European Journal of Human Genetics (2007) 15, 1186–1189

www.nature.com/ejhg

SHORT REPORT

A functional mutation in the *LDLR* promoter (−139C>G) in a patient with familial hypercholesterolemia

Andrew JP Smith*[,1], Fayha Ahmed[2], Devi Nair[2], Ros Whittall[1], Darrell Wang[3], Alison Taylor[3], Gail Norbury[3] and Steve E Humphries[1]

[1]*Department of Medicine, Centre for Cardiovascular Genetics, University College London, London, UK;* [2]*Department of Clinical Biochemistry, Royal Free Hospital, London, UK;* [3]*Regional Molecular Genetics Laboratory, Great Ormond Street Hospital for Children, London, UK*

A novel sequence change in repeat 3 of the promoter of the low-density lipoprotein receptor (*LDLR*) gene, −139C>G, has been identified in a patient with familial hypercholesterolemia (FH). *LDLR* -139G has been passed to one offspring who also shows an FH phenotype. Transient transfection studies using luciferase gene reporter assays revealed a considerable reduction (74±1.4% SEM) in reporter gene expression from the −139G variant sequence compared to the wild-type sequence, strongly suggesting that this change is the basis for FH in these patients. Analysis using electrophoretic mobility shift assay demonstrated the loss of Sp1 binding to the variant sequence *in vitro*, explaining the reduction of transcription.
European Journal of Human Genetics (2007) **15,** 1186–1189. doi:10.1038/sj.ejhg.5201897; published online 11 July 2007

Keywords: familial hypercholesterolemia; *LDLR* gene; promoter mutation

Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by an impaired clearance of cholesterol from the plasma, with the resulting hypercholesterolaemia leading to an accelerated risk of atherosclerosis and coronary heart disease. In the majority of cases the disease is caused by mutations in the gene coding for the low-density lipoprotein receptor (*LDLR*), but mutations in the apolipoprotein-B, and proprotein convertase subtilisin/kexin type 9 (*PCSK9*), genes produce a similar phenotype.[1] Of the *LDLR* mutations, nearly all occur within coding regions and are summarized at www.ucl.ac.uk/fh. This report describes a novel sequence change, -139C>G in repeat 3 of the *LDLR* promoter.

*Correspondence: Dr AJP Smith, Department of Medicine, Centre for Cardiovascular Genetics, University College London, 5 University Street, London WC1E 6JF, UK.
Tel: +44 (0)20 7679 6964; Fax: +44 (0)20 7679 6212;
E-mail: Andrew.J.P.Smith@ucl.ac.uk
Received 16 March 2007; revised 9 June 2007; accepted 15 June 2007; published online 11 July 2007

Materials and methods

FH proband and family

The proband is a female born in Turkey in 1955, referred to the Royal Free Hospital lipid clinic, London, UK, with pretreatment fasting total cholesterol, 10.3 mmol/l; LDL-cholesterol (LDL-C) 8.1 mmol/l. On examination, the patient showed tendon xanthoma in the left Achilles tendon. The patient had a body mass index of 33.3 kg/m^2, had no previous history of ischaemic heart disease (IHD), was normotensive, and non-diabetic. Following treatment of 40 mg Atorvastatin daily, total cholesterol reduced to 5.3 mmol/l and LDL-C to 2.6 mmol/l. The proband's father had possible IHD and throat cancer, and died at the age of 57; her mother suffered 3 myocardial infarctions (MI) and died at the age of 54. Her eldest sister had IHD and died at the age of 47, and one of her brothers had an MI and coronary artery bypass graft surgery and died at the age of 50. The two remaining brothers and sister were found to have high cholesterol, and are now on treatment. The proband has two daughters and a son. Only the son, aged 20, had indications of FH, with total cholesterol of

8.8 mmol/l and LDL-C 6.7 mmol/l, and is currently on treatment with 20 mg Simvastatin daily.

DNA extraction and sequencing

DNA was extracted from blood by standard methods,[2] and for the full screen of all coding exons, including intron–exon junctions and of the promoter region of the *LDLR*, SSCP analysis was carried out essentially as described,[3] and dHPLC as described.[4] The presence of the *LDLR* c.−139C>G mutation was confirmed by sequencing and subsequently determined by PCR-RFLP using *BsmF* I (NEB, Hertfordshire, UK).

