Mutations in the very low-density lipoprotein receptor *VLDLR* cause cerebellar hypoplasia and quadrupedal locomotion in humans

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Quadrupedal gait in humans, also known as Unertan syndrome, is a rare phenotype associated with dysarthric speech, mental retardation, and varying degrees of cerebrocerebellar hypoplasia. Four large consanguineous kindreds from Turkey manifest this phenotype. In two families (A and D), shared homozygosity among affected relatives mapped the trait to a 1.3-Mb region of chromosome 9p24. This genomic region includes the *VLDLR* gene, which encodes the very low-density lipoprotein receptor, a component of the reelin signaling pathway involved in neuroblast migration in the cerebral cortex and cerebellum. Sequence analysis of *VLDLR* revealed nonsense mutation R257X in family A and single-nucleotide deletion c2339delT in family D. Both these mutations are predicted to lead to truncated proteins lacking transmembrane and signaling domains. In two other families (B and C), the phenotype is not linked to chromosome 9p. Our data indicate that mutations in *VLDLR* impair cerebrocerebellar function, conferring in these families a dramatic influence on gait, and that hereditary disorders associated with quadrupedal gait in humans are genetically heterogeneous.

genetics | Unertan syndrome

Obligatory bipedal locomotion and upright posture of modern humans are unique among living primates. Studies of fossil hominids have contributed significantly to modern understanding of the evolution of posture and locomotion (1–5), but little is known about the underlying molecular pathways for development of these traits. Evaluation of changes in brain activity during voluntary walking in normal subjects suggests that the cerebral cortices controlling motor functions, visual cortex, basal ganglia, and the cerebellum might be involved in bipedal locomotor activities (6). The cerebellum is particularly important for movement control and plays a critical role in balance and locomotion (7).

Neurodevelopmental disorders associated with cerebellar hypoplasias are rare and often accompanied by additional neuropathology. These clinical phenotypes vary from predominantly cerebellar syndromes to sensorimotor neuropathology, ophthalmological disturbances, involuntary movements, seizures, cognitive dysfunction, skeletal abnormalities, and cutaneous disorders, among others (8). Quadrupedal locomotion was first reported when Tan (9, 10) described a large consanguineous family exhibiting Unertan syndrome, an autosomal recessive neurodevelopmental condition with cerebellar and cortical hypoplasia accompanied by mental retardation, primitive and dysarthric speech, and, most notably, quadrupedal locomotion. Subsequent homozygosity mapping indicated that the phenotype of this family was linked to chromosome 17p (11). Thereafter, three additional families from Turkey (12–14) and another from Brazil (15) with similar phenotypes have been described, and video recordings illustrating the quadrupedal gait have been made (10–12). Here, we report that *VLDLR* is the gene responsible for the syndrome in two of these four Turkish families and report additional gene mapping studies that indicate the disorder to be highly genetically heterogeneous.

Fig. 1. Phenotypic (*A*) and cranial radiologic (*B*) presentation of quadrupedal gait in families A and D. (*A*) Affected brothers VI:20 and VI:18 and cousin VI:25 in family A (*Upper*) and the proband II:2 in family D (*Lower*) display palmigrate walking. This is different from quadrupedal knuckle-walking of the great apes (2). The hands make contact with the ground at the ulnar palm, and consequently this area is heavily callused as exemplified by VI:20. Strabismus was observed in all affected individuals. (*B*) Coronal and midsagittal MRI sections of VI:20, demonstrating vermial hypoplasia, with the inferior vermial portion being completely absent. Inferior cerebellar hypoplasia and a moderate simplification of the cerebral cortical gyri are noted. The brainstem and the pons are particularly small (*Left* and *Center*). Similar findings are observed for II:2 (*Right*).

