Iraqi-Jewish kindreds with optic atrophy plus (3-methylglutaconic aciduria type 3) demonstrate linkage disequilibrium with the CTG repeat in the 3′ untranslated region of the myotonic dystrophy protein kinase gene

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Iraqi-Jewish optic atrophy plus is an autosomal recessive condition characterized by infantile optic atrophy, an early onset movement disorder, and 3-methylglutaconic aciduria. Other features include spastic paraplegia, mild ataxia, mild cognitive deficiency and dysarthria. This disorder was identified in inbred Iraqi-Jewish kindreds in which relationships between most of the affected individuals were unknown. In this study we identify linkage to chromosome 19q13.2–q13.3 by using a DNA pooling strategy to perform a genome wide screen followed by a high density search for shared segments among affected individuals in candidate regions identified in the initial genome wide screen. A significantly high positive lod score of 6.14 at zero recombination was obtained for the CTG repeat in the 3′ untranslated region of the myotonic dystrophy protein kinase gene. The existence of multiple recombinant individuals indicates the disease interval can be further narrowed with additional markers. Linkage disequilibrium was seen in six polymorphic markers across a 1 Mb interval. This region is well characterized and contains several candidate genes.

INTRODUCTION

Several syndromes with optic atrophy as a component of the phenotype have been identified (1–4). This appears to be a heterogeneous condition in which X-linked, autosomal dominant, and recessive forms are documented (5–8). One autosomal dominant form, OPA1, has been mapped to chromosome 3q (9,10). In this study we report the mapping of an autosomal recessive form of optic atrophy identified in Iraqi-Jewish kindreds (11). This syndrome, Iraqi-Jewish optic atrophy plus (OMIM#258501), also known as 3-methylglutaconic (3-MGC) aciduria type 3, is characterized by infantile bilateral optic atrophy and 3-methylglutaconic aciduria. An early onset choreiform movement disorder is seen in most cases. Mild ataxia is seen in half the cases and a mild cognitive deficit in a few. All of the above characteristics are of early onset, most likely congenital, and non-progressive. Most patients develop non-progressive spastic paraparesis by the second decade of life (12).

The 3-MGC aciduria seen in these patients is suggestive of an inborn error of metabolism. 3-MGC-CoA is an intermediate of leucine degradation which would suggest an enzymatic deficiency in this pathway. Several disorders associated with 3-MGC aciduria have been identified (13). 3-MGC aciduria type 1 has been associated with a deficiency in 3-MG-CoA hydratase activity, which is involved in the conversion of 3-MG-CoA to 3-HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) (14). However, cultured fibroblasts from Iraqi-Jewish patients have normal 3-MG-CoA hydratase activity (12). In addition, 3-MGC aciduria type 1 is clinically distinct from 3-MGC aciduria type 3 in both phenotype and degree of 3-MGC aciduria (patients with type 1 excrete ∼10-fold more 3-MGC than patients with type 3) (13). In the absence of a strong candidate gene we undertook a genome wide screen to search for linkage.

Eleven patients from four families were initially evaluated for the purpose of linkage analysis. Three of the families were nuclear, and one family was an extended kindred with consanguineous marriages. Two additional nuclear families with members affected with the same condition were ascertained later.

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in the study. All six families were of Iraqi-Jewish descent. The patients were born in Israel, though most of their parents originated from Baghdad. All affected individuals were assumed to descend from a common founder due to the relative cultural isolation of the Iraqi-Jewish population, the presence of consanguineous marriages, and the rarity of the disorder. Based upon the assumption of identity by descent at the disease locus (IBD), a DNA pooling strategy was used to perform a genome wide screen to search for regions of homozygosity in affected individuals. This was followed by a shared segment analysis to confirm linkage among the candidate regions identified in the primary genome wide screen.

RESULTS

Clinical findings

A total of 41 patients of Iraqi-Jewish origin are known (23 female, 18 male), of which 36 have been clinically evaluated. Early onset bilateral optic atrophy was present in all cases associated with prolonged visual evoked potential (VEP) latencies. Electroretinograms (ERG) were normal in all patients. Most of the patients had an early-onset choreiform movement disorder. Twenty seven of the patients had developed a non-progressive spastic paraparesis between the ages of 6 and 16. About half of the patients had a mild non-progressive ataxia. Half of the patients had dysarthria, although not always the same patients that had ataxia. All patients had excessive excretion of 3-methylglutaconic acid and 3-methylglutaric acid in their urine, although activity of 3-methylglutaconyl-CoA hydratase measured in cultured skin fibroblasts was normal. Additionally, urinary creatinine levels were usually higher in patients than in age-matched controls (12).

