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L-2-Hydroxyglutaric aciduria: identification of a mutant gene *C14orf160*, localized on chromosome 14q22.1

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L-2-Hydroxyglutaric aciduria (L-2-HGA) is characterized by progressive deterioration of central nervous system function including epilepsy and macrocephaly in 50% of cases, and elevated levels of L-2-hydroxyglutaric acid in urine, blood and cerebrospinal fluid (CSF). Nuclear magnetic resonance imaging shows distinct abnormalities. We report the identification of a gene for L-2-HGA aciduria (MIM 236792) using homozygosity mapping. Nine homozygous mutations including three missense mutations, two nonsense mutations, two splice site mutations and two deletions were identified in the gene *C14orf160*, localized on chromosome 14q22.1, in 21 patients from one non-consanguineous and 14 consanguineous Turkish families. We propose to name the gene *duranin*. *Duranin* encodes a putative mitochondrial protein with homology to FAD-dependent oxidoreductases. The functional role of this enzyme in intermediary metabolism in humans remains to be established.

INTRODUCTION

L-2-Hydroxyglutaric aciduria (L-2-HGA) is a rare autosomal recessive disorder (MIM 236792), which belongs to the group of organic acidurias (1,2). Since the first description of the disease in 1980 (3), ~75 patients have been reported from different parts of the world (4–16). Clinically, the disease is characterized by mild psychomotor delay in the first years of life, followed by progressive cerebellar ataxia, dysarthria and moderate to severe mental deterioration (3–16). Pyramidal and extrapyramidal signs, seizures, macrocephaly and stunting may be additional findings. Episodes of acute metabolic decompensation do not occur and brain damage is not related to acidosis or other metabolic imbalance, as in other forms of organic acidurias (1,2,17).

Magnetic resonance imaging (MRI) characteristically shows lesions in the subcortical white matter, with cerebellar atrophy and abnormal signals in the dentate nuclei and putamens. The centripetal extension of the white matter involvement constitutes a distinct feature that differentiates this disorder from other leukodystrophies (4–16,18,19). MR spectroscopy shows a decrease in the neuronal marker *N*-acetylaspartate (NAA) and in choline, and an increase in the *myo*-inositol peaks (18). The increase of *myo*-inositol, which is a precursor of phosphatidylinositol, suggests an inhibition of phosphatidylinositol synthesis, whereas the reduction of choline may reflect a progressive stage of neurodegeneration (18).

The diagnosis is confirmed by the finding of an excess of L-2-hydroxyglutaric acid in urine, blood and cerebrospinal fluid (CSF) by gas chromatography and mass spectrometry, with 90% of isoforms representing the L-form (20–24). L-2-Hydroxyglutaric acid is increased >10-fold in urine (800–1300 mmol/mol creatinine) compared with normal levels in control subjects of under 52 mmol/mol creatinine (3–16,20–22). The plasma levels are 4–6-fold higher than

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in normal controls, and >10 times higher in CSF (3–16, 20–22). Concentrations of glycolate, glycerate, pyruvate, laurate, 2,4-hydroxybutyrate, citrate, isocitrate, palmitate, myristate, oleate and stearate have also been reported to be increased, albeit moderately (2-fold in plasma and/or in CSF) (3–16,21). Some patients show increased levels of L-lysine and pipecolate in CSF (7,11,14,24,25), suggesting a defect in the alternative degradation pathway of lysine. By analysis of Krebs cycle intermediates (succinate, 2-ketoglutarate and citrate) in culture medium from cells of two patients with L-2-HGA, concentrations of 2-ketoglutarate were found to be increased relative to controls (26). A NAD^+-dependent L-2-hydroxyglutaric acid dehydrogenase is present in human liver, but normal activity of this enzyme in patients excluded a pathogenic role for this protein (27). The precise role and relevance of L-2-hydroxyglutaric acid in normal intermediary metabolism in human beings and the underlying enzymatic defect in L-2-HGA has remained obscure so far.

Using a genome-wide scan of five consanguineous families, each with two affected children, we localized a gene for L-2-HGA on chromosome 14q22.1. This localization was found in a total of 18 families. To refine the initial interval of 5.4 Mb, we analyzed 21 microsatellite markers from public databases, and developed 20 new microsatellites (Fig. 1, Table 1), and further reduced the region by haplotype analysis to 1.07 Mb. Five candidate genes were excluded by sequencing, before we identified mutations in a new gene, *C14orf160*, that we propose to name *duranin* in honor of Dr Marinus Duran, who first described the disorder in 1980 (3). *Duranin* encodes a putative mitochondrial protein with homology to FAD-dependent oxidoreductases. The exact catalytic activity of this enzyme and its role in intermediary metabolism remain to be established.

RESULTS

Patient origins; clinical and biochemical features

All 18 families were from rural areas of Turkey in Central and South-East Anatolia. Consanguinity was known in 17 families (Table 2). A total of 89 individuals including 28 affected subjects (15 males and 13 females) were analyzed. The mean age at the time of diagnosis was 13.4 years (2.5–32 years). The main clinical findings were mental retardation and symptoms related to cerebellar involvement such as ataxic gait and intentional tremor. IQ levels were between 50 and 75 in all patients, and before the age of 10, short attention span and hyperactivity were observed. Additional findings were macrocephaly, febrile or afebrile and partial or generalized seizures, which were well controlled with anticonvulsants. EEG findings included irregular background activity and slowing, focal or generalized spikes and slow wave discharges. MRI findings were suggestive of a spongiform encephalopathy; myelin breakdown was predominantly subcortical rather than periventricular, and there was centripetal extension of white matter involvement. On follow-up examinations, cerebellar involvement became more prominent around the age of 12 years. There was no marked deterioration after the age of 14. The patients all followed a static encephalopathy course. Urinary excretion of L-2-hydroxyglutaric acid was increased in all patients ranging from 1000 to 5520 mmol/mol creatinine (N: 1.3–19 mmol/mol creatinine). A more complete description of clinical and biochemical characteristics of these patients will be published elsewhere (28).