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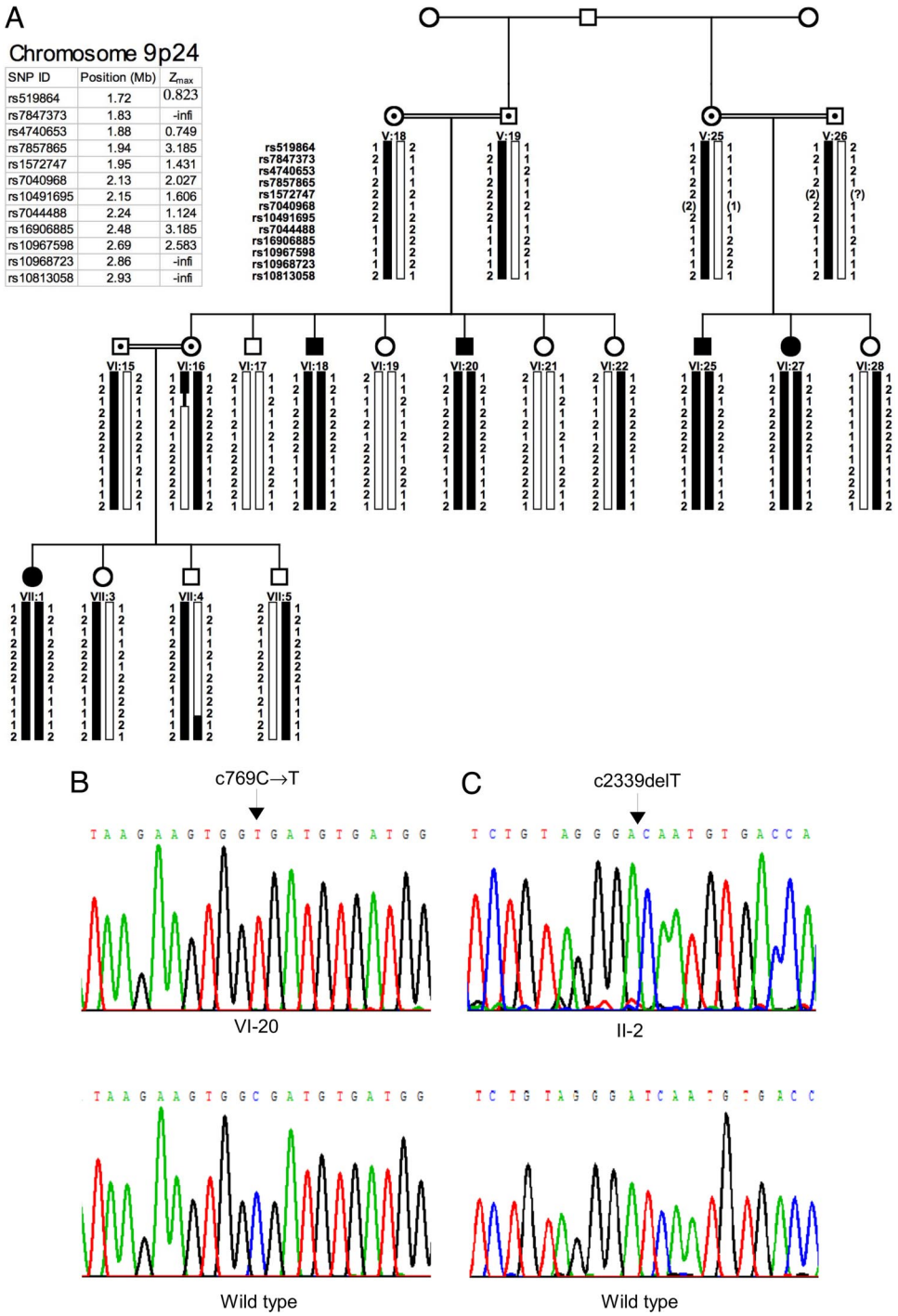

Fig. 2. Homozygosity mapping of cerebellar hypoplasia and quadrupedal locomotion to chromosome 9p24 (*A*) and identification of the *VLDLR* c769C → T mutation in family A (*B*) and of the *VLDLR* c2339delT mutation in family D (*C*). (*A*) Pedigree of family A; filled symbols represent the affected individuals. Squares indicate males, and circles indicate females. Black bars represent the haplotype coinherited with the quadrupedal phenotype in the family. Recombination events in individuals VI:16 (obligate carrier) and VII:4 (normal sibling) positioned the disease gene between markers rs7847373 and rs10968723. Physical positions and pairwise lod scores for each marker are shown on the upper left. Z_{max} represents the maximum lod score obtained at $\theta = 0.00$ cM. (*B* and *C*) Sequences of critical regions of *VLDLR* for wild-type and homozygous mutant genotypes.