Homozygosity mapping using pooled DNA samples

In order to screen for markers linked to the disease, we performed a genome wide screen on a subset of affected individuals for which DNA samples were available by using a DNA pooling...
strategy (15–17). This was followed by shared segment analysis (18–19) to rapidly confirm linkage to markers identified in the DNA pooling screen as candidates. The DNA pooling strategy relies on the assumption of IBD (that affected individuals will share a chromosomal region inherited from a common ancestor). Equimolar amounts of DNA from 11 affected individuals from families A, B, C and D (Fig. 1a and b) were combined into a single pool. A second pool was made from DNA from 13 unaffected siblings and parents. The pools were used as templates for PCR amplification with short tandem repeat polymorphisms (STRPs) developed by the Cooperative Human Linkage Center (CHLC) (20). A modified version of screening set 6.0 in which all dinucleotide markers in the set were replaced with a nearby trinucleotide STRP was used.

The two pools were screened with >300 STRPs. PCR products were analyzed on denaturing polyacrylamide gels and visualized by silver staining. The banding patterns between affected and unaffected pools were visually compared. A pool of genomic DNA from affected individuals will show a reduction in the number of alleles towards homozygosity for markers at the disease locus when compared with a pool of unaffected samples, whereas unlinked markers will show nearly identical allelic banding patterns between the two pools. The initial genome wide screen identified a total of 14 markers that appeared to show a shift toward homozygosity.

**Shared segment analysis**

In order to rapidly identify linkage among the candidate markers we searched for shared segments in these regions between affected individuals. Two flanking markers were chosen for each of the 14 candidate markers. These markers were used to genotype 10 of the 11 affected individuals included in the DNA pool. One of the candidate regions identified in the DNA pooling screen showed allele sharing among affected individuals. The positive from the DNA pooling screen in this region was GATA29B01 on chromosome 19. Eleven of 20 chromosomes shared an allele at the flanking marker, A TA28H02. Another marker, GATA84G04, showed evidence of allele sharing among affected individuals from the largest kindred. The other 13 candidate regions demonstrated some extent of allele sharing in the marker used in the genome wide screen, but were inconsistent with linkage when flanking markers were analyzed.

Based upon the above evidence, additional markers in the region between GATA84G04 and ATA28H02 were analyzed (Fig. 2). For D19S908, a dinucleotide marker 6 cM distal to GATA84G04, 20 of 20 chromosomes shared the same allele. Data obtained with other markers in this region were also consistent with linkage.

**Statistical analysis**

In order to confirm linkage, all available members from the four kindreds A, B, C and D were genotyped with markers in the region between GATA84G04 and ATA28H02, and two-point lod score analysis was performed (Table 1). A peak lod score of 6.14 at \( \theta = 0 \) was obtained for the CTG repeat in the 3′ untranslated region (3′UTR) of the myotonic dystrophy kinase gene (DMK). This marker was informative in all matings, and all patients were homozygous for the disease allele.

**Table 1.** Pairwise linkage data between optic atrophy plus and most closely linked chromosome 19 markers for families A, B, C and D

<table>
<thead>
<tr>
<th>Marker</th>
<th>Recombination fraction</th>
</tr>
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<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>D19S918</td>
<td>4.83</td>
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<td>D19S908</td>
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<td>ERRC2C06</td>
<td>4.16</td>
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<tr>
<td>ERC1C02A</td>
<td>3.88</td>
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<tr>
<td>DMK CTG</td>
<td>6.14</td>
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Table 2. Genotypes of all 15 affected members subdivided into their respective families are shown

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<tr>
<th>Marker</th>
<th>V1</th>
<th>V4</th>
<th>V5</th>
<th>V7</th>
<th>V8</th>
<th>I1</th>
<th>I2</th>
<th>I3</th>
<th>I4</th>
<th>I5</th>
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</thead>
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<tr>
<td>D19S220</td>
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<td>41</td>
<td>11</td>
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<td>16</td>
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<td>25</td>
<td>23</td>
<td>23</td>
<td>-</td>
<td>25</td>
<td>45</td>
</tr>
</tbody>
</table>

The families are listed in order of decreasing region of allele sharing, which is outlined.

