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APOE p.Leu167del mutation in familial hypercholesterolemia

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ABSTRACT

Background: Autosomal dominant hypercholesterolemia (ADH) is caused by mutations in the low density lipoprotein receptor (*LDLR*), its ligand apoB (*APOB*) or proprotein convertase subtilisin/kexin type 9 (*PCSK9*) genes. Yet DNA sequencing does not identify mutations in these genes in a significant number of cases, suggesting that ADH has multiple genetic etiologies.
Methods: Through a combination of clinical examination, biochemical analysis, candidate gene approach and next-generation exome sequencing we investigated the genetic basis of an ADH phenotype in a proband of an Italian origin.
Results: The proband presented with an acute myocardial infarction at age 43. He had tendinous xanthomas, xanthelasmas and elevated levels of total and LDL cholesterol, at 11.2 and 9.69 mmol/L, respectively, with normal levels of HDL cholesterol and triglycerides at 1.62 and 1.13 mmol/L, respectively. HPLC lipoprotein profile showed selective increase in LDL-C. DNA sequencing did not identify any mutation in the *LDLR*, *PCSK9*, *LDLRAP1* and *APOB* gene. We then performed exome sequencing on three individuals from the family. The strongest evidence of association was found for the previously identified apolipoprotein E mutation (*APOE*, chromosome 19:45412053-55) known as *APOE* Leu167del, an in-frame three base-pair deletion. Computational biology confirmed the deleterious nature of this mutation. The Leu167del mutation is predicted to alter the protein structure of apoE near the α-helix within the receptor binding domain.
Conclusions: This report confirms a previous report that ADH can be caused by mutations within the *APOE* gene and represents the 4th loci causing ADH. Standard screening for ADH should include *APOE* gene.

1. Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by cutaneous xanthomas and xanthelasmas, a marked increase in low density lipoprotein (LDL)-cholesterol (LDL-C) and premature coronary artery disease. The genetic basis of FH is primarily from mutations within the *LDLR* gene, coding for the LDL receptor (LDLR) [1,2]. At least two other genes other than the *LDLR* have been found to cause a phenotype clinically similar to FH. These include the *APOB* [3] and *PCSK9* [4,5] genes. The term Autosomal Dominant Hypercholesterolemia (ADH) thus captures the genetic diversity of FH. Mutations in the *LDLRAP1* [6], coding for the LDLR adaptor protein 1 cause the rare autosomal recessive hypercholesterolemia disorder. In population-based studies, ~20% of patients with the FH phenotype do not have identifiable mutations in these genes [7]. Genome-wide association studies (GWAS) had broadened the search for genes contributing to LDL-C levels; this technique has allowed the identification of several genes that contribute to small variation in LDL-C levels [8]. Remarkably, mutations in several of the genes identified by GWAS have been

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previously identified in patients with FH. Contemporary techniques in high-throughput sequencing allow the sequencing of all exons expressed in the human genome. Using exome sequencing has allowed the identification of the genetic basis of rare diseases, usually of an extreme phenotype. Here, we present a mutation of the *APOE* gene in a kindred with a classical ADH phenotype.

ApoE is a multi-functional glycosylated protein (34 KDa) secreted from a variety of tissues, including the liver, brain, kidney, adrenals, adipocytes, macrophages and immune cells. It is a key component of all lipoproteins, but especially of triglyceride-rich lipoproteins (chylomicrons and chylomicron remnants), VLDL (very low density lipoproteins) and IDL (intermediate density lipoproteins), and participates in their catabolism through interacting with receptors belonging to the LDLR superfamily. ApoE is recognized by most receptors of this class, but especially the LDLR (also known as the apoB/E receptor), LDL-receptor related peptide-1 (LRP1) and LRP8 (apoE receptor 2) [9]. Three common polymorphisms at the *APOE* gene, apoE3, E4 and E2 have been recognized. ApoE2 binds with much less affinity to the LDLR. Homozygosity for the E2 allele is seen in approximately 0.5% of the population and a small percentage of these subjects will develop type III dyslipidemia, characterized by accumulation of remnant lipoprotein particles in plasma and premature vascular disease. A "second hit" is postulated to contribute to the expression of the apoE2/E2 genotype into a clinical dyslipidemia phenotype [10]. Rare mutations of *APOE* also lead to a rare form of lipid glomerulopathy [11]. Genome-wide association studies have shown that apoE is strongly associated with LDL-C levels [8]. Marduel et al. [12] were the first to report the strong genetic link between *APOE* p.Leu167del and the ADH phenotype in a large family.

