

ONLINE MUTATION REPORT

The intron 14 2140+5G>A variant in the low density lipoprotein receptor gene has no effect on plasma cholesterol levels

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Familial hypercholesterolaemia (FH) is an autosomal dominant inherited lipoprotein disorder characterised by raised plasma low density lipoprotein (LDL) levels, xanthomas, premature coronary artery disease, and a family history of one or more of these.¹ Homozygous FH occurs in one in a million people and they are severely affected, while heterozygotes are moderately affected and occur at a frequency of 1 in 500 in genetically heterogeneous populations. FH is caused by a mutation in the LDL receptor gene (*LDLR*) and over 700 have been reported to date (<http://www.ucl.ac.uk/fh>).² However, it is not always possible to predict the pathogenicity or the clinical phenotype of a detected *LDLR* gene sequence variation. This is particularly the case for variants causing missense mutations, promoter regulatory element variations, or splice site junction variants where it is difficult to predict the degree or type of aberrant splicing.³

Since 1996, a molecular genetic diagnostic service for FH has been running at Great Ormond Street Hospital.⁴ Over the last year several probands were found to have one of two variants in the *LDLR* gene, one in the promoter, -269G>T (numbering -1 from the base immediately upstream from the first base of the initiator methionine codon), which was originally reported as -175G>T, in a region known to contain important *cis* acting elements,⁵ and another at a moderately conserved base in a consensus splice site, 2140+5G>A, in intron 14. Both of these variants have been previously reported in FH patients.^{6,7} Here we determine the frequency of these variants in UK FH patients and investigate the likely pathogenicity of these two variants by determining their frequency in healthy non-FH subjects in the UK general population.

MATERIALS AND METHODS

An assay was designed for the -269G>T variant where an *NruI* restriction site was introduced into the PCR product of the common g allele by a mismatch in the sense primer (underlined), sense primer 5'-CAA CAA ATC AAG TCG CCT GCC CTC GC-3' and antisense primer 5'-ACC TGC TGT GTC CTA GCT GG-3'. On digestion of the PCR with *NruI*, the T allele is uncut (252 bp) and the G allele is cut (227 bp + 25 bp). The primers for amplification of exon/intron 14 were: sense primer 5'-CTT CCA CAA CCT CAC CCA GCC A-3' and antisense primer 5'-GCA GAG AGA GGC TCA GGA GG-3'. The 2140+5G>A variant was detected using a natural *MslI* restriction enzyme site, where the rare A allele is uncut (381 bp) and the G allele is cut (351 bp + 66 bp). PCR conditions were as described previously.⁸ Both enzymes were added directly to the PCR product, incubated for two hours or overnight at 37°C, and the products separated on a 7.5% MADGE⁹ stained with ethidium bromide.

The samples studied consisted of 511 FH patients from the Simon Broome Register,¹⁰ and 745 white men from two general practices, Halesworth and North Mymms, from the Northwick Park Heart Study (NPHS-II),¹¹ considered to be

Table 1 Characteristics, mean (SD), of intron 14, 2140+5G>A variants, carriers and non-carriers in 745 healthy white men from the NPHS II study

	2140+5G/A (n=19)	Wild type (n=726)
Age (years)	56.05 (3.31)	56.27 (3.23)
BMI (kg/m ²)	25.92 (3.40)	26.31 (3.37)
Chol (mmol/l)	5.43 (1.09)	5.69 (0.95)
ApoB (mg/dl)	0.79 (0.23)	0.96 (0.32)
Tg (mmol/l)	1.79 (1.14)	1.70 (0.87)
HDL (mol/l)	0.81 (0.21)	0.81 (0.24)
ApoA1 (mg/dl)	1.67 (0.27)	1.54 (0.33)

non-FH subjects. Exclusion criteria for NPHS-II included non-white origin, a history of unstable angina or myocardial infarction, regular medication with aspirin or anticoagulants, cerebrovascular disease, malignancy (except skin cancer other than melanoma), diseases exposing staff to risk of infection, mental disorder, or other conditions precluding informed consent or regular attendance for examination.¹¹ Plasma cholesterol and triglycerides were measured as previously reported¹¹ and genomic DNA was isolated by a standard method.¹²

RESULTS

No carriers of the -269T allele were found in 745 NPHS-II healthy men or in the cohort of 511 FH patients. For the 2140+5A allele, 19 carriers were found in the NPHS-II sample and 22 in the FH cohort giving allele frequencies of 0.013 (0.01-0.02) and 0.022 (0.01-0.03), respectively. Although the frequency was higher in the FH patients than in healthy subjects, the difference was not statistically significant ($p=0.23$).

In the NPHS II men, data were available for various lipid traits. As can be seen from table 1, no statistically significant differences were observed between the mean total cholesterol, triglyceride levels, or other characteristics when carriers of the 2140+5A rare allele and non-carriers were compared.