Table 3. Primers obtained from chromosome 19 genomic sequences for fine mapping purposes

<table>
<thead>
<tr>
<th>Marker</th>
<th>Forward</th>
<th>Reverse</th>
<th>Size</th>
<th># of Alleles in Iraqi-Jewish Population</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>XRC1CA1</td>
<td>cetaggtcacccagtaagtc</td>
<td>tctggtgggccagtaact</td>
<td>159</td>
<td>6</td>
<td>Cosmids f5050, Acc #L34079, bps 36120-36279</td>
</tr>
<tr>
<td>ERR1CA2</td>
<td>ttaaggtgagagccacat</td>
<td>agaagctcagaggggttg</td>
<td>215</td>
<td>7</td>
<td>Cosmids f0080, Acc #M98651, bps 16601-16816</td>
</tr>
<tr>
<td>ERR2CA6</td>
<td>tgtgaactagctgtggcag</td>
<td>ttagaagctacgagtaacc</td>
<td>244</td>
<td>4</td>
<td>Cosmids f25251, f19186, Acc #L47234, bps 20073-20317</td>
</tr>
<tr>
<td>ERR1C4TA</td>
<td>tatactgctagctgtgc</td>
<td>tcttggtgggccagtaact</td>
<td>184</td>
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<td>Cosmids f5123, Acc #M67396, bps 30801-30985</td>
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<tr>
<td>ERR1CA3</td>
<td>gacgctagggagacatc</td>
<td>ttatagcggctagggca</td>
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<td>1</td>
<td>Cosmids f0080, Acc #M98651, bps 24442-24609</td>
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<tr>
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<td>caactgtaataagctgag</td>
<td>aactgtaataagctgag</td>
<td>226</td>
<td>1</td>
<td>Cosmids f25251, f19186, Acc #L47234, bps 46685-45911</td>
</tr>
</tbody>
</table>

The genomic sequences were generated by Lawrence Livermore National Labs, the cosmid clone number is given as well as the GenBank accession number and the base pair number of the 5’ end.

**Linkage disequilibrium**

Additional CA markers were developed from genomic sequences generated by Lawrence Livermore National Laboratory (LLNL) (Fig. 2). The sequences were accessed through the LLNL web page, which links to GenBank at the National Center for Biotechnology Information (NCBI). Repeat sequences were found simply by text word searches using the ‘find’ command. Unique primer sequences flanking the repeat were selected. Three of the six markers identified in this manner were informative (Table 2), of which two were linked with no recombinant individuals.

DNA samples from two additional nuclear families (families E and F), were genotyped with markers across the region (Table 3). All affected individuals are homozygous for an identical haplotype with six markers across a 1 Mb region. None of the unaffected siblings or parents are homozygous for this haplotype. The narrowest region, as defined by allele sharing as well as observed and historical recombination events, is flanked by D19S412 and D19S918, a genetic distance of 1 cM. Family E has the smallest region of allele sharing, being the only family with affected individuals not homozygous for allele 1 at D19S918. The absence of allele sharing between families at D19S412 suggests that the disease interval can be further narrowed with additional markers.

**Analysis of muscle type creatine kinase**

The gene for muscle type creatine kinase maps within the disease interval (Fig. 2) (21,22). This gene was considered a good candidate based upon the higher than normal levels of creatinine in the urine. Primers were selected from genomic sequence data (accession nos M21487-M21494). Near full coverage (98%) of exon sequence was obtained, the only exceptions being primers which partially overlapped with coding regions and/or splice sites. PCR fragments were analyzed for mutations by a single
stranded conformational polymorphism (SSCP) assay. However, no variants were found in the Iraqi-Jewish population. Products from affected individuals were sequenced in order to confirm the SSCP results. Sequences from affected individuals and obligate carriers matched the published sequence.

