superior rectus muscle and hypertrophy of the inferior rectus muscle. A forced duction test done under general anaesthesia showed no passive restriction of eye movements, and the patient was diagnosed as having right double elevator palsy. He had absent fifth fingers bilaterally, and the proximal part of his right hand...
was markedly hypoplastic compared with the left. While the fourth finger on the right hand was incurved and hypoplastic (clinodactyly), the fourth finger of the left hand was normally developed and had a very small (~3–4 mm) fifth finger bud emerging from the ulnar side at the metacarpophalangeal level. On x ray, the metacarpal and phalangeal bones of the fifth fingers were absent bilaterally, and were hypoplastic for the right fourth finger. The right ulnar styloid process was also hypoplastic. In addition, the carpals of the right hand were abnormal, the triquetrum and pisiform bones were absent, and the capitate and hamate carpal bones of the right hand were abnormal, the triquetrum and lunate bones were fused. The scaphoid bone of the left hand was bipartite (fig 2).

Patient 2 (II-14)
This was a 26 year old man who had right hemiptosis and divergent strabismus. He had 12 Δ of exotropia and 25 Δ of hypotropia OD without measurable torsional deviation. Elevation of the right eye was restricted, and this was more significant in abduction. The Bielschowsky head tilt test was slightly positive on the right and he had a chin up head posture. His visual acuity was 20/200 (OD) and 20/20 (OS). Cranial MRI was similar to that of patient II-9, with volume loss of the superior rectus muscle and hypertrophy of the inferior rectus muscle of the right eye. Under general anaesthesia, the forced duction test showed no passive restriction in ocular movements. The fifth fingers of both hands were absent, and the fourth finger of the right hand was slightly thinner than on the left, although the patient was right handed. On x-ray, both hands were symmetrical, and the metacarpal and phalangeal bones of the fifth fingers were absent. The ulnar styloid processes were hypoplastic, and the triquetrum and lunate bones were fused bilaterally (fig 2).

Patient 3 (III-10)
This was a four year old girl who had 20/20 visual acuity bilaterally, 16 Δ of exotropia OD, and restricted elevation on adduction of both eyes, which was more marked on the right. Head posture appeared normal, but a slight head tilt to the right shoulder occurred occasionally. Cranial MRI was normal. The forced duction test was normal, and the patient was diagnosed as having pseudo-Brown syndrome because of bilateral inferior oblique muscle dysfunction. The fifth finger of the right hand was absent, and she had syndactyly of the fourth and fifth fingers on the left, both of which were hypoplastic, and the fifth finger had no nail. On x-ray, the metacarpal and phalangeal bones of the fifth fingers were absent bilaterally, and the metacarpal and phalangeal bones of the left fourth finger were hypoplastic (fig 2).

Figure 3

Graphical presentation of multipoint LOD scores. Plot of the scores obtained by the SimWalk2 program for chromosome 21. Insert: Plot of the score obtained by Linkmap program in the critical region only.

Patient 4 (III-11)
This was a three year old boy who had a visual acuity of 20/200 (OD) and 20/20 (OS) with no significant refractive error. He had 18 Δ exotropia and 20 Δ hypotropia OD. Restricted elevation during adduction was more marked on abduction. He had a normal head posture, but a slight head tilt toward the right occurred intermittently. He had a positive forced duction test, showing a markedly restricted elevation during adduction resembling that found in Brown syndrome. Cranial MRI was normal. His fifth fingers were absent bilaterally, and the right fourth finger was slightly incurved. On x ray, the metacarpal and phalangeal bones of the fifth fingers were absent bilaterally (fig 2).

Patient 5 (IV-1)
This 2 1/2 year old girl was the most severely affected. She had total ptosis and enophthalmia OD, and the right cornea was smaller (radius, R = 11 mm) than the left (R = 12 mm). She could not fixate and follow objects with her right eye owing to profound amblyopia. Ocular movements of the right eye were restricted in all directions, while they were normal on the left. Examination of the fundus revealed a tilted disc OD. The MRI confirmed enophthalmia but did not reveal any additional abnormalities. The right third, fourth, and fifth fingers and the left fourth and fifth fingers were absent. On x-ray, the relevant metacarpal and phalangeal bones were absent, while the other bony structures were normal (fig 2).