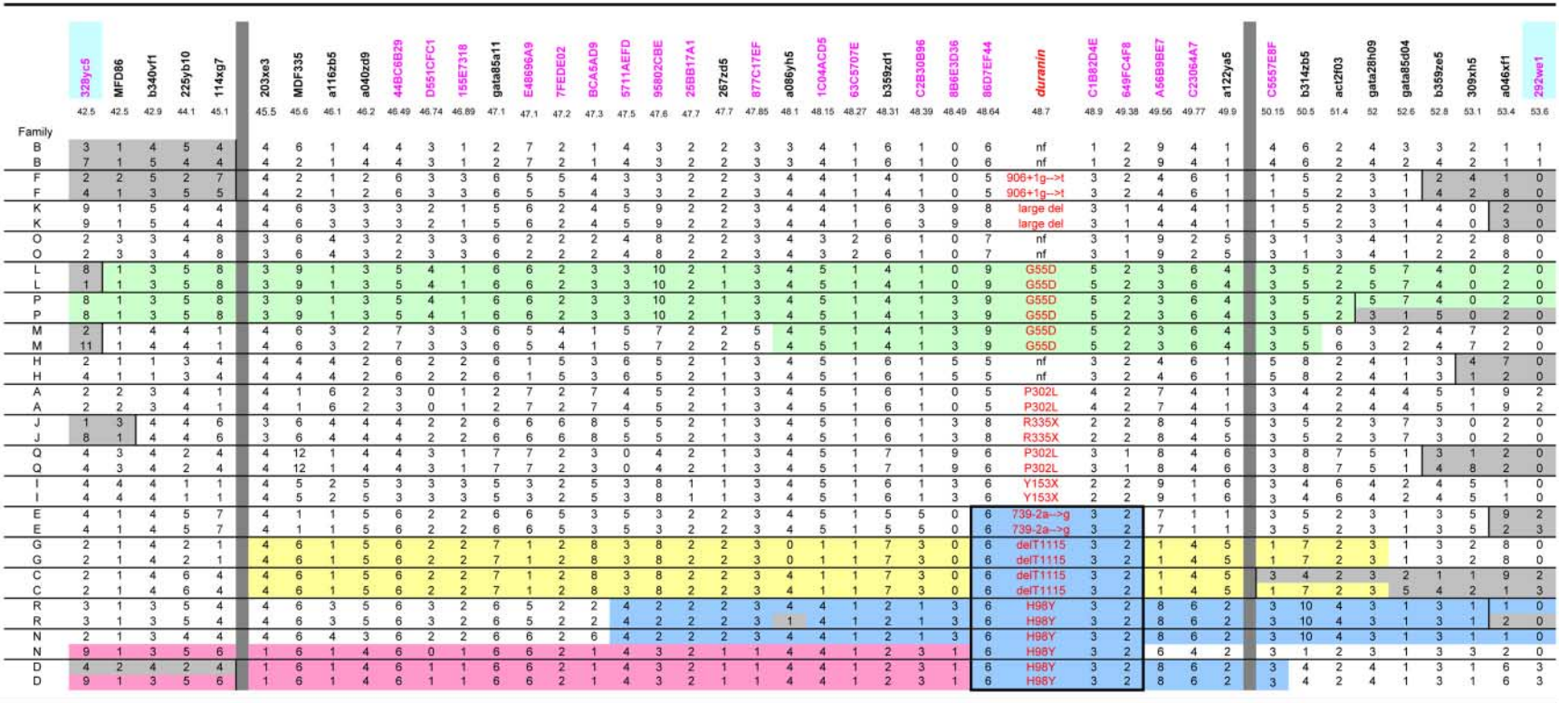
Linkage, linkage disequilibrium and haplotype analysis

A genome-wide scan of five consanguineous families (A–E), each with two affected individuals, revealed eight potential homozygous regions, which were tested with additional microsatellite markers. Linkage was confirmed in all these families for a homozygous region between the markers D14S288 (AFM328yc5) and D14S276 (AFM292we1) used for the genome-wide scan (in pink on a blue background in Fig. 1). We then performed fine mapping in a total of 18 families using 20 microsatellites from public databases (marker names in black in Fig. 1). The initial interval of 5.4 Mb was defined by recombination events with loss of homozygosity (shaded in gray in Fig. 1) in families B, D and F for the marker AFM114xg7 (D14S1068), and in family C for the marker AFMb314zb5 (D14S1018). We developed 20 additional microsatellites within this interval (Table 1, marker names in pink in Fig. 1) for linkage disequilibrium and haplotype analysis. The maximum pairwise LOD score at $\Theta = 0.00$ for the marker 95802CBE was 15.06.

