



Supporting Online Material for

LRP6 Mutation in a Family with Early Coronary Disease and Metabolic Risk Factors

Arya Mani,* Jayaram Radhakrishnan, He Wang, Alaleh Mani, Mohammad-Ali Mani,
Carol Nelson-Williams, Khary S. Carew, Shrikant Mane, Hossein Najmabadi, Dan Wu,
Richard P. Lifton*

*To whom correspondence should be addressed.

E-mail: arya.mani@yale.edu (A.M.); richard.lifton@yale.edu (R.P.L.)

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Methods

Clinical evaluation of kindred CAD-100 and human subjects. Kindred IR-100 is of Iranian descent with members living in Iran and North America. All members of the extended kindred who were available and willing to participate were recruited for the study. Subjects provided a medical history, access to medical records, and underwent a physical examination. Venous blood samples were collected from 19 kindred members for DNA extraction and measurement of fasting glucose, insulin, triglycerides, total LDL and HDL cholesterol. Blood pressures were measured 3 times at 5 minute intervals in the recumbent position using a mercury sphygmomanometer and the average of the last two measurements were used for each subject. Individuals taking medications for the treatment of hypertension or having blood pressure $> 140/90$ mm Hg were classified as hypertensive. For individuals on antihypertensive medication, blood pressure values prior to initiation of treatment were used where available. Similarly, for subjects on HMGCoA reductase inhibitors, lipid levels prior to initiation of treatment were used. Diabetes was defined using American Diabetes Association (ADA) criteria as fasting blood sugar greater than 126 mg/dl (*S1*, *S2*). Unrelated subjects with myocardial infarction were recruited and used to examine frequency of LRP6 mutation, and anonymized samples from unrelated subjects ascertained for other studies were used as controls to estimate mutant allele frequencies. Informed consent was obtained from study participants and the study protocol was approved by the Yale Human Investigation Committee and the Teheran University Medical Sciences Investigational Review Board. Statistical comparisons of differences between groups were performed using the non-parametric Mann-Whitney U test for quantitative traits and Fisher's exact test for categorical traits.

Genotyping and analysis of linkage. Genomic DNA from venous blood of nineteen family members was prepared (*S3*). Genome-wide SNP genotyping of each subject was performed using Affymetrix 10K Gene-Chips following the manufacturer's protocol. An average of 10,200 genotypes were called per subject. In addition, 11 highly polymorphic di- and tetranucleotide repeat markers were genotyped across the 35 cM interval encompassing the linked interval on 12p. Analysis of linkage was performed using two pre-specified models of the trait locus- a conservative model, specifying the

trait locus as autosomal dominant with 90% penetrance, a phenocopy rate of 1% and a disease allele frequency of 0.001, and a stringent model specifying 99% penetrance, 0.1% phenocopies and allele frequency of 0.0001. Pairwise and multipoint analysis of linkage using all SNPs was performed across the genome using Allegro software (*S4*) on a Sun Sparcstation 20 (Sun Microsystems, Mountain View, CA). Further analysis of the 12p interval was performed using SNPs and microsatellite genotypes using SimWalk 2.9 (*S5,S6*).

Mutation Screening. Mutations were sought in *LRP6* and other genes in the lod-1 interval by direct DNA sequencing of the index case. The coding exons and their flanking exon–intron boundaries were amplified by PCR using 28 pairs of specific primers (Table S2). Identified variants were confirmed by sequencing of both DNA strands in independent amplifications, and the segregation of the variant in the kindred was determined by direct DNA sequencing. The significance of the identified mutation was assessed using the PolyPhen and Sift programs [<http://genetics.bwh.harvard.edu/pph/>, <http://www.blocks.fhcrc.org/sift/SIFT.html>].

The *LRP6*_{R611C} mutation was screened for in DNA of 200 unrelated Iranian controls and 1800 US Caucasian controls by PCR amplification followed by denaturation and reannealing and analysis via temperature gradient denaturation gel electrophoresis using a Spectrumedix 96-capillary Reveal Mutation Discovery System (Spectrumedix, State College, PA) as previously described (*S7*); mutation carriers from CAD-100 served as positive controls. In addition, we screened the entire *LRP6* gene for mutations in 400 unrelated subjects with CAD from Iran and the United States using the Spectrumedix system. Identified variants were subjected to DNA sequencing.