Histopathological studies
Histological examination of the right superior rectus from patient III-11 showed primarily fibroadipose tissue and no muscle cells. In contrast, the skeletal muscle from the right inferior oblique biopsy had mild non-specific variation in fibre size on light microscopy, but no abnormalities were observed on histochemical analysis or electron microscopy (data not shown).

Genome scan and linkage to 21qter
A genome scan with 422 autosomal microsatellite markers was carried out using genomic DNA from 13 family members, and multipoint linkage analysis was used to map the disease locus. A location score of 3.13 was obtained at marker D21S1259 on chromosome 21q22.3. The addition of the polymorphic markers D21S1260 and D21S1897—which were centromeric and telomeric to D21S1259, respectively—increased the location score to 4.53. The Linkmap program gave a LOD score of 4.03 at D21S1259 (fig 3). Initial calculations were made assuming that II-8 and II-9 were not consanguineous. This assumption, and the limited informativeness of the most telomeric marker, D21S1446, resulted in the highest location score being more centromeric, located among more informative markers. Haplotype analysis assuming non-consanguinity between II-8 and II-9 confined the critical region between D21S1260 and the telomere, a critical region of ~5.2 Mb (fig 1). Only the most telomeric marker, D21S1446, was homozygous in all affected individuals. Thus the critical region was interrogated using four additional microsatellite markers (D21S1890, D21S1912, D21S171, and D21S1903), and four SNPs...
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because of bilateral inferior oblique dysfunction. The fifth
test, which was interpreted as a pseudo-Brown syndrome

elevation during adduction with a negative forced duction

positive forced duction test, as observed in patients with

III-11 was distinctive in having a more marked mechanical

perhaps caused by a superior rectus muscle paresis. 27 Patient

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new autosomal recessive syndrome of CFEOM with ulnar

DISCUSSION

The consanguineous Turkish patients described here define a

new autosomal recessive syndrome of CFEOM with unilateral

hand abnormalities (designated CFEOM/U). The affected

individuals all had CFEOM of varying severity, which was

mainly confined to the right eye, and bilateral postaxial

oligodactyly/oligosyndactyly of the hands, which was more

severe on the right. Although the clinical findings were

variable between affected individuals, penetrance was com-

plete. Interestingly, the severity of the eye and hand

involvement was consistent in the same individual. This

was clearly observed in the most and least severely affected

individuals, IV-1 and III-10, respectively.

All five patients had superior rectus and inferior oblique

muscle involvement, and three (II-14, III-11, and IV-1) also

did levator palpebrae dysfunction. Phenotypically, three

patients (II-9, II-14, and III-11) had a double elevator palsy,

perhaps caused by a superior rectus muscle paresis. 27 Patient

III-11 was distinctive in having a more marked mechanical

restriction of elevation during adduction, resulting in a

positive forced duction test, as observed in patients with

Brown syndrome; and patient III-10 had bilateral restricted

elevation during adduction with a negative forced duction
test, which was interpreted as a pseudo-Brown syndrome