2. Material and methods

Patients with an FH phenotype were selected from the McGill University Health Centre (MUHC). In all patients, a cascade screening of first- and second-degree relatives is offered. Clinical FH were suspected when total cholesterol is above 7 mmol/L, LDL-cholesterol is above 5 mmol/L and triglycerides are below 3 mmol/L. The protocol for blood sampling, family studies and DNA analysis has been approved by the Research Ethics Board of the research institute of the MUHC. Follow-up data for a period of 3 years was retrieved. Proper informed consent was obtained prior to clinical photography. A lipid profile is obtained in the fasted state, preferably without lipid-lowering drugs. Such medications are not withheld in subjects at high cardiovascular risk. In all cases, medical records are sought to determine previous lipid profiles.

Plasma samples (150 μl) from subjects were separated into lipoprotein fractions using high performance liquid chromatography (HPLC) with a Superose 6 10/300 GL column (GE Healthcare) attached to a Beckman Coulter System Gold™ apparatus. A 150 mM NaCl mobile phase with a flow rate of 0.4 ml/min was used for the separation of the sample into 72 × 400 μl-HPLC fractions that were collected in a 96-well plate using the ProteomeLab™ automated fraction collector (Beckman Coulter). Total cholesterol and triglyceride concentrations were subsequently analyzed on each fraction using the Infinity™ Cholesterol and Triglyceride Liquid Stable Reagents (Thermo Electron Corporation) following the manufacturer's instructions.

Using a candidate gene approach, we sequenced all exons of the *LDLR*, *PCSK9* and *LDLRAP1* genes as well as part of exon 26 of the *APOB* gene, associated with the apoB -LDLR interaction. In addition, a search for common copy number variants (CNV) at the 5′ region of the LDLR was performed. We then performed whole exon sequencing as previously described [12,13]. All identified SNPs in non-coding regions or not expected to impart functional defects on PolyPhen-2 [14] and SIFT softwares [15] were excluded. We used the following filters: we removed variants unlikely to be causal in the LDL-C genome-wide association studies, variants with a minor allele frequency >5%, synonymous or intronic variants and variants that did not fit a dominant model of transmission.

Exome sequencing of individuals from the proband family are performed to confirm association. Suspected gene are then