Results

The proband of Family A (12) is a 37-year-old male with habitual quadrupedal gait (Fig. 1*A Upper Left* and Fig. 2*A*, VI:20). He did not make the transition to bipedality during his childhood despite the efforts of his healthy parents. He has dysarthric speech with a limited vocabulary, truncal ataxia, and profound mental retardation. He was not aware of place or of the year, month, or day. His MRI brain scan revealed inferior cerebellar and vermial hypoplasia, with the inferior vermial portion being completely absent. Whereas corpus callosum appeared normal, a moderate simplification of the cerebral cortical gyri accompanied by a particularly small brainstem and the pons was observed (Fig. 1 *B Left* and *Center*). Subsequently, we studied the proband's affected brother and cousin (Fig. 1*A Upper Center* and

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Table 1. Physical, radiological, and genetic characteristics of the Turkish families in this study and of Hutterite family DES-H (27)

Characteristic	Family A	Family B	Family C	Family D	DES-H
Chromosomal location	9p24	17p	Not 9p or 17p	9p24	9p24
Gene and mutation	*VLDLR* (c769C → T)	Unknown	Unknown	*VLDLR* (c2339delT)	Deletion including *VLDLR* and *LOC401491*
Gait	Quadrupedal	Quadrupedal	Quadrupedal	Quadrupedal	Bipedal
Speech	Dysarthric	Dysarthric	Dysarthric	Dysarthric	Dysarthric
Hypotonia	Absent	Absent	Absent	Absent	Present
Barany caloric nystagmus	Normal	Cvs defect	Pvs defect	Not done	Not done
Mental retardation	Profound	Severe to profound	Profound	Profound	Moderate to profound
Ambulation	Delayed	Delayed	Delayed	Delayed	Delayed
Truncal ataxia	Severe	Severe	Severe	Severe	Severe
Lower leg reflexes	Hyperactive	Hyperactive	Hyperactive	Hyperactive	Hyperactive
Upper extremity reflexes	Vivid	Vivid	Vivid	Vivid	Vivid
Tremor	Very rare	Mild	Present	Absent	Present
Pes-planus	Present	Present	Present	Present	Present
Seizures	Very rare	Rare	Rare	Absent	Observed in 40% of cases
Strabismus	Present	Present	Present	Present	Present
Inferior cerebellum	Hypoplasia	Hypoplasia	Mild hypoplasia	Hypoplasia	Hypoplasia
Inferior vermis	Absent	Absent	Normal	Absent	Absent
Cortical gyri	Mild simplification	Mild simplification	Mild simplification	Mild simplification	Mild simplification
Corpus callosum	Normal	Reduced	Normal	Normal	Normal

Cvs, central vestibular system; Pvs, peripheral vestibular system.

Upper Right and Fig. 2*A*, VI:18 and VI:25) and other branches of the family living in nearby villages in southeastern Turkey. All affected individuals were offspring of consanguineous marriages (Fig. 2*A*). With the exception of one female (VII:1), who was an occasional biped with ataxic gait, all affected persons in family A had quadrupedal locomotion.

The proband of family D (14) is a 38-year-old male (Fig. 1*A Lower Left* and *Center*). Like all other quadrupedal individuals in these families, he did not make the transition to bipedality during his early childhood. He is profoundly retarded and exhibits dysarthric speech along with truncal ataxia. His MRI brain scan images are consistent with moderate cerebral cortical simplification and inferior cerebellar and vermial hypoplasia (Fig. 1*B Right*). The 65-year-old aunt and 63-year-old uncle of the proband are both mentally retarded and continue to walk on their wrists and feet despite their advanced ages. The family is consanguineous; all relatives were raised in neighboring villages on the western tip of the Anatolian peninsula.

All patients in these four families had significant developmental delay noted in infancy (Table 1). They sat unsupported between 9 and 18 months, and began to crawl on hands and knees or feet. Whereas normal infants make the transition to bipedal walking in a short period, the affected individuals continued to move on their palms and feet and never walked upright. All patients had severe truncal ataxia affecting their walking patterns. They can stand from a sitting position and maintain the upright position with flexed hips and knees. However, they virtually never initiate bipedal walking on their own and instead ambulate efficiently in a quadrupedal fashion. All patients had hyperactive lower leg and vivid upper extremity reflexes. Normal tone and power were observed in motor examination. All affected persons were mentally retarded to the degree that consciousness of place, time, or other experience appeared to be absent. However, no autistic features were expressed. The affected individuals all had good interpersonal skills, were friendly and curious to visitors, and followed very simple questions and commands. Additional clinical information on families A and D is provided in supporting information (SI) Table 2.