Wnt signaling. cDNAs encoding human wild-type *LRP6* or *LRP6*_{R611C} were produced by PCR with specific primers using the high fidelity polymerase *Pfu* (Stratagene) and were cloned into plasmids driving their expression from the cytomegalovirus promoter. Segments encoding HA epitope tags were introduced into the C-termini of both constructs. The constructs were verified by DNA sequencing. A well-established Wnt signaling reporter system was utilized as previously described (*S8,S9*). In each experiment, plasmids encoding LEF-1 (expressed from a

cytomegalovirus promoter), luciferase (under control of an LEF-1-responsive promoter), green fluorescent protein and either the wild-type LRP6 or LRP6_{R611C} constructs were introduced by transfection using Lipofectamine Plus (Invitrogen, San Diego, Calif.) into NIH 3T3 mouse fibroblasts maintained in DMEM with 10% FBS cells in 24-well plates seeded at 5×10^4 cells/well. Cells were transfected with a total of 0.5 μ g of DNA/well. Twenty hours after transfection, cells were incubated with purified mouse Wnt 3a protein (R&D Systems, Minneapolis, MN) for 8 hr and then lysed for determination of luciferase activity and GFP level. Luciferase activities were normalized for transfection efficiency using GFP levels; background luciferase levels seen in the absence of added LRP6 were subtracted. Each experiment was conducted in quadruplicate. Total cellular expression of wild-type and mutant LRP6 was determined by lysis of cells, mixing with sample buffer, and boiling followed by fractionation via SDS-PAGE and Western blotting with anti-HA antibodies (Santa Cruz Biotechnology); the membranes were stripped and stained with anti-tubulin antibodies (Sigma) to ensure equal loading of gel lanes. Surface expression of LRP6 was determined by specific binding to Dkk-1. The binding assay was carried out as previously described (S10). Alkaline-phosphatase-linked Dkk-1 (Dkk-AP) was expressed in HEK293T cells and conditioned medium (CM) was collected 48 h later. Cells were transfected with LRP6 or LacZ constructs; one day later, the cells were washed and incubated with mouse DKK1-AP CM on ice for 2 h. The cells were washed three times with the washing buffer and lysed. The lysates were heated at 65°C for 10 minutes, and the AP activity was determined using a Tropix luminescence AP assay kit (Applied Biosystems). Dkk-specific binding was determined by subtracting the binding to cells expressing LacZ.

Table S1. Clinical characteristics of members of kindred CAD-100

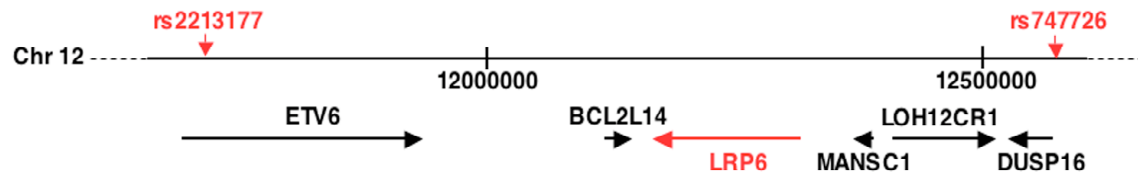
Kindred ID	Age Dx CAD/ Current age	CAD status	Blood Pressure, mmHg	Fasting Glucose	LDL, mg/dl	TG, mg/dl	HDL, mg/dl	BMI	Bone density, Hip fracture history
I-1	48/50†	MI x 3, SCD	NA	NA	NA	NA	NA	24	Hip fx, 48
I-2	52/56†	SCD	170/90	180	NA	NA	NA	25	NA
II-3	48/80†	3VD, SCD	170/100	105	NA	NA	NA	26	Hip fx, 68
II-4	48/51†	3VD, SCD	190/120	220	163	237	52	25	NA
II-5	45/56†	3VD, SCD	180/110	178	176	212	49	23	NA
II-6	47/61	1VD	170/90	180	187	175	63	24	Z= -1.15
II-7	45/72†	CABG,stroke	170/100	212	195	250	55	24	Hip fx 62, Z= -3.4
II-8	47/50†	3VD, SCD	180/100	91	182	220	45	26	NA
II-9	53/74	MI	180/120	197	164	140	58	26	NA
III-1	44/54	CABG,stroke	190/90	211	168	290	45	24	Z= -2.2
III-6	44/45	1VD	160/90	128	185	353	59	25	Z= -1.2
III-11	54†	SCD	140/90	156	171	189	65	25	NA
III-12	35/52	3VD/PTCA	190/120	167	173	335	57	23	NA
II-1	82	Unaffected	120/80	83	105	65	51	23	NA
II-2	85	Unaffected	120/80	78	92	62	54	24	NA
II-10	55	Unaffected	110/90	76	97	97	62	27	Z= +0.3
III-10	50	Unaffected	110/70	82	101	73	65	23	NA
III-15	57	Unaffected	120/85	81	94	43	49	25	NA
III-2	30	Unknown	130/85	97	192	174	63	23	NA
III-3	27	Unknown	130/90	92	163	187	53	23	NA
III-4	36	Unknown	120/80	94	159	172	64	24	NA
III-5	29	Unknown	140/87	97	157	276	67	24	NA
III-7	42	Unknown	130/80	118	158	188	65	23	NA
III-8	34	Unknown	130/80	86	160	179	63	23	NA
III-9	34	Unknown	120/80	92	162	93	41	24	NA
III-13	53	Unknown	140/90	113	160	100	69	25	Z= -1.2
III-14	32	Unknown	120/80	87	165	NA	NA	24	NA