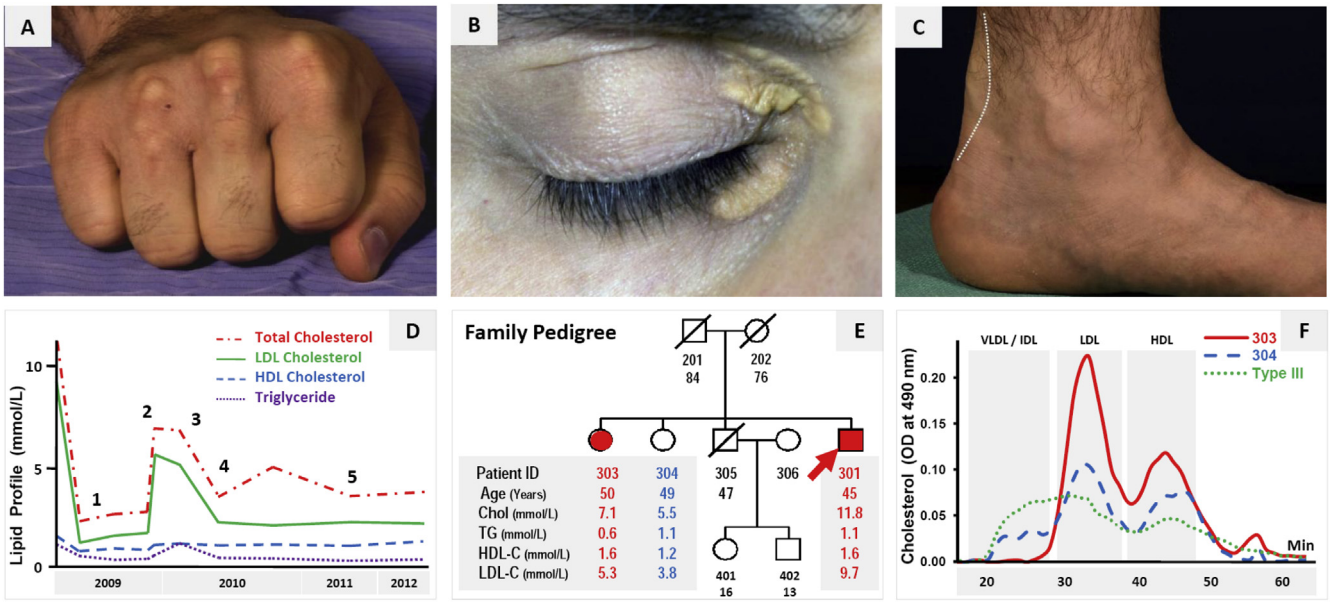

Fig. 1. A. Tendinous xanthomas in the proband, at the time of initial diagnosis. B. Xanthelasmas around both eyes in the proband. C. Achilles tendon xanthomas in the proband are demarcated with the dash line. D. Lipid and lipoprotein lipids over a three-year period in response to change of medication. The proband developed myalgias to high-dose atorvastatin and finally tolerated rosuvastatin (Medication history indicated by numbers: 1-Atorvastatin 80 mg and Ezetimibe 10 mg, 2-No medication, 3-Atorvastatin 20 mg, 4-Atorvastatin 20 mg and Ezetimibe 10 mg, 5-Rosuvastatin 10 mg and Ezetimibe 10 mg). E. Family tree of the kindred with *APOE* Leu167del. The proband (#301) is indicated by an arrow. The heterozygotes were subjects in red (#301 and #303) while the sister in blue (#304) was normal. F. HPLC profiles of the proband's affected sister subjects #303, unaffected sister r#304 and a type III hyperlipidemias associated with apoE2/E2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sequenced in 39 independent individuals with a clinical diagnosis of ADH (primarily diagnosed based on the Simon Broome criteria) from a tertiary lipid referral clinic in Southwestern Ontario; mutations in known ADH genes, namely *LDLR*, *APOB* and *PCSK9* were excluded in these patients.

Finally we used computer prediction and modeling software to identify and illustrate the impact of a mutation on a protein function. Protein Variation Effect Analyzer (PROVEAN) was used to determine if a given mutation is deleterious to the protein structure in multiple nucleotide involvement [16]. Template-based protein structure modeling using the I-TASSER server was accessed and then illustrated with the PyMol viewer to show structural impact of the mutation [17,18].

3. Results

Our proband is a 43 year-old man of Italian origin. He presented initially with an acute coronary syndrome; a coronary angiogram revealed severe three-vessel coronary artery disease. He underwent coronary artery bypass surgery. Because of the presence of tendinous xanthomas, xanthelasmas, Achilles tendons xanthomas, elevated total cholesterol and LDL-C levels (Fig. 1A,B and C), he was selected for further evaluation. The initial lipid and lipoprotein lipid profile revealed total cholesterol of 11.2 mmol/L (457 mg/dL), LDL-C of 9.69 mmol/L (374 mg/dL), HDL-C of 1.62 mmol/L (63 mg/dL) and triglyceride level of 1.13 mmol/L (100 mg/dL). He did not have any cause of secondary hypercholesterolemia nor was there any sign of a hematological disease and his abdominal ultrasound was unremarkable—specifically the spleen was not enlarged.

Compliance issues, including the development of myalgias on atorvastatin 80 mg/day led to unsatisfactory results during the initial year of treatment. On a combination of rosuvastatin 10 mg/day and ezetimibe 10 mg/day, the lipoprotein profile dropped to a total cholesterol of 3.87 mmol/L (150 mg/dL), a and LDL-C of 2.3 mmol/L (90 mg/dL), a respective 67% and 76% reduction from baseline (Fig. 1D). While a target level of an LDL-C < 2.0 mmol/L ($\sim$80 mg/dL) was not achieved, these results are well within the current recommended Canadian Cholesterol guidelines [19].