The disease-associated haplotype is shown for one patient per family from maternal and paternal chromosomes in Figure 1. Some families shared an extended common haplotype (yellow in C and G, green in L, P and M). A smaller common haplotype was observed in patients from most families between markers 95802CBE and AFMb359zd1 (D14S1031), but this haplotype was also present in non-affected individuals, and no linkage disequilibrium was detected. The non-consanguineous family N displayed a heterozygous haplotype for most microsatellites. The patient shared an identical haplotype on the paternal chromosome with family R (blue in Fig. 1) between the marker BCA5AD9 and AFMa046xf1 (D14S1057); the haplotype on the maternal chromosome was the same as in family D between AFM328yc5 (D14S288) and A56B9BE7. These three families (D, N and R) displayed the same alleles (6-3-2) for the markers 86D7EF44, C1B82D4E and 649FC4F8. This smallest co-segregating haplotype (6-3-2) of 1.07 Mb was also present in patients from families C, E and G, and partly present (6-2 or 3-2) in patients from three other families (F, H and Q). The complete haplotype 6-3-2 was never observed in non-transmitted chromosomes of unaffected individuals or controls. However, linkage disequilibrium using one marker at a time for the 6-3-2 haplotype entity was not statistically significant.

Exclusion of candidate genes and identification of mutations in a novel gene *C14orf160* (*duranin*)

The initial 5.4 Mb interval contains about 40 genes, out of which we sequenced five candidate genes (*ATP5S*, *GNG2*, *AK129564*, *C14orf138* and *TXNDC*). No mutations were found in their coding regions and exon–intron boundaries. Ten genes were known in the reduced 1.07 interval, but one of them, *C14orf160*, was particularly interesting because of functional similarities to the *aspartoacylase* gene, which has been found to be mutated in Canavan disease (29). Sequencing of the