because of bilateral inferior oblique dysfunction. The fifth

patient had a more generalised and severe pattern of extracocular muscle involvement with markedly restricted eye movements in all directions and a total palsy resembling an atypical fibrosis syndrome. The third patient (II-14) exhibited a classical CFEOM (that is, CFEOM1) usually have bilateral ptosis and restrictive ophthalmoplegia, with their eyes fixed below the horizontal neutral position with or without secondary esotropia or exotropia. Necropsy examinations of individuals affected by classical CFEOM revealed the absence of the superior division of the oculomotor nerve, which normally innervates the superior rectus and levator palpebrae superioris muscles. 23 In contrast, atypical patients with CFEOM2 and CFEOM3 have a restrictive ophthalmoplegia, caused by deficient function of the muscles innervated by the third or fourth cranial nerves. Unlike the classical type, they can raise their eyes above the horizontal line or have unilateral, atypical CFEOM, marked phenotypic variability has been reported. 4 The patients described here can be classified as atypical CFEOM as they have involvement of the superior and inferior divisions of the third cranial nerve, although patient IV-1 may also have involvement of the fourth and sixth cranial nerves, indicating the variability in the ocular phenotype. In addition to the ocular abnormalities, these patients had oligodactyly/oligosyndactyly of the hands. The only ocular motility disorder with upper limb defects is the Duane-radial ray syndrome with radial ray abnormalities ranging from hypoplasia of the thanar eminence to absence of the radial bone or forearm. In the family presented here, neither the ocular nor the skeletal findings resembled the clinical findings of DRRS. Moebius syndrome, which is characterised by congenital paresis or paralysis of the seventh (facial) cranial nerve frequently accompanied by dysfunction of other cranial nerves, may also be associated with arthrogryposis and hand abnormalities but is quite distinctive from the syndrome we present here.

It is estimated that approximately 1/600 newborn infants have a congenital abnormality of the upper limb. 29 Postaxial limb deficiencies are most often unilateral and sporadic. They also occur as a feature of various syndromes. An autosomal dominant, non-syndromic postaxial oligodactyly which affects all four extremities has also been described (MIM 176240). 30 However, the association of a congenital fibrosis syndrome with postaxial oligodactyly/oligosyndactyly is novel and has not been reported to date. Of note, only a few causative genes for this group of disorders have been mapped or identified. 30 By multipoint linkage analysis, the disease locus for CFEOM/U1 was mapped to chromosome 21 between the new microsatellite marker 2044K and the chromosome 21 telomer, a critical region spanning 1.5 Mb. Initial calculations were made assuming consanguinity between II-8 and II-9, and with the inclusion of the new microsatellite markers and SNPs. Using the Simwalk2 program, a multipoint location score of 3.28 was obtained at microsatellite D21S1446 through SNP rs881827. This result was confirmed with the Linkmap program, which gave a multipoint LOD score of 3.12 at D21S1446.

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Based on the current Human May 2004 (hg17) Assembly (NCBI Build 35) on the UCSC human genome browser, this region of 3.5 Mb contains 17 genes (C2orf1123, COL18A1, SLC19A1, PCBP3, COL6A1, COL6A2, FTCD, C2orf56, LSS, MCM3APAS, AFA2626, C2orf57, C2orf58, PCNT2, C2orf1106, S100B, and HRTMT1L1), which have corresponding entries in PDB or SWISS-PROT, or are NCBI reference sequence mRNAs with a "reviewed" status. As there may be unrecognised genes in this 3.5 Mb region, it may contain about 20 genes. However, there were no obvious candidate genes, and no obvious motifs. Efforts are under way to further refine the region of homozygosity and to identify the disease causing gene.
Conclusions

A new autosomal recessive ocular motility syndrome with postaxial oligodactyly and syndactyly (designated CFEOM/U1) was identified and its locus mapped to the most telomeric 1.5 Mb of chromosome 21. Future identification and functional studies of the gene causing this new syndrome may provide insights into the development of the extraocular muscles and their cranial motor nuclei, as well as anterior-posterior limb development.

ELECTRONIC DATABASE INFORMATION

- Center for Medical Genetics, Marshfield Clinic Research Foundation, http://research.marshfieldclinic.org/genetics/Map_Markers/maps/MapIndexMaps.html
- Primer3 primer design program, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi
- University of California Santa Cruz (UCSC), Human Genome Browser, http://genome.ucsc.edu/cgi-bin/hgGateway

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Supplementary tables 1 and 2 can be found on our web site, www.jmedgenet.com/supplemental

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Supplementary Information

REFERENCES


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Further evidence for LBP-1c/CP2/LSF association in Alzheimer’s disease families

L Bertram, M Parkinson, M B McQueen, K Mullin, M Hsiao, R Menon, T J Moscarillo, D Blacker, R E Tanzi

**Objectives:** Several studies suggested chromosome 12 harbours an Alzheimer’s disease (AD) risk factor gene. Significant association of a single nucleotide polymorphism (SNP) in the 3' UTR of transcription factor CP2 (LBP-1c/CP2/LSF or TFCP2) at 12q13 was reported in three independent case-control studies, but no family based analyses have been performed to date.