We then conducted a family study and performed DNA sequencing for the *LDLR*, *PCSK9*, *LDLRAP1* and exon 26 of the *APOB* genes; in addition, to common CNV in *LDLR* in the proband. No known mutation was identified and the several polymorphisms found were either in non-coding regions or were not expected to impart functional defects on PolyPhen [14] and SIFT software [15].

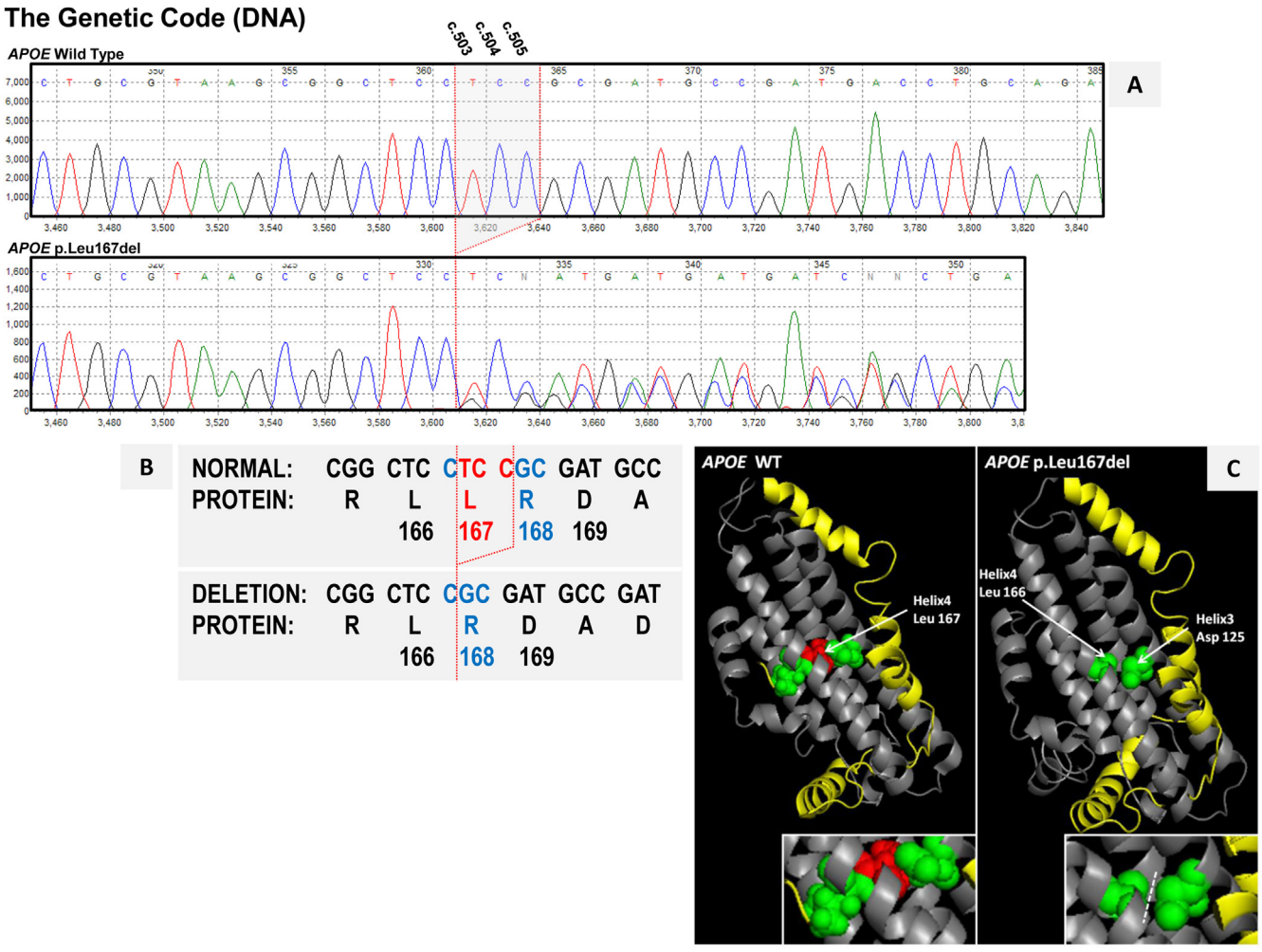

Fig. 2. A. Shows chromosome 19 sequence data of the affected subject using next-generation sequencing. B. Part of *APOE* coding gene. The deleted leucine residue is shown in red creating an in-frame deletion (codon TCC). C. The predicted crystal structure of apoE protein with Leu167 deletion in compare to the wild type. Notice proximity of Leu167 residue (red) from LDL receptor binding site spanning between 136 and 150 residues. The N-terminal portion (gray ribbon) contains the 4th α-helical region in which the leucine zipper motif is destabilized (dash line) by the Leu167del. The C-terminal portion of the protein is indicated by a yellow ribbon. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Multiplex ligation-dependent probe amplification was not performed for the *LDLR* gene. The *AOPE* gene was not considered as a candidate for the initial screening strategy. Until the report by Marduel et al. [12], there was little biological plausibility to include *APOE* as causal in the ADH phenotype.

We then proceeded with exome sequencing on three individuals from the same family (proband #301 and both sisters #303 and #304). Approximately 54,000 variants now were identified. We used the previously mentioned filters in the method section and ended with 49 missense mutations, 1 frameshift and 2 in-frame deletions in 52 genes. The *APOE* gene Leu167del mutation was the most likely candidate gene since it was recently discovered in a large family with FH [12]. The proband was found to be heterozygous for Leu167del as well as his sister (# 303). The other sister (#304) was found not to carry the *APOE* mutation (Fig. 1E). The *APOE* gene was then Sanger sequenced to confirm results obtained from the next-generation sequencing strategy. *APOE* genotyping showed that the proband and both sisters (Fig. 1D) were homozygous for the apoE3 allele.

HPLC profile on the subject's affected sister (#303) was performed who was not on lipid-lowering medication at the time. Because the apoE2/E2 phenotype is not associated with an isolated LDL elevation but rather a "broad beta" fraction, we sought to contrast the lipoprotein profile—as determined by HPLC separation-between a normal relative (#304), an *APOE* mutation subject (#303) and a subject with known type III hyperlipoproteinemia (apoE2/E2). Therefore the major particle abnormality in the affected subject (#303) resides in the LDL fraction and hence the selective increase in LDL-sized lipoproteins in the proband's sister is compatible with FH (type IIa hyperlipoproteinemia) (Fig. 1F).