	328yc5	MFD86	b340vf1	225yb10	114xg7	203xe3	MDF335	a116zb5	a040zd9	44BC6B29	D551CFC1	155E7318	gata85a11	E48696A9	7FEDE02	BCA5AD9	5711AEFD	95802CBE	25BB17A1	267zd5	877C17EF	a086yh5	1C04ACD5	63C5707E	b359zd1	C2B30B96	8B6E3D36	86D7EF44	*duranin*	C1B82D4E	649FC4F8	A56B9BE7	C230644A7	a122ya5	C5557E8F	b314zb5	act2f03	gata28h09	gata85d04	b359ze5	309xh5	a046xf1	292we1
	42.5	42.5	42.9	44.1	45.1	45.5	45.6	46.1	46.2	46.49	46.74	46.89	47.1	47.1	47.2	47.3	47.5	47.6	47.7	47.7	47.85	48.1	48.15	48.27	48.31	48.39	48.49	48.64	48.7	48.9	49.38	49.56	49.77	49.9	50.15	50.5	51.4	52	52.6	52.8	53.1	53.4	53.6
Family																																											
B	3	1	4	5	4	4	6	1	4	4	3	1	2	7	2	1	4	3	2	2	3	3	4	1	6	1	0	6	nf	1	2	9	4	1	4	6	2	4	3	3	2	1	1
B	7	1	5	4	4	4	2	1	4	4	3	1	2	7	2	1	4	3	2	2	3	3	4	1	6	1	0	6	nf	1	2	9	4	1	4	6	2	4	2	4	2	1	1
F	2	2	5	2	7	4	2	1	2	6	3	3	6	5	5	4	3	3	2	2	3	4	4	1	4	1	0	5	906+1g-->t	3	2	4	6	1	1	5	2	3	1	2	4	1	0
F	4	1	3	5	5	4	2	1	2	6	3	3	6	5	5	4	3	3	2	2	3	4	4	1	4	1	0	5	906+1g-->t	3	2	4	6	1	1	5	2	3	1	4	2	8	0
K	9	1	5	4	4	4	6	3	3	3	2	1	5	6	2	4	5	9	2	2	3	4	4	1	6	3	9	8	large del	3	1	4	4	1	1	5	2	3	1	4	0	2	0
K	9	1	5	4	4	4	6	3	3	3	2	1	5	6	2	4	5	9	2	2	3	4	4	1	6	3	9	8	large del	3	1	4	4	1	1	5	2	3	1	4	0	3	0
O	2	3	3	4	8	3	6	4	3	2	3	3	6	2	2	2	4	8	2	2	3	4	3	2	6	1	0	7	nf	3	1	9	2	5	3	1	3	4	1	2	2	8	0
O	2	3	3	4	8	3	6	4	3	2	3	3	6	2	2	2	4	8	2	2	3	4	3	2	6	1	0	7	nf	3	1	9	2	5	3	1	3	4	1	2	2	8	0
L	8	1	3	5	8	3	9	1	3	5	4	1	6	6	2	3	3	10	2	1	3	4	5	1	4	1	0	9	G55D	5	2	3	6	4	3	5	2	5	7	4	0	2	0
L	1	1	3	5	8	3	9	1	3	5	4	1	6	6	2	3	3	10	2	1	3	4	5	1	4	1	0	9	G55D	5	2	3	6	4	3	5	2	5	7	4	0	2	0
P	8	1	3	5	8	3	9	1	3	5	4	1	6	6	2	3	3	10	2	1	3	4	5	1	4	1	3	9	G55D	5	2	3	6	4	3	5	2	5	7	4	0	2	0
P	8	1	3	5	8	3	9	1	3	5	4	1	6	6	2	3	3	10	2	1	3	4	5	1	4	1	3	9	G55D	5	2	3	6	4	3	5	2	3	1	5	0	2	0
M	2	1	4	4	1	4	6	3	2	7	3	3	6	5	4	1	5	7	2	2	5	4	5	1	4	1	3	9	G55D	5	2	3	6	4	3	5	6	3	2	4	7	2	0
M	11	1	4	4	1	4	6	3	2	7	3	3	6	5	4	1	5	7	2	2	5	4	5	1	4	1	3	9	G55D	5	2	3	6	4	3	5	6	3	2	4	7	2	0
H	2	1	1	3	4	4	4	4	2	6	2	2	6	1	5	3	6	5	2	1	3	4	5	1	6	1	5	5	nf	3	2	4	6	1	5	8	2	4	1	3	4	7	0
H	4	1	1	3	4	4	4	4	2	6	2	2	6	1	5	3	6	5	2	1	3	4	5	1	6	1	5	5	nf	3	2	4	6	1	5	8	2	4	1	3	1	2	0
A	2	2	3	4	1	4	1	6	2	3	0	1	2	7	2	7	4	5	2	1	3	4	5	1	6	1	0	5	P302L	4	2	7	4	1	3	4	2	4	4	5	1	9	2
A	2	2	3	4	1	4	1	6	2	3	0	1	2	7	2	7	4	5	2	1	3	4	5	1	6	1	0	5	P302L	4	2	7	4	1	3	4	2	4	4	5	1	9	2
J	1	3	4	4	6	3	6	4	4	4	2	2	6	6	6	8	5	5	2	1	3	4	5	1	6	1	3	8	R335X	2	2	8	4	5	3	5	2	3	7	3	0	2	0
J	8	1	4	4	6	3	6	4	4	4	2	2	6	6	6	8	5	5	2	1	3	4	5	1	6	1	3	8	R335X	2	2	8	4	5	3	5	2	3	7	3	0	2	0
Q	4	3	4	2	4	4	12	1	4	4	3	1	7	7	2	3	0	4	2	1	3	4	5	1	7	1	9	6	P302L	3	1	8	4	6	3	8	7	5	1	3	1	2	0
Q	4	3	4	2	4	4	12	1	4	4	3	1	7	7	2	3	0	4	2	1	3	4	5	1	7	1	9	6	P302L	3	1	8	4	6	3	8	7	5	1	4	8	2	0
I	4	4	4	1	1	4	5	2	5	3	3	3	5	3	2	5	3	8	1	1	3	4	5	1	6	1	3	6	Y153X	2	2	9	1	6	3	4	6	4	2	4	5	1	0
I	4	4	4	1	1	4	5	2	5	3	3	3	5	3	2	5	3	8	1	1	3	4	5	1	6	1	3	6	Y153X	2	2	9	1	6	3	4	6	4	2	4	5	1	0
E	4	1	4	5	7	4	1	1	5	6	2	2	6	6	5	3	5	3	2	2	3	4	5	1	5	5	0	6	739-2a-->g	3	2	7	1	1	3	5	2	3	1	3	5	9	2
E	4	1	4	5	7	4	1	1	5	6	2	2	6	6	5	3	5	3	2	2	3	4	5	1	5	5	0	6	739-2a-->g	3	2	7	1	1	3	5	2	3	1	3	5	2	3
G	2	1	4	2	1	4	6	1	5	6	2	2	7	1	2	8	3	8	2	2	3	0	1	1	7	3	0	6	delT1115	3	2	1	4	5	1	7	2	3	1	3	2	8	0
G	2	1	4	2	1	4	6	1	5	6	2	2	7	1	2	8	3	8	2	2	3	0	1	1	7	3	0	6	delT1115	3	2	1	4	5	1	7	2	3	1	3	2	8	0
C	2	1	4	6	4	4	6	1	5	6	2	2	7	1	2	8	3	8	2	2	3	4	1	1	7	3	0	6	delT1115	3	2	1	4	5	3	4	2	3	2	1	1	9	2
C	2	1	4	6	4	4	6	1	5	6	2	2	7	1	2	8	3	8	2	2	3	4	1	1	7	3	0	6	delT1115	3	2	1	4	5	1	7	2	3	5	4	2	1	3
R	3	1	3	5	4	4	6	3	5	6	3	2	6	5	2	2	4	2	2	2	3	4	4	1	2	1	3	6	H98Y	3	2	8	6	2	3	10	4	3	1	3	1	1	0
R	3	1	3	5	4	4	6	3	5	6	3	2	6	5	2	2	4	2	2	2	3	1	4	1	2	1	3	6	H98Y	3	2	8	6	2	3	10	4	3	1	3	1	2	0
N	2	1	3	4	4	4	6	4	3	6	2	2	6	6	2	6	4	2	2	2	3	4	4	1	2	1	3	6	H98Y	3	2	8	6	2	3	10	4	3	1	3	1	1	0
N	9	1	3	5	6	1	6	1	4	6	0	1	6	6	2	1	4	3	2	1	1	4	4	1	2	3	1	6	H98Y	3	2	6	4	2	3	1	2	3	1	3	3	2	0
D	4	2	4	2	4	1	6	1	4	6	1	1	6	6	2	1	4	3	2	1	1	4	4	1	2	3	1	6	H98Y	3	2	8	6	2	3	4	2	4	1	3	1	6	3
D	9	1	3	5	6	1	6	1	4	6	1	1	6	6	2	1	4	3	2	1	1	4	4	1	2	3	1	6	H98Y	3	2	8	6	2	3	4	2	4	1	3	1	6	3

Figure 1. Patients' haplotypes. Loss of homozygosity is indicated in gray. The initial interval of 5.4 Mb is marked by gray columns. Microsatellites are indicated in the first row: flanking markers in pink on blue background are from the genome-wide scan, those in black are from public databases; in pink newly developed markers. The names of the new microsatellites came form the corresponding BAC sequences. Positions are in megabases from Golden Path UCSC (July 2003). The smallest segregating interval (6-3-2) between markers 8B6E3D36 and A56B9BE7 is shaded in blue and boxed. The allele number is indicated as 0 when no genotyping result is available. Paternal and maternal alleles for one affected child per family are presented; nf indicates mutation not found.