To identify the chromosomal locale of the gene or genes responsible for this phenotype, we carried out genome-wide linkage analysis and homozygosity mapping in families A–C (see *Materials and Methods* below). Although the families lived in isolated villages 200–300 km apart and reported no ancestral relationship, the rarity of the quadrupedal gait in humans led us to expect a single locus shared by affected individuals in all families. Instead, the trait mapped to three different chromosomal locales. In family A, linkage analysis and homozygosity mapping positioned the critical gene on chromosome 9p24 between rs7847373 and rs10968723 in a 1.032-Mb region (Fig. 2*A* and SI Fig. 4). In family B, the trait mapped to chromosome 17p13, confirming a previous study (11). In family C, highly negative logarithm of odds (lod) scores were obtained for both chromosomes 9p24 and 17p13 (SI Figs. 5 and 6); gene mapping in this family is ongoing. In family D, polymorphic markers from the critical intervals of chromosomes 9p24 and 17p13 were genotyped, and homozygosity was detected with markers on 9p24. Together, these results indicate that the syndrome including quadrupedal gait, dysarthric speech, mental retardation, and cerebrocerebellar hypoplasia is genetically heterogeneous.

The chromosome 9p24 region linked to the trait in families A and D includes *VLDLR*, the very low-density lipoprotein receptor. We hypothesized that a gene involved in neural development, cell positioning in brain, and cerebellar maturation could be involved in the pathogenesis of quadrupedal gait. In addition, cerebellar hypoplasia with cerebral gyral simplification was shown to be associated with a genomic deletion that includes *VLDLR* (16). We therefore considered *VLDLR* (17) to be a prime positional candidate for our phenotype and sequenced the gene in genomic DNA from probands of the four families (SI Table 3). The *VLDLR* sequence of affected members of family A was homozygous for a nonsense mutation in exon 5 (c769C → T; R257X) (Fig. 2*B*). The *VLDLR* sequence of the proband of family D was homozygous for a single-nucleotide deletion in exon 17 resulting in a stop codon (c2339delT; I780TfsX3) (Fig. 2*C*). *VLDLR* sequences excluded the possibility of compound heterozygosity in families B and C (SI Fig. 7). In families A and D, homozygosity for the *VLDLR* mutations was perfectly coinherited with quadrupedal gait (SI Figs. 8 and 9). Both mutations were absent from 100 unaffected individuals who live in the same local areas of southeastern and western Turkey as families A and D (SI Fig. 10).

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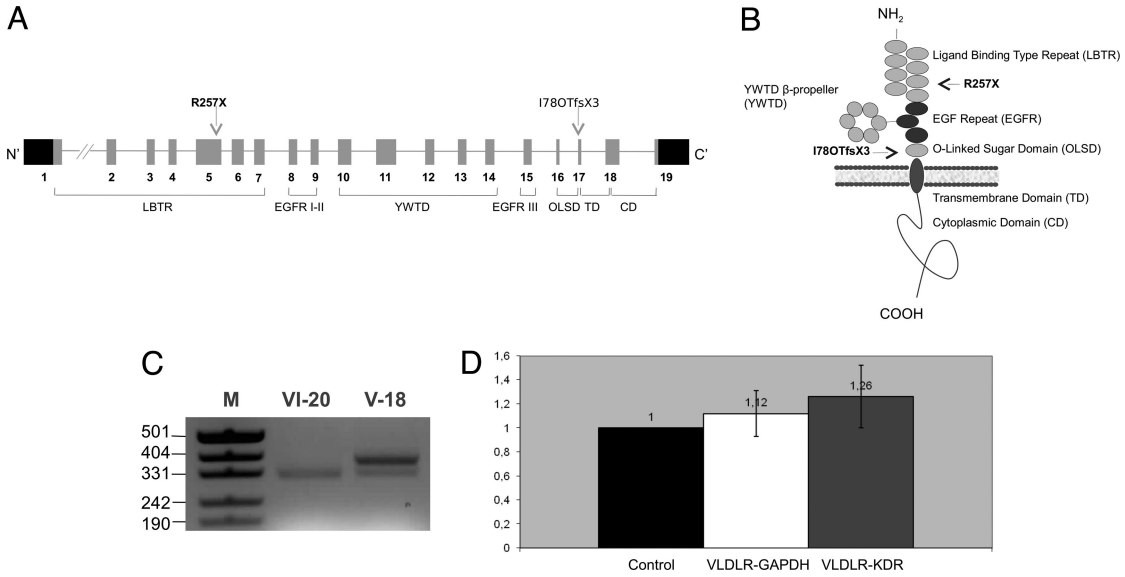