Mutagenesis of *LDLR* reporter plasmid

A 595 bp fragment of the 5′ flanking region of the *LDLR* gene, from position −600 to −5 was cloned into the pGL3 reporter plasmid (Promega, Southampton, UK).[5] Using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) as directed, a C>G mutation was created at position −139 (mutagenesis primers available on request). The 595 bp fragment was sub-cloned into WT pGL3 plasmid, and the mutation verified by direct sequencing using the DYEnamic Dye Terminator kit protocol (GE Healthcare, Buckinghamshire, UK) on the MegaBACE 1000 (GE Healthcare).

Transfection assays

Huh7 cells were seeded at a density of 2.15×10^4/well in a 96-well plate and grown to confluence overnight in high-glucose Dulbecco's modified Eagle's medium (PAA, Paschning, Austria) supplemented with 2 mM L-glutamine and 10% fetal bovine serum. Cells were transfected with 250 ng pGL3 reporter construct with 10 ng pRLTK as a transfection control. Transfection was carried out in Opti-Mem serum-free media (Sigma, Dorset, UK) using Lipofectamine 2000 (Invitrogen, Paisley, UK) as described in the manual. Media was replaced 24 h following transfection, with serum-containing media described above, and the cells left for 2 days before harvesting. Cells were lysed using Passive Lysis Buffer (Promega) and luciferase expression was determined using the Dual Luciferase Reporter Assay System (Promega), and measured in the Tropix TR717 Microplate Luminometer (PE Applied Biosystems, Warrington, UK). Luciferase activity was determined as the mean of eight transfections with the assay performed in triplicate.

Electrophoretic mobility shift assay

Purified Sp1 protein (Active Motif, Rixensart, Belgium) derived from HeLa nuclear extract was used to bind DNA sequences. Probes sequences for the 22 bp sequences are available on request, and comprised Sp1 consensus

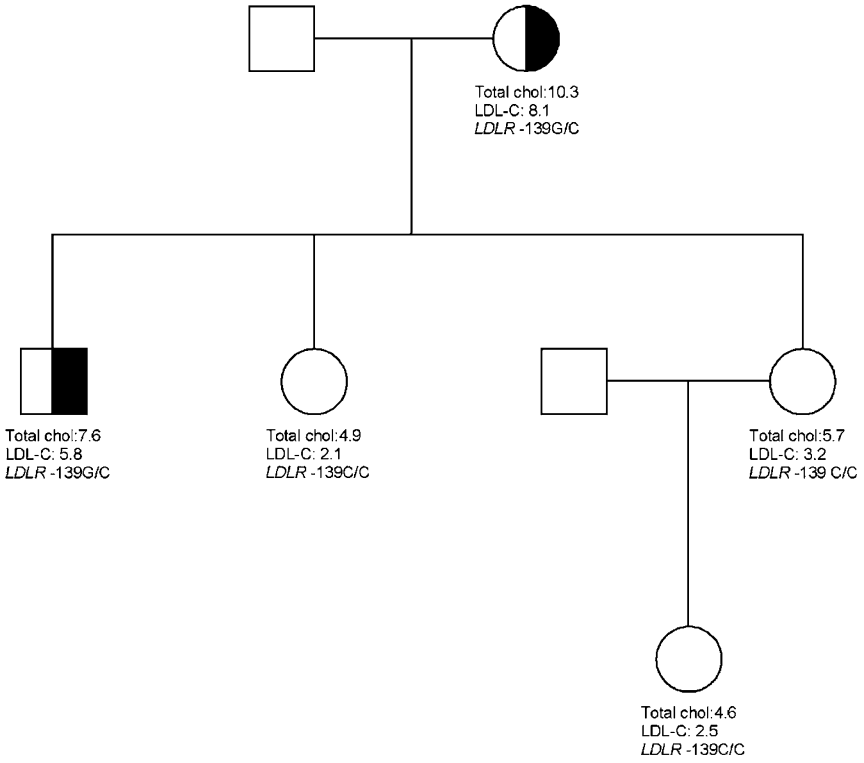

Figure 1 Pedigree of proband's family. Untreated total and LDL-C levels (mmol/l) are shown in the tested individuals. Individuals who fulfil the clinical diagnosis of FH are shown half-filled. The *LDLR* −139G>C genotype is shown.