DISCUSSION

The intron 14, 2140+5G is a moderately conserved base in the donor splice site consensus sequence, being a G in over 80% of introns examined.¹³ Although the effect of this intron variant on *LDLR* mRNA splicing has not been investigated, a similar mutation 1592+5G>A in intron 10 of *LDLR* has been studied at the mRNA level.¹⁴ This mutation was detected in two Danish families both in hypercholesterolaemic and normocholesterolaemic subjects. 1592+5A was found to generate two aberrant mRNA species owing to alternative splicing, either skipping of exon 10 and deletion of 75 amino acids, or activation of a cryptic splice site in intron 10 resulting in the insertion of 66 intronic base pairs resulting in the insertion of 22

novel amino acids in the protein.¹⁴ The intron 14 variant is thus potentially pathogenic.

In the original report of the 2140+5G>A variant in an Austrian patient, it was found together with a missense mutation in the coding region of the *LDLR* D333A.¹⁵ Other patients have been found with the 2140+5A variant together with another mutation in the coding region of *LDLR*. In a group of Danish FH patients it was found with missense mutation E207K in exon 4 of *LDLR* in one family but also alone where it cosegregated with clinical signs of FH in the absence of another detected *LDLR* mutation.⁶ Analysis of 100 control subjects did not show any 2140+5A carriers, suggesting that this variant was indeed FH causing.⁶ In a group of 36 unrelated Spanish patients, it was found in *cis* with a frameshift mutation 518delG, also in exon 4, which would render the 2140+5A irrelevant, as a stop codon would have been encountered before the intron 14 variant.¹⁶ In the Simon Broome patients, we have found a deletion of 2 bp (680delAC) in exon 4 of *LDLR*, together with 2140+5A (unpublished), which would also render the intron 14 variant irrelevant. However, the 2140+5A allele has been found in FH patients without any other detected mutation in *LDLR*, both in patients referred for diagnosis in London (unpublished) as well as in Holland where the variant was found to cosegregate with the FH phenotype in families.⁶

The promoter of the *LDLR* gene is well studied, and all essential regulatory elements lie within 200 bp upstream of the transcription start site.¹⁷ The -269G>T occurs in the FP2 *cis* acting regulatory element which spans nucleotides -281 to -269 (-187 to -175) in the *LDLR* gene promoter, with both FP1 and FP2 being essential for maximal induction of transcription.⁵ The -269G>T variant would disrupt this conserved site and is thus potentially pathogenic. The -269G>T variant was first reported in a black South African FH family (as -175G>T). The proband was found to be heterozygous for the -269G>T and a recycling deficient mutation E387K in the coding region of the *LDLR*. One of the proband's children who had inherited the 387K allele was found to be homozygous for the -269T. Other members of the family were found to be heterozygous for -269T but not carriers of 387K. Some of these -269T heterozygotes had cholesterol levels within the normal range, while others were hypercholesterolaemic. The carrier frequency of -269T in the general black population was found to be 6% and in FH patients 20%. It was also found in Lipid Clinic patients without the FH phenotype at an intermediate frequency (13%). The authors concluded that despite its location within an important region of the promoter, the -269T change was having little, if any, effect on lipid levels and was not a primary cause of FH in this family. The -269T allele was not found in over 300 whites investigated.⁷

The -269T allele was not found in 745 non-FH white controls or in 511 white FH patients. On investigation of the ethnicity of the two -269T carriers found in the molecular genetic diagnostic laboratory at Great Ormond Street Hospital, one, who is Afro-Caribbean, was also found to have the exon 4 E207Q mutation. The other patient was also reported to be of mixed ancestry (Irish, Maltese, English) and -269T was the only *LDLR* gene variant found. Thus, this variant has, to date, only been found in people of black African descent.

Taken together, the published reports and the screening data reported here suggest that neither of these variants has a major effect on *LDLR* function and are not FH causing. We have previously reported two other non-pathogenic variants of *LDLR*, both of which are missense variants, T705I¹⁸ and A370T,¹⁹ and which occur at low to moderate frequency in healthy UK subjects. Ideally, functional cellular studies should be carried out on all novel variants identified in the genetic diagnostic setting, but this is currently impractical in a diagnostic laboratory because of time and cost constraints. In the diagnostic laboratory it is recommended²⁰ that at least 50

normal subjects should be screened from the particular ethnic group to determine the frequency of the novel variant. Although the intron 14 variant is common enough to have been detected in 100 subjects with a high probability, increasing the number of normal subjects screened to 200 or 300 would be a more stringent test. The promoter variant is essentially absent in UK whites and would only have been detected if the correct ethnic group was identified and if normal subjects of this group were available. These results emphasise that care must be taken in reporting sequence variants as FH causing, even when the variant has been reported to cosegregate with the FH phenotype in a family.⁶