Fig. 3. Functional domains of *VLDLR* with positions of the mutations relative to the exons (*A*), domains (*B*), and the analysis of VLDLR transcript (*C* and *D*). (*A*) The gene consists of 19 exons. Arrows indicate the locations of the mutations. (*B*) VLDLR consists of ligand-binding type repeat (LBTR), epidermal growth factor repeat (EGFR) I–III, YWTD β-propeller (YWTD), *O*-linked sugar domain (OLSD), transmembrane domain (TD), and cytoplasmic domain (CD) (34) (www.expasy.org/uniprot/P98155). (*C*) Restriction-based analysis with HphI revealed the presence of only the mutant (347 bp) and both the mutant and wild type (396 and 347 bp; please note that the 49-bp fragment is not visible) VLDLR transcripts in patient VI:20 and carrier V:18 (both from family A), respectively. M is a DNA size marker. (*D*) Quantitative RT-PCR analysis of VLDLR transcript from peripheral blood samples of all probands in families A and D and controls was performed. Relative expression ratios were normalized according to the housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and the endothelial marker *KDR* (kinase insert domain receptor). ΔCt values were calculated from duplicate samples and were converted to linear scale (35). Control denotes ''VLDLR expression in controls,'' VLDLR-GAPDH denotes ''VLDLR expression in patients normalized to GAPDH,'' and finally VLDLR-KDR denotes ''VLDLR expression in patients normalized to KDR.''

VLDLR_R257X is in the ligand-binding domain, and *VLDLR_I780TfsX3* is in the *O*-linked sugar domain of the VLDLR protein (Fig. 3 *A* and *B*). Mutant VLDLR transcripts were expressed in endothelial cells from blood of affected individuals (Fig. 3*C*), and in these cells, levels of mutant and wild-type transcript expression appeared approximately equal (Fig. 3*D*; please also see *SI Text*). Because the stop codons of both mutations are located in the extracellular domain of VLDLR (Fig. 3*B*), the encoded mutant proteins could not be inserted into the membrane and could not function as receptors for reelin.

We propose *VLDLR*-associated Quadrupedal Locomotion (VLDLR-QL) or Unertan Syndrome Type 1 to describe the phenotype of families A and D.

Discussion

The identification of these *VLDLR* mutations provides molecular insight into the pathogenesis of neurodevelopmental movement disorders and expands the scope of diseases caused by mutations in components of the reelin pathway (18). Reelin is a secreted glycoprotein that regulates neuronal positioning in cortical brain structures and the migration of neurons along the radial glial fiber network by binding to lipoprotein receptors VLDLR and APOER2 and the adapter protein DAB1 (19). In the cerebellum, reelin regulates Purkinje cell alignment (20), which is necessary for the formation of a well defined cortical plate through which postmitotic granual cells migrate to form the internal granular layer (21). Homozygous mutations in the reelin gene (*RELN*) cause the Norman–Roberts type lissencephaly syndrome, associated with severe abnormalities of the cerebellum, hippocampus, and brainstem (OMIM 257320) (22). Mutation of *Reln* in the mouse results in the *reeler* phenotype and disrupts neuronal migration in several brain regions and gives rise to functional deficits such as ataxic gait and trembling (23). In contrast, mice deficient for *Vldlr* appear neurologically normal (24), but the cerebellae of these mice are small, with reduced foliation and heterotopic Purkinje cells (17).

In humans, homozygosity for either of two *VLDLR* truncating mutations leads to cerebrocerebellar hypoplasia, specifically vermial hypoplasia, accompanied by mental retardation, dysarthric speech, and quadrupedal gait. In the Hutterite population of North America, homozygosity for a 199-kb deletion encompassing the *VLDLR* gene leads to a form of Disequilibrium Syndrome (DES-H, OMIM 224050), characterized by nonprogressive cerebellar hypoplasia with moderate-to-profound mental retardation, cerebral gyral simplification, truncal ataxia, and delayed ambulation (16). The designation Disequilibrium Syndrome was originally given to cerebral palsy characterized by a variety of congenital abnormalities, including mental retardation, disturbed equilibrium, severely retarded motor development, muscular hypotonia, and perceptual abnormalities (25, 26). Neither DES-H nor other disequilibrium syndromes have been reported to include quadrupedal gait. The movement of most DES-H patients was so severely affected that independent walking was not possible. Those who could walk had a wide-based, nontandem gait (27).