Table 1. Primer sequences for *C14orf160* (*duranin*) exon amplification, RT–PCR and surrounding microsatellite markers

Name	Forward sequences	Reverse sequences	PCR-conditions	Length of amplicon (bp)
Exon amplification				
1	CTAAAGAGCGCGGGTCCT	GAAATACGAACAGGGGCACA	III	306
2	GCCGAATACGTTTTCATGATT	CTTGTCCCACACTCTGCTTTT	I	371
3	GGCAGAATTTTACATGATGTGG	GGCGAATTGTATGGTGCAT	I	483
4	CAACTTTTCTTGGTGGAGGAAG	CCCTCTTGTGTCTGTCACTTCA	I	467
5	GCTTGGCAAAATCAACTCCT	CCGAATGCAATGTAAATGCT	II	467
6	TCAGAATGAACTGTAAGGTGCAA	AATACAGCCCTGTGGGTTAATTT	I	248
7	GCTGCCACCTAAATGCTAAAG	GCTTACAAAAACGGTCCTTCA	I	459
8	CCAGCATCAGGGTTTCTTGT	CTACCCCATCCACTCCAAAG	I	510
9	CACTCCTGGCCTTGATTTTCT	CTGCAAAGGGAATTCAACAAC	I	448
10_BC006117	GGAGTGGGGAGGCATACTT	GATCCAAGCCCAATTAAAATGT	I	428
10_AK022680	GGCTACAACTGACAGCCTTTG	ATTGATTCTCCTGCCTCAGC	I	592
RT–PCR				
RT_1_7	GGGTCAAGTGGCTTCTTCTG	TGGTACAATTCGAGGATCAGG	IV	890
RT_4_9	ATGAGAAAGGCCTCCAGAATG	CACTGTTGCACCAAGAAAACA	IV	689
Genotyping of newly developed microsatellites				Size range
44BC6B29	GGTTTGTTATTTTGTTGAGGAT	CTCCCTAACAAATTCTGCAC		145–175
D551CFC1	TATCTGCCATACCAGACCTT	TCATTCCTGCTTAAGATACCA		340–365
155E7318	ATGTCCTGTAGCCAAATTGT	TGTTGGGGAGGATAGGTAG		180–205
E48696A9	AGTTTTCTATCAGTGCTGCAA	TCCATAACATCTCTCTGTCTCC		155–185
7FEDE02	TCTCTTAGCAGATTGCCTGT	TTTATCCAGATTCCCCAAA		255–285
BCA5AD9	CATGACAATATAACAGTGGACAG	ATGTAACACCCATGGTATTTAAG		105–135
5711AEFD	CTTCAAGAGGAAAGGTTTAATG	ATACCTGGAATGAAAACGTAAG		310–340
95802CBE	CCAGACTACCTTCACACCAC	TAGTAGGTGGCAGAGCCTTA		200–235
25BB17A1	TTAATTCTGGGGTTTTTGG	TCACATCTGTGGAATGAAGA		165–185
877C17EF	TCAGAGCTGAACTTAAGGAAAT	AAGATCTGATGTCTGGTTTGTT		85–110
1C04ACD5	ACTGATTAGGGTGGTTGTTG	TGGTGCACACCTGTAATTC		260–285
63C5707E	CTCTCCCTTCAATTCTGTCA	ATTATTTGAGGCCAGGAGTT		295–315
C2B30B96	GCATCTGTAGTCCCAGCTAC	ACAGAGAGGTTAAGAAACTTGC		200–220
8B6E3D36	TTTCAAGGTAGCCCTGACT	CCTGATTGAGATTGCTGTG		145–180
86D7EF44	CCTGTCTCGACCAAAAATAC	TATTTCCCACCTTCAACAAC		300–330
C1B82D4E	CTGAACTATGACTGCACTACTGA	TTTCAGGAAATGTGTGTTTCTA		200–235
649FC4F8	ACAACCAAGAATCAATAGCAGT	AGACAGAGATTGCAGTGAGC		135–160
A56B9BE7	CCAGCATATGGGATATTTTACT	GGACTTCAATAACTCCACTATCA		270–300
C23064A7	AAAGTAGTGCCATGCTTGATA	GTTGGGTTATAGGTGATGTTTT		90–120
C5557E8F	CTGAATAAAAATCGACAATGTG	GATTAGCCTTCCCTTCACTTA		290–315

PCR conditions—I: TM 60°C using Hot Master *Taq* (Eppendorf); II: TM 59°C using Hot Master *Taq* (Eppendorf); III: TM 56°C using Advantage GC genomic Polymerase Mix (Clontech) and IV: TM 60°C using Titanium *Taq* DNA Polymerase (Clontech). Two couples of primers have been designed for exon 10, derived from two different cDNA variants. AK022680 and BC006117. PCR conditions for genotyping are described in Fischer *et al.* (34).

coding regions and exon–intron boundaries of *C14orf160* revealed nine different homozygous mutations in 15 of the 18 affected families. We propose to name this gene *duranin*.

Structure of the *duranin* gene and its cDNA

Two cDNAs of different length have been described for *C14orf160* (FLJ12618). Both are described as full-length human cDNAs (30,31). The reference sequence NM_024884 (2064 bp) was derived from AK022680, and codes for a protein of 463 amino acids (BAB14174). A second human sequence, BC006117 (1404 bp), codes for a protein of only 441 amino acids (AAH06117), which is identical with respect to the first 399 amino acids, but differs from AK022680 at the 3′ end from amino acid 400 to the stop codon (amino acid 441) by alternate splicing and use of an alternative last exon. BLAST analysis between the mRNA (AK022680) and the genomic sequence supported the existence of 10 exons as described in other public databases such as Ensembl (http://www.ensembl.org/) and Golden Path (UCSC; http://genome.ucsc.edu). We checked the mRNA by overlapping RT–PCRs, sequenced the products and compared them with the sequences from public databases.