**Methods:** Genotypes for three SNPs were generated in two independent AD family samples. A meta-analysis on all published case-control studies was also performed.

**Results:** The A allele of the 3' UTR SNP was associated with increased risk for AD in one sample (odds ratio (OR) 2.1, 95% confidence interval (95% CI) 1.1 to 4.3), but not in the other, possibly due to low power. Haplotype analyses showed that this allele is part of a putative risk haplotype overtransmitted to affected individuals in one sample and in both samples combined. Meta-analysis of the previously associated 3' UTR SNP showed a trend towards a protective effect of the A allele in AD (OR 0.73, 95% CI 0.5 to 1.1).

**Conclusions:** This is the first study to examine LBP-1c/CP2/LSF in AD families, and the fifth to independently show significant association. While our results support a role of this gene in AD pathogenesis, the direction of the effect remains uncertain, possibly indicating linkage disequilibrium with another variant nearby.

**Abbreviations:** AD, Alzheimer’s disease; CAG, Consortium on Alzheimer’s Genetics; CLR, conditional logistic regression; LD, linkage disequilibrium; OR, odds ratio; PDT, pedigree disequilibrium test; SNP, single nucleotide polymorphism; 95% CI, 95% confidence interval.
LSF in two independent and carefully ascertained and evaluated AD family samples, and provide further support for a significant role of this gene in contributing to overall AD risk.

METHODS

Samples

The NIMH AD genetics initiative study sample

Subjects were collected following a standardised protocol applying NINCDS/ADRDA criteria for the diagnosis of AD. Over the 10 years that the participating families have been followed, a clinical diagnosis of AD has been ascertained at autopsy in 94% of the cases. The NIMH sample includes 1439 individuals (69% female) from 437 families with at least two affected individuals (994 affected individuals (mean SD) age of onset 72.4 (7.7) years, range 50–97 years), 411 unaffected individuals, and 34 with unknown phenotype.

Consortium on Alzheimer’s Genetics (CAG) study sample

Subjects for this second, independently ascertained, AD family sample were collected under the auspices of the Consortium on Alzheimer’s Genetics, a collaborative effort of the Massachusetts AD Research Center, the University of California, Los Angeles, the University of California, San Diego, and the University of Rochester Medical Center. NINCDS/ADRDA criteria were used for a clinical diagnosis of AD, and probands were included only if they had at least one unaffected living sibling willing to participate in this study. Unlike the NIMH sample, no affected individual beyond the proband was required; thus, the vast majority of families are not multiplex. Currently, data and specimen collection is completed for 489 individuals (62.6% female) from 217 sibships in which all affected individuals displayed an onset age ≥ 50 years (n = 224 affected individuals (mean SD) age of onset 71.2 (9.1) years, range 50–89 years), n = 265 unaffected individuals). Most sibships consisted of just one discordant sibpair, but in 41 families there were more than two sibships available.

Genotyping

Genotypes for a total of three polymorphisms (that is, the original 3' UTR SNP, rs4438107 (~10 kb proximal), and rs10876135 (773 bp distal)) in LBP-1c/CP/LSF were generated using fluorescent polisation detected single base extension (FP-SBE) on a Criterion Analyst AD (Molecular Devices, Sunnyvale, CA). PCR primers were designed to yield a product of approximately 250 bp in length and added to ~10 ng of genomic DNA using individually optimised PCR conditions (sequences available on request). PCR primers and unincorporated dNTPs were degraded by the direct addition of exonuclease I (0.1–0.15 U/rxn) and shrimp alkaline phosphatase (1 U/rxn). The single base extension step was carried out using Thermosequenase (0.4 U/rxn) and the appropriate mix of R110-ddNTP, TAMRA-ddNTP (3 mM), and all four unlabeled ddNTPs (22 or 25 μM) to the Exol1/SAP treated PCR product. To assess genotyping quality and ensure consistency of the genotyping calls, ~10% of the samples were randomly duplicated and genotyped twice. For all three SNPs combined, average genotyping efficiency was 97.8%, and the discrepancy rate (based on comparison to blinded duplicated samples) was below 0.3% in both samples.