DISCUSSION

We have linked Iraqi-Jewish optic atrophy plus to chromosome 19q13.2–13.3 by using a DNA pooling approach to perform a genome-wide screen followed by a shared segment analysis. Significantly positive lod scores were obtained with several markers. The (CTG)n repeat in the 3'UTR of the DMK gene gave a lod score of 6.14 at zero recombination. Further support for linkage is demonstrated by the extensive allele sharing between affected individuals from multiple kindreds with six STRPs in a 1 Mb interval. The fact that all affected individuals share a common haplotype support our original assumption of identity by descent at the disease locus. This assumption was originally made based upon the known consanguineous marriages, the relative cultural isolation seen in the Iraqi-Jewish population, and the rarity of the disease in the general population.

Optic atrophy plus is distinguished from other optic atrophy syndromes by the clear autosomal recessive pattern of inheritance and an early onset movement disorder. OPA1 is clinically distinct from optic atrophy plus in that OPA1 has no neurologic abnormalities. OPA1 is linked to chromosome 3q28–29, and is the only other autosomal optic atrophy that has been mapped. Optic atrophy plus was originally thought to be a form of Behr's syndrome, a similar disorder with compatible neurological involvement (23). Ataxia, which is an essential element of Behr's syndrome, is seen only in half of the Iraqi-Jewish patients, and 3-methylglutaconic aciduria was not found in patients with Behr's syndrome (13). However, the possibility that Behr's syndrome and optic atrophy plus are allelic has not been excluded.

The DNA pooling method has been used to efficiently link several disease loci (15,16,24–27). For example, in one study there was a single striking positive marker out of >300 markers screened (27). However, in this study 14 positive markers were identified in the DNA pooling screen. In order to find linkage among the positive markers, a high density secondary screen for allele sharing among affected individuals was performed. When individuals were genotyped with the 14 positive markers there was a predominance of a single allele for each marker, which supports the DNA pooling data. However, only one of the candidate regions demonstrated allele sharing in flanking markers, the other 13 candidate regions did not show allele sharing among affected individuals in the flanking markers. This study demonstrates that the DNA pooling strategy can be used to detect areas of potential linkage even in distantly related individuals. Candidate areas identified by DNA pooling can then be rapidly screened for allele sharing.

The fact that the families used in this study are distantly related allowed us to define a small disease region of ~1 cM. The disease region is defined by multiple recombinant individuals and extensive allele sharing. Due to the number of recombinant individuals both observed and historical, and the lack of allele sharing in D19S412, this region can be further narrowed with additional markers. Family E provides us with the smallest disease interval, putatively because members of this family are only distantly related to the original founder. Additional families would potentially allow us to narrow the interval further either by recombination events or allele sharing.

The chromosome 19 sequence data, and physical map generated by LLNL has been a valuable resource (28). The sequence data allowed the generation of three polymorphic markers, two of which have shared alleles in all affected individuals. The physical map allowed us to order several markers which otherwise would have been difficult to order genetically. The physical map also allowed us to identify the following candidate genes: muscle-type creatine kinase, myotonic dystrophy protein kinase (DMK), and phosphoprotein phosphatase 5C (PPPS5). The initial biochemical candidate for this disorder, 3-HMG-CoA hydratase, was not pursued due to the fact that this enzyme has normal activity in fibroblast cell lines from patients. Two other enzymes, HMG-CoA lyase, which catalyzes the final step in leucine degradation, and HMG-CoA reductase (HMGR), which is involved in cholesterol biosynthesis are both excluded due to the map position of these genes, 1pter–p33 and 5q13.3–q14 respectively (29,30).

The creatine kinase gene was chosen as a candidate based upon excessive urinary excretion of creatinine. This enzyme catalyzes the reversible reaction of the addition of a phosphoryl group to creatine to form phosphocreatine. We postulated that a defect in this enzyme could lead to the higher creatine levels, and therefore higher creatinine levels and excretion (creatinine undergoes non-enzymatic rearrangement to form creatinine). We also postulated that the atrophy of the optic nerve could be due to a toxic effect from the abnormally high creatinine levels. However, no sequence variants were found in the coding sequence of this gene. Additionally, the activity of creatine kinase was found to be normal in an affected patient (Costeff, personal communication).