The mouse and rat orthologs are both described to have 10 exons also. The full-length mouse cDNA (NM145443, BC016226) is 3289 bp long and codes for a protein of 464 amino acids (NP663418, AAH16226), whereas the rat mRNA is 1515 bp long (XM234264) and codes for 504 amino acids. BLAST analysis of orthologs from human, mouse and rat revealed strong homologies of 86% for the nucleotide sequence, and of 77% (mouse) and 69% (rat) for the protein sequence. Multiple nucleotide alignments (http://prodes.toulouse.inra.fr/multalin/) of orthologs from human, mouse and rat showed also a highly conserved coding sequence with a homology of ~85%.

Table 2. Mutations and consanguinity of 18 Turkish families

Family number	Number of patients	Consanguinity degree	Mutation	Effect	Exon
A	2	First	905C→T	P302L	7
B	2	First	—	—	
C	2	First	delT1115	Stop codon	9
D	2	First	292C→T	H98Y	3
E	2	First	739-2 a→g	Splice site ag	7
F	2	Second	906+1 g→t	Splice site gt	7
G	1	First	delT1115	Stop codon	9
H	2	First	—	—	
I	1	First	459T→A	Y153X	4
J	1	First	1003C→T	R335X	8
K	1	Third	Large deletion		Including exons 1–9
L	1	First	164G→A	G55D	2
M	1	First	164G→A	G55D	2
N	1	No	292C→T	H98Y	3
O	2	First	—	—	
P	2	First	164G→A	G55D	2
Q	1	Second	905C→T	P302L	7
R	1	First	292C→T	H98Y	3

Mutation analysis of the *duranin* gene

Sequencing of the coding regions and exon–intron boundaries of *C14orf160* including the alternate last exon revealed nine different homozygous mutations in 15 families. There were three missense mutations, two nonsense mutations, two splice site mutations, one small deletion and one large deletion of at least several hundred kilobases (Table 1, Figs 1 and 2). Some of these mutations were present in more than one family: the 164G→A (G55D) mutation in exon 2 is shared by three families, L, M and P; the 292C→T (H98Y) mutation in exon 3 is the same in three families, D, N and R; the 905C→T (P302L) mutation in exon 7 is common to two families, A and Q; the mutation 459T→A (Y153X) in exon 4 is present in family I; the mutation 1003C→T (R335X) is in exon 8 in family J; families C and G have a single base deletion of T at position 1115, which leads to a premature stop codon in the protein. Two different splice site mutations were identified in exon 7, 739-2a→g and 906 + 1g→t, in families E and F, respectively. The large deletion in family K was at least several hundred kilobases, with an absence of genomic amplification of exons 1–9, but normal amplification of exon 10 and of all the surrounding microsatellites. None of these sequence variations was found in 100 normal chromosomes from a Mediterranean control population. No mutations were found in families B, H and O, but successful amplification of all exons, including the alternative form of exon 10, excludes a large deletion in this gene.

Expression analysis

We analyzed tissue expression using *C14orf160*-specific RT–PCR with primer pairs RT_1_7 and RT_4_9 (Table 1) for two fragments of 890 and 689 bp, respectively. We used RNAs from brain, bladder, testis, small intestine, liver, muscle, bone marrow, lymphocytes, fibroblasts, keratinocytes and placenta (data not shown). The transcripts were found to be expressed in all tissues tested; strong expression was found in brain (++++), and in testis and muscle (+++). Expression was weaker in lymphocytes, fibroblasts, keratinocytes and placenta (++), and in bladder, small intestine, liver and bone marrow (+).

Characterization of the protein and consequence of mutations

The predicted protein of 463 amino acids has a calculated molecular weight of 50.3 kDa and a pI of 8.45, and the variant of 441 amino acids, a molecular weight of 48.2 kDa and a pI of 8.99. They were submitted to: (1) topology and domain prediction programs for sequence and targeting signals, and transmembrane domains which are available through the ExPASy server (WWW.expasy.org); (2) protein analysis tools from the Biology WorkBench (http://biowb.sdsc.edu/); and (3) homology searches through protein databases (http://www.ncbi.nlm.nih.gov/). A mitochondrial targeting peptide is predicted by iPSORT, TargetP and MitoProtII (0.79–0.90 probability of export to mitochondria), but only weakly predicted by PSORTII. Starting at amino acid position 50 and ending at amino acid position 422, a domain (PFAM: DAO, KOG2665) was identified, which defines a family of various FAD-dependent oxidoreductases, known to be located in mitochondria or peroxisomes. A weaker homology (1/10 of the former) was found with the deaminating domain (COG0665) of glycine/D-amino acid oxidases (between amino acids 61 and 381).

The deletion and nonsense mutations in the *duranin* gene have obvious consequences for the synthesis of the protein; all other mutations we found in patients with L-2-HGA are situated in parts of the sequence which is conserved between the two variants of the protein (463 versus 441 amino acids) and which are also conserved between different species (*C. elegans*, drosophila, *Arabidopsis thalania*, mouse, rat and human).