Statistical analyses

Single locus and haplotype based tests of association were done in FBAT (v1.5.3). FBAT uses a generalised score statistic to perform a variety of TDT type tests and despite the true underlying genetic model, FBAT performs best assuming an additive genetic model, which was used here. We used the empirical variance function of the program to account for the presence of linkage in the area as suggested by previous studies, and an equal weight offset correction to incorporate genotypes from both affected and unaffected individuals. All analyses were performed on the full NIMH sample, the CAG sample, and on the two samples combined. All single locus analyses were repeated using the pedigree disequilibrium test (PDT) to confirm results obtained with FBAT (note that the PDT currently does not accommodate haplotype tests). While both tests compute valid p values under the null hypothesis of linkage but no association, the PDT statistic can be less efficient under some circumstances as it only includes discordant sibships and ignores families where only affected siblings are available. Further, to assess the magnitude of any potential effect on disease risk for the 3' UTR SNP, we performed conditional logistic regression (CLR) stratified on family, comparing carriers of the A allele to carriers of the GG genotype. All OR are adjusted for age, gender, and APOE ε4 allele status. Note that 95% confidence intervals may be too narrow because CLR may slightly underestimate the standard errors when multiplex affected and unaffected subjects are included in each family. However, the magnitude of this effect is expected to be small unless genetic effects are very large. Finally, to assess whether families showing association with the 3' UTR SNP in LBP-1c/CP/LSF overlap with families associated with the intron 18 deletion in A2M, we determined for both polymorphisms which of the NIMH pedigrees showed

<table>
<thead>
<tr>
<th>Study</th>
<th>AD cases</th>
<th>Normal controls</th>
<th>Results</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Subjects, n (% women)</td>
<td>Onset age, mean (SD)</td>
<td>Subjects, n (% women)</td>
</tr>
<tr>
<td>Panza et al</td>
<td></td>
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<tr>
<td>Italy</td>
<td>166 (63%)</td>
<td>69.4 (10.3)</td>
<td>225 (68%)</td>
</tr>
<tr>
<td>Lambert et al</td>
<td>239 (64%)</td>
<td>81.2 (7.8)</td>
<td>342 (59%)</td>
</tr>
<tr>
<td>All combined</td>
<td>1139 (64.6%)</td>
<td>70.5 (6.6)</td>
<td>1317 (62.5%)</td>
</tr>
<tr>
<td>France</td>
<td>684 (63%)</td>
<td>69.4 (8.4)</td>
<td>650 (63%)</td>
</tr>
<tr>
<td>Taylor et al</td>
<td>159 (67%)</td>
<td>65.7 (11.1)</td>
<td>205 (51%)</td>
</tr>
<tr>
<td>UK</td>
<td>296 (67%)</td>
<td>75.7 (7.0)</td>
<td>462 (67%)</td>
</tr>
<tr>
<td>Italy</td>
<td>159 (67%)</td>
<td>65.7 (11.1)</td>
<td>205 (51%)</td>
</tr>
</tbody>
</table>

Studies are shown in chronological order, with the most recent study listed first. Odds ratios (ORs) and 95% confidence intervals (CI) as reported by authors; some ORs are adjusted for co-variables (like age, gender, and APOE ε4 status) and might thus vary slightly from the crude ORs presented in fig 1, which were used to calculate summary ORs.
transmission of at least two risk alleles to affected individuals (using the Viewstat option in FBAT).