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REFERENCES

- Goldstein JL, Hobbs HH, Brown MS. Familial hypercholesterolaemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The metabolic basis of inherited disease*. 2nd ed. New York: McGraw-Hill, 1995:1981-2030.
- Wilson DJ, Gahan M, Haddad L, Heath K, Whittall RA, Williams RR, Humphries SE, Day INM. A World Wide Web site for low-density lipoprotein receptor gene mutations in familial hypercholesterolaemia: sequence based, tabular and direct submission data handling. *Am J Cardiol* 1998;**81**:1509-11.
- Sun XM, Patel DD, Bhatnagar D, Knight BL, Soutar AK. Characterisation of a splice site mutation in the gene for the LDL receptor associated with an unpredictably severe phenotype in english patients with heterozygous FH. *Arterioscl Thromb Vasc Biol* 1995;**15**:219-27.
- Heath KE, Humphries SE, Middleton-Price H, Boxer M. A molecular genetic service for diagnosing individuals with familial hypercholesterolaemia (FH). *Eur J Hum Genet* 2001;**9**:244-52.
- Mehta KD, Chang R, Underwood J, Wise J, Kumar A. Identification of a novel cis-acting element participating in maximal induction of the human LDL-receptor gene transcription in response to low cellular cholesterol levels. *J Biol Chem* 1996;**271**:33616-22.
- Lombardi MP, Redeker EJW, Defesche JC, Kamerling SWA, Trip MD, Mannens MMAM, Havekes LM, Kastelein JJP. Molecular genetic testing for FH: spectrum of LDL receptor gene mutations in the Netherlands. *Clin Genet* 2000;**57**:116-24.
- Thiart R, Scholtz CL, Vergotine J, Hoogendijk CF, de Villiers JNP, Nissen H, Brusgaard K, Gaffney D, Hoffs MS, Vermaak WJH, Kotze MJ. Predominance of a 6 bp deletion in exon 2 of the LDL receptor gene in Africans with FH. *J Med Genet* 2000;**37**:514-19.
- Day INM, Whittall RA, O'Dell SD, Haddad L, Bolla MK, Gudnason V, Humphries SE. Spectrum of LDL receptor gene mutations in heterozygous familial hypercholesterolaemia. *Hum Mutat* 1997;**10**:116-27.
- Day INM, Humphries SE, Richards S, Norton D, Reid M. High-throughput genotyping using horizontal polyacrylamide gels with wells arranged for microplate array diagonal gel electrophoresis (MADGE). *Biotechniques* 1995;**19**:830-5.
- Simon Broome Steering Committee. Risk of fatal coronary heart disease in familial hypercholesterolaemia. Scientific Steering Committee on behalf of the Simon Broome Register Group. *BMJ* 1991;**303**:893-6.
- Miller GJ, Bauer KA, Barzegar S, Cooper JA, Rosenberg RD. Increased activation of the haemostatic system in men at high risk of fatal coronary heart disease. *Thromb Haemost* 1996;**75**:767-71.
- Nicolaides NC, Stoeckert CJ. A simple, efficient method for the separate isolation of RNA and DNA from the same cells. *Biotechniques* 1990;**8**:154-6.
- Krawczak M, Reiss J, Cooper DN. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Hum Genet* 1992;**90**:41-54.

- 14 **Jensen HK**, Jensen LG, Holst HU, Andreassen PH, Hansen PS, Larsen ML, Kølvrå S, Bolund L, Færgeman O. Normolipidemia and hypercholesterolaemia in persons heterozygous for the same 1592+5G>A splice site mutation in the LDL-receptor gene. *Clin Genet* 1999;**56**:378-88.
- 15 **Varret M**, Rabès JP, Thiar R, Kotze MJ, Baron H, Cenarro A, Descamps O, Eberhardt M, Hondelijn JC, Kostner GM, Miyake Y, Pocovi M, Schmidt H, Schuster H, Stuhmann M, Yamamura T, Junien C, Bérout C, Boileau C. LDLR database (second edition): new additions to the database and software, results of the first molecular analysis. *Nucleic Acids Res* 1998;**26**:248-52.
- 16 **Mozas P**, Cenarro A, Civeira F, Castillo S, Ros E, Pocovi M. Mutation analysis in 36 unrelated Spanish subjects with FH: identification of 3 novel mutations in the LDL receptor gene. *Hum Mutat* 2000; Mutations in brief No 326 online.
- 17 **Sudhof TC**, Van der Westhuyzen DR, Goldstein JL, Brown MS, Russell DW. Three direct repeats and a TATA-like sequence are required for regulated expression of the human LDL receptor gene. *J Biol Chem* 1987;**262**:10773-9.
- 18 **Heath KE**, Whittall RA, Miller GJ, Humphries SE. I705 variant in the low density lipoprotein receptor gene has no effect on plasma cholesterol levels. *J Med Genet* 2000;**37**:713-15.
- 19 **Gudnason V**, Patel D, Sun XM, Humphries S, Soutar AK, Knight BL. Effect of the Stul polymorphism in the LDL receptor gene (Ala 370 to Thr) on lipid levels in healthy individuals. *Clin Genet* 1995;**47**:68-74.
- 20 **Cotton RGH**, Scriver CR. Proof of "disease causing" mutation. *Hum Mutat* 1998;**12**:1-3.