The *APOE* gene mutation consists of a TCC deletion (c.500_502delTCC; p.Leu167del) coding for a leucine at position 167 in exon 4 of the *APOE* gene (chromosome 19:45412053-55) (Fig. 2A and B). The *APOE* p.Leu167del mutation was absent from 39 individuals with a clinical diagnosis of ADH ascertained in Ontario, Canada who had no mutation in any of the known ADH genes; indeed no rare mutations in *APOE* were found in this cohort. Using the protein structure algorithm online PROVEAN software to predict the effect of an amino acid loss on the protein functionality, *APOE* Leu167del scored −7.41 (default threshold −2.5) marking it deleterious to the protein structure [16]. Using established template-based protein structure modeling (I-TASSER server) [17] followed by 3-D structure alignment of both reference and Leu167del protein using PyMol viewer we could identify a leucine zipper disruption (Fig. 2C).

4. Discussion

Recently *APOE* p.Leu167del was reported in a kindred from France with ADH [12]. The *APOE* mutation was shown to segregate in a large family with elevated LDL-C. Based on kinetic studies, it was postulated that this mutation may cause a decrease in LDL clearance [12]. Furthermore, apoE is not only a ligand for the LDLR but also for many other LRPs in the LDLR family that may assist in LDL clearance and may be more affected by the mutation seen in our patient [9]. Interestingly recent data have shown that LDLR-mediated LDL uptake is regulated by LRP6 (another LDLR family member) that can potentially bind apoE and thus the Leu167del mutation may interfere with LDL internalization [20]. This mutation has also been previously reported in a patient with sea-blue histiocytosis [21] and was known as *APOE* Leu149del, due to different numbering based on exclusion of the pro-peptide sequence. Thus, *APOE* p.Leu167del is associated with a range of clinical phenotypes, including classical ADH, hypertriglyceridemia with splenomegaly and sea-blue histiocytosis and familial combined hyperlipidemia [12,21–24]. The reasons behind expression of such a diverse range of phenotypes in carriers of this mutation, but not seen in our proband is unclear, but mechanism may be related to differences in genetic background of different carriers, gene–gene interactions, gene–environment interactions, or perhaps epigenetic and other non-mendelian interacting effects [22–24]. Thus our report confirms the observation of Marduel et al. [12], and confirms that *APOE* is the 4th locus identified in ADH.