**Haplotype block predictions**

Haplotype blocks were estimated using the program Haplovie based on the four gamete rule (fourth gamete at 0.02 frequency; see Haplovie website for details at http://www.broad.mit.edu/personal/jcbarret/haplovie/index.php). Haplovie was also used for the calculation of pairwise linkage disequilibrium (LD) measures across all three SNPs.

**Meta analysis**

Study specific crude ORs and 95% CIs were calculated from the raw data for each of the case-control studies investigating the association between the LBP-1c/CP2/LSF 3' UTR SNP and AD. The Q statistic, a test for heterogeneity among the study specific ORs, that is distributed approximately as $\chi^2$ with $k-1$ degrees of freedom ($k$ = number of studies), resulted in a p value <0.1, suggesting significant between-study heterogeneity. Therefore, to calculate a summary OR for all studies, we used the DerSimonian and Laird random effects model, which utilises weights that incorporate both the within study and between study variance. Note the ORs estimated from the CLR in our family based analyses are adjusted for a number of co-variables (see above), which is why we elected not to combine them with the crude or differently adjusted ORs from the case-control studies. Statistical Analysis System (SAS) was used for the statistical analyses and resulting graphs.

**RESULTS**

The results of the single locus and haplotype association analyses are shown in table 2. Allele frequencies, as estimated by FBAT, were very similar for all three SNPs in both samples, and for the 3' UTR SNP are comparable to the previous reports (see legend to table 2). Genotype frequencies for all SNPs were in Hardy-Weinberg equilibrium ($p>0.90$). Testing the 3' UTR SNP in FBAT revealed significant overtransmission of the A allele to affected individuals in the NIMH families ($p = 0.05$). This was confirmed using the PDT ($p = 0.04$), and resulted in a significant risk increase in carriers of the A allele (OR 2.1, 95% CI 1.0 to 4.3) using CLR stratified on family. Due to the low numbers, heterozygous and homozygous A allele carriers could not be examined separately, but all three subjects carrying the A/A heterozygous and homozygous A allele carriers could not be considered to be carriers of the A allele and non-carriers (OR 2.1, 95% CI 1.1 to 4.3) using CLR stratified on family. Due to the low numbers, heterozygous and homozygous A allele carriers could not be examined separately, but all three subjects carrying the A/A genotype were affected (two autopsy confirmed, one clinical AD diagnosis), in accordance with the observation that the A allele confers risk in this sample. While the same allele was also overtransmitted to affected individuals in families of the CAG sample resulting in a similar effect size estimate as for the NIMH families, this did not reach statistical significance in any of the analyses (OR 2.1, 95% CI 0.5 to 8.5), possibly due to low power in this overall smaller sample. Also, there were no homozygous A allele carriers in these families. However, combining both samples yielded slightly decreased p values (FBAT = 0.03; PDT = 0.02) and narrower confidence intervals in the effect size estimates (OR 2.1, 95% CI 1.1 to 3.7). In addition to these findings, one of the other two tested SNPs (that is, rs10876135, located 773 bp further 3') also showed significant association in the NIMH but not in the CAG sample ($p = 0.04$ and 0.2, respectively; table 2). All three SNPs showed strong pairwise LD and were estimated to reside within the same haplotype block, which is in good agreement with predictions from the International HapMap Project (http://www.hapmap.org/). Thus, all three SNPs were combined in the haplotype analyses which showed evidence for one rare haplotype (H4) being significantly overtransmitted to affected individuals in the NIMH sample ($p = 0.03$; table 2). A similar frequency and transmission pattern for this haplotype were observed in the CAG families, although the overtransmission to affected individuals did not reach statistical significance, again possibly due to low power. However, as for the 3' UTR SNP, the association signal of this haplotype was strongest when both samples were combined ($p = 0.01$). None of these SNPs showed a significant interaction with ApoE 44, gender, or onset age in our CLR analyses (data not shown). This is noteworthy because most of the previous studies demonstrating a protective role for the A allele of the 3' UTR SNP were comprised of late onset AD families. In this study, however, effect size estimates are quite comparable in families of late (OR 1.8 (1.0–3.2)) and early/mixed onset (OR 2.2 (0.7–7.2); combined sample).