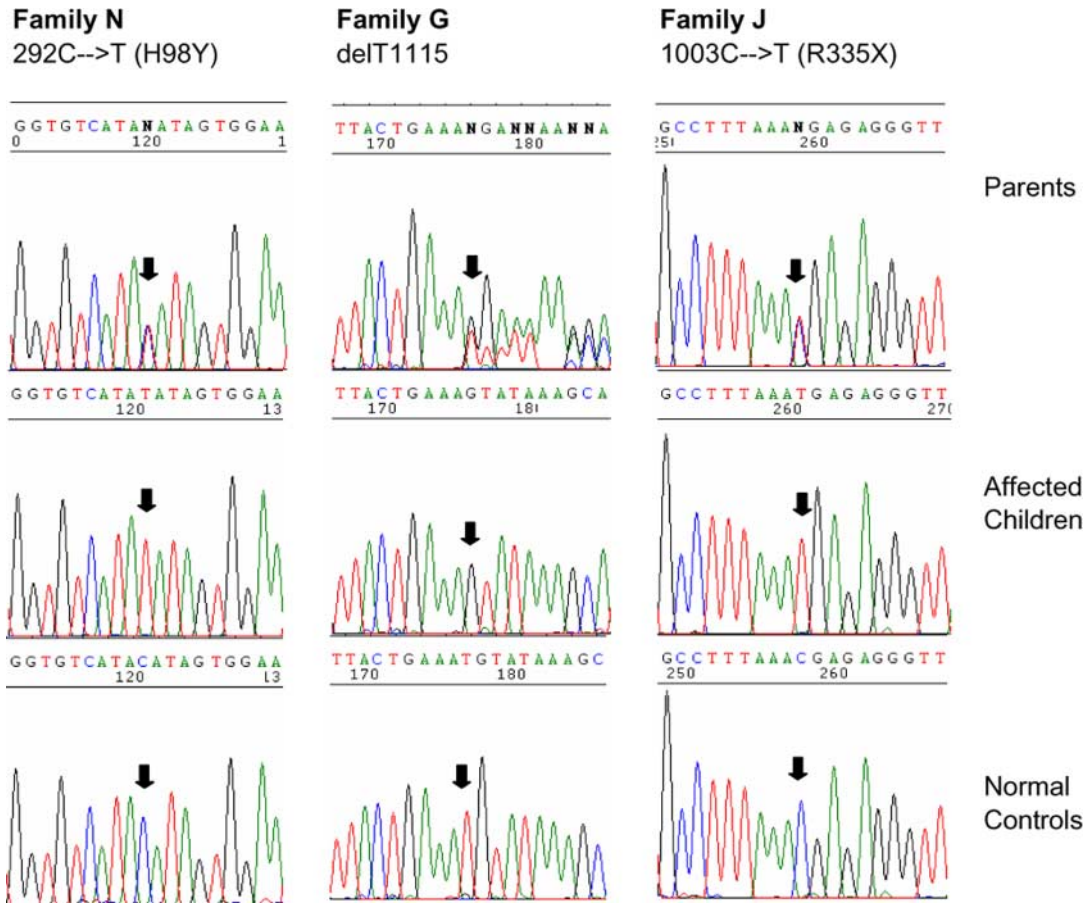


Figure 2. Extracts of sequences from a missense mutation (292C→T; H98Y), a deletion (delT1115) and a nonsense mutation (1003C→T; R335X) from families N, G and J, respectively, are shown, including one parent (top) and one affected patient (middle) from each family. The normal control is shown at the bottom.

DISCUSSION

The principal difficulties encountered in gene discovery in rare disorders include a limited number of patients and families, often with diverse ancestral origins, and sometimes a poorly defined phenotype, which leads to the impression of clinical and genetic heterogeneity in the disorder. Although less than one hundred patients with L-2-HGA have been described in the literature, we had the opportunity to study 28 patients from 18 mostly consanguineous families from the same country. Furthermore, these patients were analyzed clinically and biochemically, and DNA was collected.

The clinical and radiologic features of L-2-HGA fit a chronic progressive neurodegenerative disease with progressive ataxia and developmental delay. Signs of cerebellar dysfunction and macrocephaly are common. MRI findings are suggestive of a spongiform encephalopathy similar to that found in Canavan disease, with white matter involvement more pronounced in the subcortical region than in the periventricular area. This differentiates L-2-HGA from other leukodystrophies. L-2-HGA and Canavan disease also cause symmetric hyperintensity in the dentate nuclei in addition to the predominantly subcortical cerebral white matter abnormality. However, Canavan disease involves the brain stem, whereas L-2-HGA does not (9).

With the help of these well-defined clinical and biochemical criteria, homozygosity mapping, as expected, was a powerful method to localize, and then to identify the implicated gene in our cohort of patients. Mutations were found in 21 patients from 15 families in a new gene named *duranin.* Haplotype and mutation analyses of these families, all from Turkey, revealed nine different mutations which exclude a founder effect, and a Mediterranean origin of the disorder, as has been suggested previously (15). The presence of so many cases in Turkey may simply indicate an effective diagnostic and reporting network. This suggest that there are probably many unrecognized cases world-wide. Some families shared a common haplotype and exhibited the same mutation; these probably do have a common ancestor. Mutations were not found in three families even though the clinical and biochemical aspects of the disorder were the same as in the other patients. Normal sequences of the 10 exons, and exon–intron boundaries exclude complete or partial deletions of the gene in these patients, but mutations such as exon skipping or creation of additional splicing sites in introns cannot be ruled out. Other possibilities include clinical and/or biochemical misdiagnosis and genetic heterogeneity of L-2-HGA. Analysis of cDNAs from these patients will be helpful in further characterization of their disorders.

The identification of mutations in a new gene in the group of organic acidurias extends the list of proteins involved in mitochondrial function and biogenesis encoded by the nuclear genome, as well as that of neurological disorders due to mitochondrial dysfunction. Among the presumed 700–1000 mitochondrial proteins, only 40–60% are estimated to have been identified to date (32).