The structure of apoE has been extensively studied. The amino terminal portion has 4 α-helical structures that contain the receptor-binding region. The major lipid binding region is located near the carboxyl terminus [25]. The deletion of Leu167 is part of this highly conserved motif within the forth helix of the apoE protein. This region besides containing the receptor binding site (residues 136–150), it contain 112 and 158 residues, sites of common polymorphisms leading to apoE2, E3 and E4 isoforms and different binding affinity. We used computer prediction and modeling softwares to predict and illustrate the impact of this deletion on the 3D-structure of apoE [16–18]. It is apparent that *APOE* p.Leu167del destabilizes a leucine zipper motif in a critical region of the protein that, in turn, would weaken the lipoprotein particle binding to the LDLR (Fig. 2C). However the proband was heterozygous therefore it was feasible to overcome this phenotype by statin therapy that is known to induce LDLR in the liver, therefore the pharmacological approach was remarkable. From a clinical point of view it would be interesting to examine the effect of upcoming class of PCSK9 inhibitors that enhances LDLR availability on the liver surface and thus lowers LDL-C, with fewer myalgic effects than those experienced by our proband while on statin [5].

In conclusion the p.Leu167del mutation was associated with ADH in a kindred of an Italian ancestry and was identified by next-generation exome sequencing; the candidate gene approach did not identify mutations in genes known to be associated with ADH or autosomal recessive hypercholesterolemia. ADH individuals with a confirmed molecular diagnosis [26] are known to have premature atherosclerotic vascular disease, especially coronary artery disease, which if left untreated will lead to clinical symptoms in the third to fourth decade of life in men, and approximately ten years later in women [27–29]. Accordingly, the proband was 43 years of age at the time of presentation and had classical and biochemical features of FH, but not recognized for an acute coronary syndrome prior to his hospitalization. This report may have an impact on large registries of FH patients and access to specialized medical care. With the unraveling of additional genes associated with FH, future generations will benefit from cascade gene screening in high risk families to prevent vascular disease.

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References

[1] Talmud PJ, Shah S, Whittall R, et al. Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: a case-control study. Lancet 2013 Apr 13;381(9874):1293–301.
[2] Rader DJ, Cohen J, Hobbs HH. Monogenic hypercholesterolemia: new insights in pathogenesis and treatment. J Clin Invest 2003 Jun;111(12):1795–803.
[3] Vega GL, Grundy SM. In vivo evidence for reduced binding of low density lipoproteins to receptors as a cause of primary moderate hypercholesterolemia. J Clin Invest 1986 Nov;78(5):1410–4.