Combining all four previously published case-control studies into one meta-analysis revealed an overall protective effect of the A allele, although this did not reach statistical significance when all six independent case-control series were considered (0.73, 95% CI 0.5 to 1.1; fig 1). Interestingly, allele-frequency estimates across studies were quite similar for the AD cases (ranging from 0.04 to 0.06), but were considerably more variable in the control populations (ranging from 0.02 to 0.09; table 1). In an attempt to reduce this variability, we removed the two populations with the most extreme allele frequency estimates in healthy controls (that is, Panza et al11 and the UK sample from Lambert et al12) and repeated the analyses. As expected, the resulting summary OR proved somewhat more stable, indicating a significant protective effect across the remaining samples (OR 0.62, 95% CI 0.5 to 0.8; fig 1).

### Table 2 Association analyses of three SNPs in LBP-1c/CP2/LSF in two independent family samples

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<thead>
<tr>
<th></th>
<th>Single locus analysis*</th>
<th>Haplotype analysis†</th>
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<tbody>
<tr>
<td></td>
<td>FBAT</td>
<td>3' UTR</td>
</tr>
<tr>
<td></td>
<td>z score (p)</td>
<td>z score (p)</td>
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<tr>
<td><strong>NIMH (n = 437)</strong></td>
<td>0.4 (0.7)</td>
<td>1.9 (0.05)</td>
</tr>
<tr>
<td><strong>CAG (n = 217)</strong></td>
<td>0.5 (0.6)</td>
<td>1.1 (0.3)</td>
</tr>
<tr>
<td><strong>Combined</strong></td>
<td>0 (1)</td>
<td>2.2 (0.03)</td>
</tr>
<tr>
<td><strong>PDT</strong></td>
<td>z² (p)</td>
<td>z² (p)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NIMH (n = 437)</strong></td>
<td>0.5 (0.5)</td>
<td>5.1 (0.02)</td>
</tr>
<tr>
<td><strong>CAG (n = 217)</strong></td>
<td>1.2 (0.3)</td>
<td>0.5 (0.5)</td>
</tr>
<tr>
<td><strong>Combined</strong></td>
<td>0 (0.9)</td>
<td>5.5 (0.02)</td>
</tr>
</tbody>
</table>

*Minor allele frequencies in the NIMH (CAG) sample are: rs4438107 (T) = 0.46 (0.47), 3' UTR (A) = 0.06 (0.07), rs10876135 (T) = 0.08 (0.06). z score for minor allele or denoted haplotype allele (positive values indicate overtransmission to affected individuals).
†Haplotype frequencies in the NIMH (CAG) sample are: H1 (C-G-C) = 0.51 (0.48), H2 (T-G-C) = 0.39 (0.43), H3 (G-T-G) = 0.06 (0.04), H4 (C-A-C) = 0.04 (0.04).

Association statistics are presented for risk associated haplotype (H4) and 1 df.
This is the first study to assess the previously reported association between LBP-1c/CP2/LSF and AD using family based methods. Examining two independently ascertained and carefully characterised AD family samples, we observed a significant risk effect of the A allele of the 3' UTR SNP in the NIMH families, and a similar but insignificant effect in the smaller CAG dataset. Combining both samples resulted in the same effect size estimate as for the NIMH families alone, with slightly narrower confidence intervals. This could indicate that the A allele is also a risk factor in the CAG families, which possibly remained undetected due to insufficient power. Interestingly, only two of the NIMH families found to be associated with LBP-1c/CP2/LSF here also show association with the intron 18 deletion in AD2 published earlier by our group using the same NIMH dataset,11 if association is assumed when at least two risk alleles are transmitted (as indicated by the Viewstat function in FBAT; see Methods) to affected individuals for each polymorphism. This suggests that these two genes, which are located ~40 Mb apart on chromosome 12, likely function as independent risk factors.