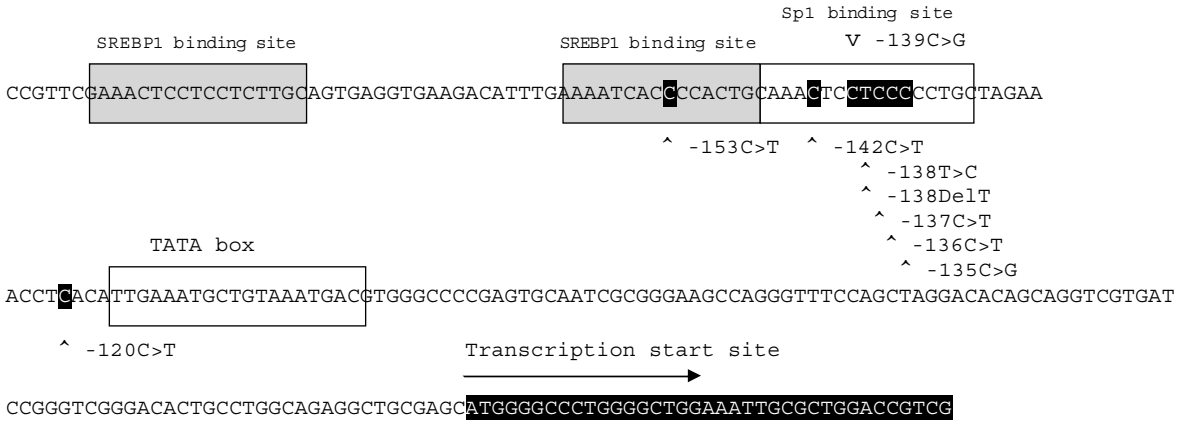

Figure 2 *LDLR* promoter and reported FH-causing sequence changes. Representation of the *LDLR* promoter, indicating location of transcription factor binding sites (SREBP: sterol response element binding protein). FH-causing mutations are highlighted: c.−153C>T;[7] c.−142C>T;[8] c.−138T>C;[6] c.−138delT;[5] c.−137C>T;[9] c.−136C>T;[10] c.−135C>G;[9] c.−120C>T.[7]

sequence, *LDLR* wild-type sequence, and corresponding *LDLR* −139G sequence.

Probes were labelled using the Biotin 3′-end DNA Labelling kit (Pierce, Rockford, IL, USA) as described in the manual. Each binding reaction consisted of 2 μl 10 × binding buffer (100 mM Tris, 500 mM KCl; pH 7.5), 25 ng purified Sp1, 2 nmol biotin-labelled DNA, made to a total of 20 μl with H_2O, and incubated at 25°C for 30 min, followed by the addition of 5 × loading buffer. Samples were electrophoresed on a 6% polyacrylamide gel for 150 min at 120 V. Transfer to nylon membrane was achieved through Southern transfer and detection using the Chemiluminescent Nucleic Acid Detection Module (Pierce, USA).

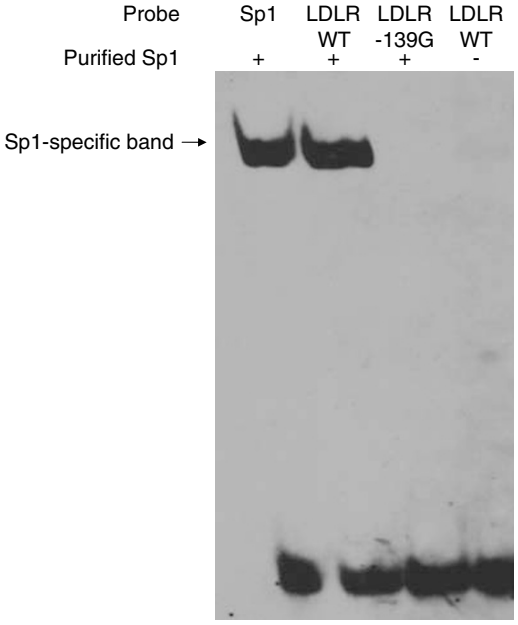

Figure 3 DNA binding of Sp1 to wild-type and −139G variant. Results of electrophoretic mobility shift assay (EMSA) using purified Sp1 protein. Lanes contain Sp1 consensus sequence, *LDLR* wild-type, and *LDLR*-139G probes as indicated.

Results

Detection of mutation

Mutation detection was carried out by SSCP and dHPLC analysis, and identified no mutations within *LDLR* coding regions. Analysis of the *LDLR* promoter identified a C>G change at position −139: a region within a putative Sp1-binding site. The mutation was subsequently detected by digestion with *BsmF* I, and all members of the proband's immediate family were found to be free of the mutation, except for the son that showed an FH phenotype (Figures 1 and 2).