Other candidate genes include myotonic dystrophy protein kinase (DMPK), and PPP5C. All myotonic dystrophy patients described thus far have a repeat expansion. There have been no point mutations found in this gene. It is possible that a point mutation in this gene would cause a different phenotype than the repeat expansion. For example, an expansion of the CAG repeat in the androgen receptor gene causes spinal and bulbar muscular atrophy while point mutations in this gene cause testicular feminization (31,32).

Another interesting candidate that maps to the disease region is the PPP5C gene. Family members of this gene, phosphoprotein phosphatase-1 and phosphoprotein phosphatase-2, activate HMGR. HMGR catalyzes the conversion of HMG-CoA to mevalonate, the rate limiting step in cholesterol biosynthesis. A phosphorylated HMGR is less active and therefore may lead to an accumulation of 3-HMG-CoA, which in turn may push the equilibrium of HMG-CoA hydratase reaction toward 3-MGC. Both candidate genes are currently being investigated.

MATERIALS AND METHODS

Patient evaluation

All patients received a thorough neurological, psychological and ophthalmological examination. Additional examinations such as computed tomography scan, magnetic resonance imaging scan,
ERG, and evoked visual potentials were also performed. Affection status was determined prior to genotyping.

**Genome wide screen with pooled DNA samples**

DNA was prepared from blood samples by a standard non-organic protocol (33). DNA concentrations were determined by spectrophotometric readings at OD_{260}. Samples were diluted to 100 ng/μl based on initial readings, and a second reading was taken to confirm the concentration. DNA from 11 affected individuals was pooled and the pool was then diluted to a final concentration of 20 ng/μl. A second pool of 12 unaffected siblings and parents was prepared in the same manner. Each DNA sample included in the pools and the pools themselves were PCR amplified with several STRPs to ensure equal amplification between samples, and to test that the pools reflected the allele frequencies of those samples included in the pools. Amplification of STRP markers was performed with 40 ng pooled DNA in an 8.3 μl PCR reaction mixture containing, 1.25 ml PCR buffer (100 mM Tris–HCl pH 8.8, 500 mM KCl, 15 mM MgCl₂, 0.01% w/v gelatin), 200 μM each dATP, dCTP, dGTP and dTTP, 2.5 pmol of each forward and reverse primer and 0.25 U Taq polymerase.

Markers were multiplexed when possible. Reaction mixtures were subjected to 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. Products were analyzed on denaturing (7.7 M urea) polyacrylamide gels (6%). The polyacrylamide gels were visualized by silver staining (34). Genetic markers and maps used in this study were developed by the Cooperative Human Linkage Center (CHLC). CHLC STRP screening set version 6.0 modified by the addition of tri- and tetranucleotide STRPs to replace dinucleotide repeat markers was used in the genome wide screen.

**Shared segment analysis and genotyping**

Markers that were either positive from the genome wide screen and markers that flanked the positives were used for shared segment analysis. Genotyping was then performed on individual samples from all families using available CHLC and Genethon markers. Genotyping was performed using the same PCR reaction mixture as above.

**Genetic fine mapping**

New markers in the linked region were generated from public sequence data generated by LLNL. This data is available through a band selection page <http://www-bio.llnl.gov/bbrp/seq19.html>. Ultimately this page links to GenBank at the NCBI. Cosmid sequences were downloaded and the text was searched for STRPs by using the find command. Primers flanking the repeat were selected manually, and purchased from Research Genetics.

**Statistical analysis**

Lod scores analysis (35) was performed on four separate families (A, B, C and D) using the MLINK routine of the LINKAGE package of programs (36). The disease was assumed to be autosomal recessive with full penetrance. Allele frequencies were assumed to be equal for each marker.

**Candidate genes analysis**

Primers were selected from genomic sequence to amplify 150–250 bp. DNA samples from affected individuals, obligate heterozygotes, and unrelated unaffected people were PCR amplified using the conditions stated above. Amplified fragments were heat denatured at 94 °C and electrophoresed at room temperature on a non-denaturing gel 6% polyacrylamide:bis, 2.5% glycerol and 0.25% TBE in 0.25% TBE running buffer at 20 W as previously described (37). DNA was visualized by silver staining. PCR fragments were sequenced using Dye terminators (Applied Biosystems Inc.) following the manufacturer’s instructions.

**ACKNOWLEDGEMENTS**

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