The neurological phenotypes in the Turkish families and in the Hutterite families appear similar, with the most striking difference being the consistent adoption of efficient quadrupedal locomotion by the affected Turkish individuals (Table 1). In our view, the movement disorder described for the Hutterite patients may be a more profound deficit, with the patients perhaps lacking the motor skills for quadrupedal locomotion. The 199-kb deletion in DES-H encompasses the entire *VLDLR* gene and part of a hypothetical gene. *LOC401491*, the hypothetical gene, is an apparently noncoding RNA that shares a CpG island and likely promoter with *VLDLR*, and is represented by multiple alternative transcripts expressed in brain. It has been suggested that the DES-H phenotype could be the result of deletion of *VLDLR* or both *VLDLR* and the neighboring gene (16).

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It has been suggested that in the Turkish families, lack of access to proper medical care exacerbated the effects of cerebellar hypoplasia, leading to quadrupedality. Although it may be true that family B lacked proper medical care, families A and D had consistent access to medical attention, and both families actively sought a correction of quadrupedal locomotion in their affected children. An unaffected individual in family A is a physician who was actively involved in the medical interventions. In family D, the proband's mother sought a definitive diagnosis and correction of the proband's quadrupedal locomotion from private medical practices and from two major academic medical centers. The parents in family A discouraged quadrupedal walking of their affected children, but without success. We conclude that social factors were highly unlikely to contribute to the quadrupedal locomotion of the affected individuals.

In conclusion, we suggest that *VLDLR*-deficiency in the brain at a key stage of development leads to abnormal formation of the neural structures that are critical for gait. Given the heterogeneity of causes of quadrupedal gait, identification of the genes in families B and C promises to offer insights into neurodevelopmental mechanisms mediating gait in humans.

Materials and Methods

Study Subjects. Parents of patients and other unaffected individuals gave consent to the study by signing the informed consent forms prepared according to the guidelines of the Ministry of Health in Turkey. The Ethics Committees of Baskent and Cukurova Universities approved the study (decision KA07/47, 02.04.2007 and 21/3, 08.11.2005, respectively).

Genome-Wide Linkage Analysis. Linkage analysis was performed by SNP genotyping with the commercial release of the GeneChip 250K (NspI digest) or 10K Affymetrix arrays as described (28). In addition, genotype data were analyzed by hand to identify regions of homozygosity. The parametric component of the Merlin package v1.01 was used for the multipoint linkage analysis assuming autosomal recessive mode of inheritance with full penetrance (29, 30). The analysis was carried out along a grid of locations equally spaced at 1 cM. Haplotype analysis was performed on chromosomal regions with positive lod scores (Fig. 2*A* and SI Figs. 4–6). Pairwise linkage was analyzed by using the MLINK component of the LINKAGE program (FASTLINK, version 3) (31–33). Markers D17S1298 (3.51 Mb) and D9S1779 (0.4 Mb), D9S1871 (3.7 Mb) were used to test for homozygosity to chromosomes 17p13 and 9p24, respectively.

Mutation Search. Sequencing primers were designed for each *VLDLR* exon by using Primer3, BLAST, and the sequence of NC_000009. DNA from all of the probands was sequenced in both directions by using standard methods. The mutations in exons 5 (c769C → T) and 17 (c2339delT) were detected in all affected (homozygous) and carrier (heterozygous) individuals of families A and D, respectively. The c769C → T mutation creates a restriction site for the enzyme HphI (5′-GGTGA(N)8 ↓ 3′), and the c2339delT mutation abolishes a restriction site for the enzyme MboI (5′-G↓ ATC-3′). Assays using these restriction enzymes were developed to test for the mutations in all four families and in 200 healthy controls from the Turkish population. Restriction based mutation and quantitative RT-PCR analyses of *VLDLR* transcript in patients and controls was also performed (please see *SI Text* relating to Fig. 3 *C* and *D*).

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