Luciferase reporter assay

The liver cell line, Huh7, was transfected with a pGL3 luciferase reporter construct containing the *LDLR* promoter −139C>G variation. As a positive control, transfection was carried out using the equivalent wild-type pGL3 construct, and as negative controls, two constructs previously reported to diminish promoter activity: −138T>C[6] and −138delT.[5] Transfection of the −139C>G-carrying construct demonstrated a 74% (±1.4% SEM) reduced mean level of luciferase activity in Huh7 cells, compared to the wild-type construct, and comparable to the levels produced by both *LDLR*-138 variants (T>C: 3.2-fold±0.6% SEM; delT: 3.7-fold±0.5% SEM reduction in luciferase activity compared to wild-type construct).

Electrophoretic mobility shift assay

A 22 bp sequence flanking the *LDLR*-139G variant and the respective wild-type sequence were used to determine

the differential binding of Sp1 to the two sequences using purified Sp1 protein. The Sp1 consensus sequence and the *LDLR* wild-type sequence created an Sp1-specific band; the *LDLR*-139G variant probe produced no Sp1-specific band (Figure 3).

Discussion

A mutation has been identified at position −139C>G in the promoter of a heterozygous FH patient originally from Turkey. This was the only mutation to be identified in the proband after examination of all coding sequences, intron/exon junctions and 245 bp of promoter sequence. Transient transfection assays in the Huh7 cell line indicated that the c.−139C>G mutation is capable of considerably reducing transcriptional activity of the *LDLR* promoter. The c.−139C>G mutation adds to the list of FH-causing mutations occurring in the *LDLR* promoter (Figure 2), particularly prevalent within repeat 3. This region is believed to enhance expression through the binding of the transcription factor, Sp1, and as with the adjacent mutation, c.−138T>C,[6] this variation has been shown to impair the binding of Sp1 *in vitro*.

Acknowledgements

This work was supported by the British Heart Foundation (RG2005/015), and by a grant from the Department of Health to the London IDEAS GKP.

References

1 Dedoussis GVZ, Schmidt H, Genschel J: LDL-receptor mutations in Europe. *Hum Mut* 2004; **24**: 443–459.
2 Miller SA, Dykes DD, Polesky HF: A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acid Res* 1988; **16**: 1215.
3 Heath KE, Humphries SE, Middleton-Price H, Boxer M: A molecular genetic service for diagnosing individuals with familial hypercholesterolaemia (FH) in the United Kingdom. *Eur J Hum Genet* 2001; **9**: 244–252.
4 Bunn CF, Lintott CJ, Scott RS, George PM: Comparison of SSCP and DHPLC for the detection of LDLR mutations in a New Zealand cohort. *Hum Mutat* 2002; **19**: 311.
5 Dedoussis GV, Pitsavos C, Kelberman D *et al*: a novel mutation in the promoter (-45delT) of the low-density lipoprotein receptor gene associated with familial hypercholesterolemia. *Clin Genet* 2003; **64**: 414–419.
6 Sun XM, Neuwirth C, Wade DP, Knight BL, Soutar AK: A mutation (T-45C) in the promoter region of the low-density-lipoprotein (LDL)-receptor gene is associated with a mild clinical phenotype in a patient with heterozygous familial hypercholesterolaemia (FH). *Hum Mol Genet* 1995; **4**: 2125–2129.
7 Francova H, Trbusek M, Zapletalova P, Kuhrova V: New promoter mutations in the low-density lipoprotein receptor gene which induce familial hypercholesterolaemia phenotype: molecular and functional analysis. *J Inherit Metab Dis* 2004; **27**: 523–528.
8 Mozas P, Galetto R, Albajar M, Ros E, Pocovi M, Rodriguez-Rey JC: A mutation (−49C>T) in the promoter of the low density lipoprotein receptor gene associated with familial hypercholesterolemia. *J Lipid Res* 2002; **43**: 13–18.
9 Hobbs HH, Brown MS, Goldstein JL: Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. *Hum Mutat* 1992; **1**: 445–466.
10 Koivisto UM, Palvimo JJ, Janne OA, Kontula K: A single-base substitution in the proximal Sp1 site of the human low density lipoprotein receptor promoter as a cause of heterozygous familial hypercholesterolemia. *Proc Natl Acad Sci USA* 1994; **91**: 10526–10530.