Kindred ID's correspond to those in Figure 1. LDL, , HDL, triglyceride, and glucose were measured after an overnight fast. †, deceased; 3VD, three vessel disease (significant stenosis of all three major coronary arteries); BMI, body mass index; SCD, sudden cardiac death; Hip fx, 48, Hip fracture at age 48; CABG, coronary artery bypass graft; PTCA, percutaneous transluminal coronary angioplasty. NA, not available.

Table S2. Primer pairs used to amplify coding segments of *LRP6*

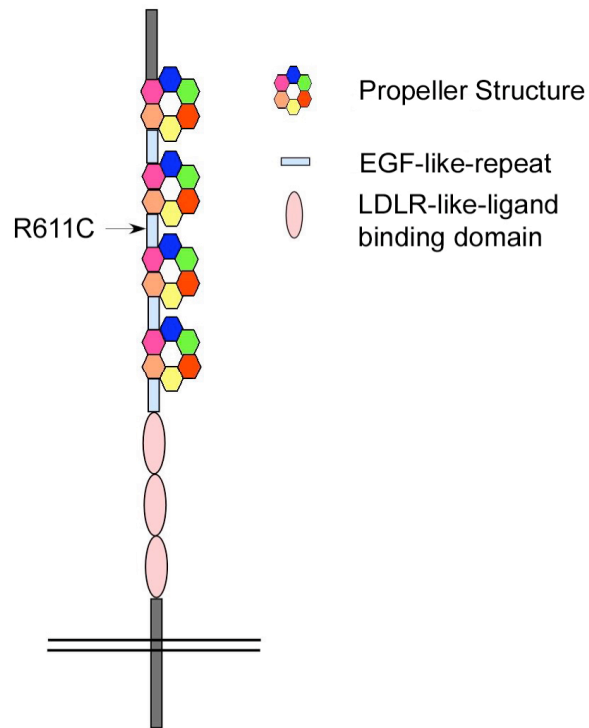
AMPLICON	FORWARD	REVERSE
1	CTGGGAAGCTGGGAAGTATG	CACATACAACAAGGCCACCTC
2	CCGATTTGAAACGCAGTTTAT	AACTTCAATCCGATTAGTTTCAGA
3	ATGTCAGCGAAGAAGCCATT	GTGGAGAAAAACCGAAAGGA
4	ATCTGCTCTGTCATGGCTTT	TCAAGGCTTCATCGAGTTTC
5	TGGGAGAGGTGACGTTATGA	CCCTCCTCCTGATCCTCACT
6	TGGCTTATCACAGTTGTTTATGT	GAGCTGATTATAGAGAAAACAAATCA
7	AAATTTTACCTCTGGGTTTCAAAG	CGTATGGCCCTCACTTCATC
8	TGCAGTTAGAAGACATCCGTCA	TCAATGTTTTTCAGGAACCTGTG
9	CCCCTTCCTTGGCATTAACT	CTGTCGACTCAAAAACCCATT
10	CAACAGGCAGTTGGATTGTT	TCATTCCTGGTCCCATTTC
11	TGAAAGCATTGTAGCCGTGA	GCAGCCACTGATATTTGCAT
12	TCTTTTGGCCAGCAAGACAC	AGGGCATCCACAAAACAAAAC
13	TCAATGTGTGTCTCCTTCTGTG	TCTCCCTTTTAGTCCCTAGCTTT
14	GCACTTCTGGATCCTCTTGC	CCATGTTCCCCGAAATGTA
15	TGAAAGCATTGTAGCCGTGA	GCAGCCACTGATATTTGCAT
16	CAGGGCCATTTGGATTATGT	TGAAAATGCTGCTTTCTCTCAT
17	TCCTCAGCCTTAGCTCTTCAA	CAGAGTGGTTGGTGAGTCCA
18	TGCCCATGTAGGTGTAAGCA	GCTGAAAAAGAAGGGTGAGG
19	TCCTCATTTTATGTGGTTTCTTACT	TGCCAAAACAATACGATGC
20	CCAAAAGTATAACTTGACCCACA	TGCATGAAAGTCTTCAAGGAAA
21	ACTTCTCTCCCCCTCCACTC	ATGTTTGCATTTAGGGTTGC
22	TGTGACCATGATTGTGTTTTTG	CCCAACTGACACTAAGCCAAG
23	AGGCACCTTTGATTCTTGC	GGAAATCTCGATAAGTAAACCTCA
24	TTAGCAAGCCCTCTTTTGATT	GCAGTCTTGCAGGACCAAA
25	TGTTGAATGAAATTGCTTGATACAT	CATTACTATCACTGATCACCCACA
26	TGAAGGAAGAAAAGTTGAAAGAAA	GACTAGTCTCTCTCTGGTGACCATC
27	TCTGAGAGGGGAAATCATTTTT	GTATTGGCTTCGGGGTGTG
28	GAATGACCTCAGTGGCAACA	CCCTCCAGATCTCAACCAAA