[4] Abifadel M, Varret M, Rabès JP, et al. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet 2003 Jun;34(2):154–6.
[5] Seidah NG. Proprotein Convertase Subtilisin Kexin 9 (PCSK9) inhibitors in the treatment of hypercholesterolemia and other pathologies. Curr Pharm Des 2013 Jan.
[6] Filigheddu F, Quagliarini F, Campagna F, et al. Prevalence and clinical features of heterozygous carriers of autosomal recessive hypercholesterolemia in Sardinia. Atherosclerosis 2009 Nov;207(1):162–7.
[7] Marduel M, Carrié A, Sassolas A, et al. Molecular spectrum of autosomal dominant hypercholesterolemia in France. Hum Mutat 2010 Nov;31(11): E1811–24.
[8] Asselbergs FW, Guo Y, van Iperen EP, et al. Large-scale gene-centric meta-analysis across 32 studies identifies multiple lipid loci. Am J Hum Genet 2012 Nov 2;91(5):823–38.
[9] Herz J, Chen Y. Reelin, lipoprotein receptors and synaptic plasticity. Nat Rev Neurosci 2006;7:850–9.
[10] Johansen CT, Kathiresan S, Hegele RA. Genetic determinants of plasma triglycerides. J Lipid Res 2011;52:189–206.
[11] Tsimihodimos V, Elisaf M. Lipoprotein glomerulopathy. Curr Opin Lipidol 2011;22(4):262–9.
[12] Marduel M, Ouguerram K, Serre V, et al. Description of a large family with autosomal dominant hypercholesterolemia associated with the APOE p.Leu167del mutation. Hum Mutat 2013 Jan;34(1):83–7.
[13] Reddy MV, Iatan I, Weissglas-Volkov D, et al. Exome sequencing identifies 2 rare variants for low high-density lipoprotein cholesterol in an extended family. Circ Cardiovasc Genet 2012 Oct 1;5(5):538–46.
[14] PolyPhen-2 prediction of functional effects of human nsSNPs. Web site. http://genetics.bwh.harvard.edu/pph2. [accessed January 2013].
[15] The J. Craig Venter Institute. The SIFT web site. http://www.jcvi.org. [accessed January 2013].
[16] The J. Craig Venter Institute. Protein Variation Effect Ananlyzer (PROVEAN) web site. http://www.provean.jcvean.org. [accessed January 2013].
[17] Zhang Y. I-TASSER server for protein 3D structure prediction. BMC Bioinform 2008;9(40).
[18] The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC. Web site. http://www.pymol.org. [accessed January 2013].
[19] Anderson TJ, Grégoire J, Hegele RA, et al. Society guidelines 2012 update of the Canadian Cardiovascular Society guidelines for the diagnosis and treatment of dyslipidemia and prevention of cardiovascular disease. 2012 recommendations. Can J Cardiol 2013;29(2):151–67.
[20] Ye ZJ, Go GW, Singh R, et al. LRP6 protein regulates low density lipoprotein (LDL) receptor-mediated LDL uptake. J Biol Chem 2012;287(2):1335e44.
[21] Nguyen T, Kruckeberg E, O'Brien F, et al. Familial splenomegaly: macrophage hypercatabolism of lipoproteins associated with apolipoprotein E mutation [apolipoprotein E (delta149 leu)]. J Clin Endocr Metab 2000;85:4354–8.
[22] Faivre L, Saugier-Veber P, Pais de Barros JP, et al. Variable expressivity of the clinical and biochemical phenotype associated with the apolipoprotein E p.Leu149del mutation. Eur J Hum Genet 2005 Nov;13(11):1186–91.
[23] Solanas-Barca M, de Castro-Orós I, Mateo-Gallego R, et al. Apolipoprotein E gene mutations in subjects with mixed hyperlipidemia and a clinical diagnosis of familial combined hyperlipidemia. Atherosclerosis 2012 Jun;222(2): 449–55.
[24] Brouwers C, van Greevenbroek M, Stehouwer D, et al. The genetics of familial combined hyperlipidaemia. Nat Rev Endocrinol 2012 Feb 14;8(6):352–6.
[25] Hatters M, Peters-Libeu A, Weisgraber H. Apolipoprotein E structure: insights into function. Trends Biochem Sci 2006 Aug;31(8):445–54.
[26] Wang J, Ban MR, Hegele RA. Multiplex ligation-dependent probe amplification of LDLR enhances molecular diagnosis of familial hypercholesterolemia. J Lipid Res 2005 Feb;46(2):366–72.
[27] Mortality in treated heterozygous familial hypercholesterolaemia: implications for clinical management. Scientific Steering Committee on behalf of the Simon Broome Register Group. Atherosclerosis 1999 Jan;142(1):105–12.
[28] Hansen PS, Defesche JC, Kastelein JJ, et al. Phenotypic variation in patients heterozygous for familial defective apolipoprotein B (FDB) in three European countries. Arterioscler Thromb Vasc Biol 1997 Apr;17(4):741–7.
[29] Jansen AC, van Aalst-Cohen ES, Tanck MW, et al. Genetic determinants of cardiovascular disease risk in familial hypercholesterolemia. Arterioscler Thromb Vasc Biol 2005 Jul;25(7):1475–81.