Despite the first description of L-2-HGA already in 1980, little is known about the source(s) of L-2-hydroxyglutaric acid as well as its breakdown. In 1993, a L-2-hydroxyglutaric acid dehydrogenase was shown to convert L-2-hydroxyglutaric acid into 2-ketoglutaric acid with NAD as a co-factor (33). The enzyme was found to be localized in the cytosol with expression only in the liver. There was no activity in brain, and normal activity of this enzyme was found in two established L-2-HGA patients (27). Interestingly, *duranin* has high homology with other oxidoreductases using either NAD or FAD as hydrogen acceptor. Expression analysis, however, has shown that *duranin* is expressed in multiple tissues, notably brain (++++), with low expression in the liver (+). These data and the presumed mitochondrial rather than cytosolic localization of *duranin* suggests that there are two different enzymes. Future studies will be needed to reveal the exact catalytic function of *duranin.* There could be a failure to catabolize L-2-HGA due to the defective enzyme or accumulation of an unknown intermediate, leading to excess conversion of this substance to L-2-HGA.

This requires expression of the cDNA followed by enzymatic studies to identify whether NAD or FAD is the co-factor and whether L-2-hydroxyglutaric acid or a derivative thereof, such as L-2-hydroxyglutaryl-CoA, is the true substrate.

MATERIALS AND METHODS

Subjects and samples

Three neurologists (T.C., M.T., C.Y.) recorded the clinical data and pedigree information from the families. Laboratory tests were performed in the biochemical screening laboratory in Hacettepe University of Ankara and in the laboratory for Genetic Metabolic Diseases in the University Hospital Amsterdam. Blood samples were collected after obtaining written informed consent from each participating family member and/or from the parents of affected children. The DNA was extracted and stored in the Tübitak DNA and Cell Bank at Hacettepe University using standard procedures.

Genetic analysis and linkage disequilibrium

Genotyping was carried out using 400 highly polymorphic microsatellite markers from the ABI panel (Linkage Mapping Set2, LMS2, Applied Biosystems) and a MegaBase capillary sequencer for the genome-wide scan. Genotyping for fine mapping with publicly available and newly developed microsatellite markers was performed on ABI 377 sequencers as described elsewhere (34). Haplotypes were constructed assuming the most parsimonious linkage phase. Linkage programs were used on the basis of the assumption of autosomal recessive inheritance, full penetrance and a disease frequency of 1/100 000 in the general population. Pairwise LOD scores were calculated with the MLINK program of the LINKAGE 5.1 package (35) incorporating consanguineous loops into the pedigree files. For linkage disequilibrium analysis, the excess of the disease-associated alleles was calculated as previously described (36).

Mutation screening

Mutation analysis was performed in 18 families in affected patients, non-affected sibs and both parents. We designed intronic oligonucleotide primers flanking the exons for amplification and sequencing of the *C14orf160* gene (Table 1) using the Primer3 program (http://www.genome.wi.mit.edu/genome_software/other/primer3.html) (37). Sets of PCR conditions were used as indicated in Table 1. The touch-down PCR reaction was performed in a 45 μl volume containing 50 ng of genomic DNA (in 5 μl) with Hot Master™ *Taq* DNA polymerase (Eppendorf): initial denaturation step at 95°C for 5 min, six cycles of amplification consisting of 40 s at 94°C and 30 s at 68°C, and a 30 s elongation step at 72°C, followed by 30 cycles of 40 s at 94°C, 30 s at optimal annealing temperature and 30 s at 72°C, and a 5 min terminal elongation step. An aliquot of 1–2 μl of purified PCR products were added to 0.5 μl of sense or antisense primer (20 μM) and 2 μl of BigDye terminator mix (Applied Biosystems) in a 15 μl volume. The linear amplification consisted of an initial 5 min denaturation step at 96°C, 25 cycles of 10 s of denaturation at 96°C and a 4 min annealing/extension step at 56–60°C. The reaction products were purified and sequenced on an Applied Biosystems Sequencer 3700. The forward or reverse strands from all patients and controls were sequenced for the entire coding region and the exon/intron boundaries. The sequences were analyzed with the Phred Phrap program on Unix.

RT–PCR

We extracted total RNA or mRNA in our laboratory from cultures of keratinocytes, fibroblasts and lymphocytes. Total RNA was isolated using the QIAamp RNA Mini Protocol for isolation of total RNA from cultured cells (QIAGEN) following the manufacturer's instructions. The mRNA was isolated following the Oligotex direct mRNA protocol as provided by the manufacturer (QIAGEN).

For the other tissues (placenta, brain, bladder, testis, small intestine, liver, muscle and bone marrow), human total RNA or human polyA + RNA was obtained from Clontech.

RT–PCR was performed using the RT–PCR kit (Life Technologies) with oligo-dT primers to generate the first strand of cDNA. Amplification of cDNA from all these tissues was performed with two primer pairs (Table 1) covering the entire coding region, the 3′- and the 5′-UTR regions.

Accession numbers

C14orf160 (synonym FLJ12618): LocusID 79944; mRNA: NM_024884 (GI:13376330), AK022680 (GI:10434214), BC006117 (GI:13543954); protein: NP_079160 (GI:13376331), BAB14174 (GI:10434215), AAH06117 (GI:13543955).

Mus musculus: LocusID 217666; mRNA: NM_145443 (GI:21703883), BC016226 (GI:16740700); protein (NP_663418) (GI:21703884), AAH16226 (GI:16740701).
Rattus norvegicus: LocusID LOC314196; mRNA: XM_234264 (GI:34865683); protein: XP_234264 (GI:34865684).

Online Mendelian inheritance in man (http://www.ncbi.nlm.nih.gov/Omim)

L-2-hydroxyglutaric acidemia (MIM 236792); Canavan disease (MIM 217900); aspartoacylase (ASPA, MIM 608034).

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