Fig. S1. Map of lod-1 interval.



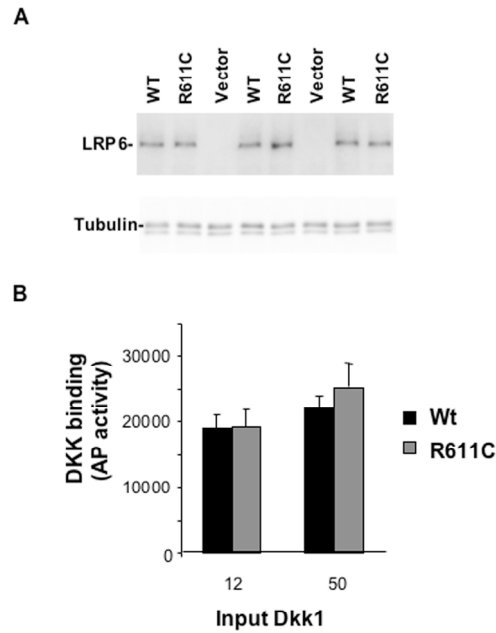
Map of the lod-1 interval showing linkage to early CAD is shown. The map is adapted from the UCSC genome browser, build 36.1 of the human genome sequence (<http://genome.ucsc.edu/cgi-bin/hgGateway>). The chromosome segment extending from 11,700,000 bp to 12,600,000 bp from 12pter is represented as a solid line. The locations of loci rs2213177 and rs747726, which define the boundaries of the lod-1 interval for the linked interval, are indicated in red at the top of the figure. Below, the locations of the six known or putative genes in the interval are shown as arrows which represent their 5' to 3' transcriptional orientations. *LRP6* is indicated in red.

Fig. S2. Location of R611C mutation in LRP6



A schematic diagram of the structure of LRP6 is shown. There is an amino-terminal signal sequence (gray), four propeller structures, each followed by an epidermal growth factor-like repeat (blue) and three low-density lipoprotein (LDL) receptor-like ligand binding domains (pink). These are followed by a single transmembrane domain and C-terminal cytoplasmic tail (gray). The R611C19 mutation lies in the second EGF-like domain.

Fig. S3. Similar total cellular and surface expression of wild-type LRP6 and LRP6_{R611C}



A. Western blotting of wild-type and mutant HA-tagged LRP6 expressed in NIH 3T3 cells used for biochemical studies of Wnt signaling was performed as described in Methods. Blots were stripped and re-probed with anti- β -tubulin antibodies to ensure similar loading of gels. The results demonstrate similar levels of expression of wild-type and mutant LRP6. B. Surface expression of wild-type and mutant LRP6 was measured by specific binding of alkaline phosphatase-linked Dkk-1 (Dkk-AP) as described in Methods. The input amount of conditioned medium containing Dkk-AP (in μ l) added to cells is indicated. The results show the mean and standard error of the mean of quadruplicate values of each group and demonstrate no significant difference in surface expression of wild-type and mutant LRP6.

Supporting References

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