In addition to the findings with the previously reported 3' UTR SNP, we also detected evidence of association with a nearby second SNP (rs10876135) in the NIMH families. It is interesting that the minor allele (that is, T) of this SNP was undertransmitted to affected individuals, reminiscent of the transmission pattern observed in all but one previous case-control study for the 3' UTR SNP. Overtransmitted alleles for both SNPs were located on a rare haplotype (H4), which was associated with risk for AD in the NIMH sample. A similar overtransmission was found in the CAG sample, although this did not reach statistical significance, possibly due to low power (that is, there were only 17 informative transmissions in both SNPs in the NIMH sample). Taken together, our findings clearly support and extend the previously suggested role of the LBP-1c/CP2/LSF gene in AD pathogenesis.

Thus far, only one study has investigated the potential consequences of the 3’ UTR SNP on LBP-1c/CP2/LSF expression and protein function.12 These experiments were somewhat preliminary and revealed that the A allele—which the same authors found to be underrepresented (that is, protective) in their AD cases as compared to controls—had a lower affinity to nuclear proteins in neuroblastoma cells using electrophoresis mobility shift assays, potentially suggesting a decreased function of this nuclear transcription factor. However, when LBP-1c/CP2/LSF mRNA expression was compared in lymphocytes from AD cases and controls, the A allele carriers showed no detectable differences. In fact, only the affected G/G carriers showed an overall reduced expression of this gene in cases v controls. However, the numbers in these latter experiments were very low (only three A allele carriers in each group) and were only performed in peripheral blood cells. Thus, no inference can be currently drawn from these data regarding any pathophysiological consequences in the brain.

Despite these promising findings, there remains discrepancy with respect to the direction of the potential effect uncovered by the genetic analyses, where two studies now show a risk effect, while three studies favour a protective role for the A allele. There are at least three conceivable scenarios to explain the observed discrepancies across studies. The first possibility is that LBP-1c/CP2/LSF is not an AD susceptibility gene, and the previously published reports constitute false-positive findings published on the basis of publication bias. While currently the possibility of publication bias cannot be assessed reliably due to the limited number of studies, this scenario appears unlikely as it is typically only an issue for the first, and usually positive, publication.13 In AD and other genetically complex diseases, the majority of promising initial findings are followed by a number of non-confirmative studies, which—at least in the past—have mostly outweighed the positive results. However and as mentioned earlier, LBP-1c/CP2/LSF has thus far been quite remarkable in that only positive studies have been published following the initial report, our study being no exception. A second explanation for the discrepant findings is that the families driving the association in our analyses (that is, NIMH) were
ascertained based on the presence of at least two AD cases in first degree relatives of the same pedigree, while all previous samples did not specifically consider family history. This could potentially lead to the sampling of genetically distinct populations, that is, samples that are governed by different genetic risk factors and risk alleles. However, the observation that at least one other investigation (by Panza et al) also described an over-representation of the A allele in their AD populations, while in the sample by Panza et al and our study it has arisen coupled with the minor allele. In the former samples the A allele would thus appear as protective (since the actual risk allele is actually in LD with the G allele at the 3′ UTR SNP), while in the latter cases it would appear as a risk factor. On the other hand, there could be several independent and rare disease modifying variants within the LBP-1c, LRP1, tumour necrosis factor α (TNFA), and butyrylcholinesterase K (BChE-K) UTR SNPs. If they do not merely represent a collection of varying false-positive findings, these differences could be attributed to the different patterns of LD across populations of different origin and/or differing degrees of population heterogeneity. While we favour this last alternative as the most likely explanation for the observed differences with the 3′ UTR SNP, clearly more studies need to be performed on the potential association of this and possibly other polymorphisms in LBP-1c/CP2/LSF and AD.

In conclusion, we provide additional and independent evidence suggesting that genetic variants in LBP-1c/CP2/LSF significantly alter the risk for developing AD. More studies will need to be performed to further establish this association, and to more definitively assess which variant(s) are actually responsible for the observed effects and how they affect disease pathogenesis.

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In figure 3 the marker D21S1400 should be D21S1440.

On page 413 in the second paragraph, the marker D21S1446 should be D21S1446.