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ARTICLE

A molecular genetic service for diagnosing individuals with familial hypercholesterolaemia (FH) in the United Kingdom

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A genetic diagnostic service for familial hypercholesterolaemia (FH) has been established over the last 4 years in the Clinical Molecular Genetics Laboratory at Great Ormond Street Hospital for Children NHS Trust (GOSH), London. In total there have been 368 referrals; 227 probands and 141 family members, which have come from a number of lipid clinics and from general practitioners. FH is caused by mutations in the low-density lipoprotein receptor gene (LDLR) and these are analysed by SSCP, DNA sequencing and direct assays. The clinically indistinguishable disorder, familial defective apolipoprotein B100 (FDB) is caused by one of three mutations in the apolipoprotein B100 gene (APOB) which are analysed by direct assays. Mutations predicted to be pathogenic were found in 76 probands, 67 in LDLR (23 previously undescribed) and nine in APOB. The mutation detection rate was 53% in paediatric probands, 32% in adults with a 'definite' FH diagnosis (tendon xanthoma positive) and 14% in adults with a 'possible' FH diagnosis (tendon xanthoma negative). The predicted loss of sensitivity that would result from reducing the number of exons tested has been assessed, and a molecular screening strategy suitable for UK patients is proposed. A similar strategy may be useful for other countries where genetic heterogeneity results in a wide mutation spectrum for FH.
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Keywords: familial hypercholesterolaemia (FH); low-density lipoprotein receptor; genetic service

Introduction

In genetically heterogeneous populations, including the UK, the estimated frequency of heterozygous familial hypercholesterolaemia (FH) is ~1 in 500, placing FH among the most common single gene diseases.[1] In the UK 110 000 people are estimated to have the heterozygous form of FH, with roughly 10% of patients currently identified based on a clinical diagnosis, while only 1% have a genetic diagnosis. The severe homozygous form affects one in a million. FH is clinically characterised by elevations in low-density lipoprotein cholesterol (LDL-C), tendon xanthomata (TX) and premature coronary heart disease (CHD). The primary genetic defect in FH is a mutation in the LDL-receptor gene (LDLR). The gene spans ~45 kb and is divided into 18 exons and 17 introns.[2] Over 700 mutations have been reported[3–5] (for further FH database information see www.ucl.ac.uk/fh).

A clinically indistinguishable disorder, familial defective apolipoprotein B100 (FDB), is due to a mutation in the apolipoprotein B gene (APOB). This encodes apolipoprotein B100, one of the ligands of the LDL-receptor. The majority of FDB cases (2–5% of hypercholesterolaemic individuals) are caused by a single mutation, R3500Q.[6] Two rare mutations are also observed, R3531C[7] and R3500W,[8] and 2.4% of Asian hypercholesterolaemic subjects are reported to have the R3500W mutation.[9] Not all cases of monogenic inherited

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hypercholesterolaemia are accounted for by mutations in LDLR or APOB.[10–12] A third locus has been identified on chromosome 1p34.1-p32[11,12] with suggestive evidence that a fourth gene exists.[11]

Studies have shown that the mortality rate of FH patients is significantly greater than in normal individuals[13,14] and that lipid-lowering treatment, for example with HMG-CoA reductase inhibitors, can reduce the degree of coronary artery stenosis[15] and mortality.[14] The early identification of individuals at risk allows changes in lifestyle, including dietary intervention[16] followed by drug treatment. These measures should lead to a better long-term prognosis.

The diagnosis of FH is usually made on clinical features; TX, high cholesterol levels, history of myocardial infarction (MI) and the presence of hyperlipidaemia in first and second degree relatives. However there are several problems with diagnosing FH solely on clinical characteristics; the onset of some FH features only occur in adulthood (eg TX), and family studies are complicated to perform. The identification of heterozygous FH on biochemical parameters is made complicated in children by the overlap in cholesterol levels between affected and normal individuals which has been shown to occur from birth,[17] such that an unequivocal diagnosis cannot be given in 8–19% of children.[18] In addition, a proportion of young children at risk of FH may initially present with lipid levels within the normal range, with elevated levels only developing at a later age.[19] A diagnosis on cholesterol levels alone is also more difficult to establish in boys than girls because they normally have lower cholesterol concentrations,[18] but later in life they are at greater risk of CHD and thus it is most important to make an early diagnosis in boys in order to start lifestyle advice and treatment.

Occasionally low cholesterol levels have been observed in some LDLR mutation carriers[20–23] but studies have not yet established whether these subjects have low risk of CHD in the long term. A DNA *vs* cholesterol diagnostic study of an extended Irish family showed that 15–20% of family members would have been incorrectly diagnosed based on cholesterol testing alone,[24] and in a Finnish study, 10–20% of relatives would have been misdiagnosed.[21] Month-to-month variability of lipids and lipoproteins and apolipoproteins has also been observed, and in a study of 63 school children, recent infection significantly lowered high density lipoprotein (HDL) whilst LDL-C was shown to be slightly elevated.[25] Thus total cholesterol, LDL-C and triglyceride levels could be falsely high after an acute infection and result in misdiagnosis.

Identification of a mutation can give an unequivocal diagnosis, although this approach in many countries is difficult due to the mutational heterogeneity of the disease. Screening is simpler in populations where there is a founder gene, such as South Africa[26] and Quebec in Canada,[27] or in populations where there are a limited number of mutations in the majority of FH individuals, such as Iceland[28] and Finland[21] or where there are good traceable family records such as Utah, USA.[29] In heterogeneous populations, a larger number of mutations are found and thus a wider screening approach must be undertaken, as reported in Denmark,[30] Netherlands[31,32] and Italy.[33]

Genetic testing of FH in the UK was established in the diagnostic laboratory at Great Ormond Street Hospital for Children (GOSH), which forms part of the UK genetic service. The laboratory operates under the UK external quality assurance (EQA) scheme (http://www.cmgs.org). This paper describes the FH testing service and the diagnostic results obtained over the first 4 years of operation.

Materials and methods

Patient selection criteria

Two hundred and twenty-seven probands and 141 family members have been referred over the last 4 years for FH genetic testing. Patients were attending adult or paediatric lipid clinics or had visited their local general practitioner.

The criteria used for the diagnosis of FH were as previously described in the UK[13,14] and are:

(a) Total cholesterol >7.5 mmol/l or LDL-C >4.9 mmol/l if >16 years and total cholesterol >6.7 mmol/l or LDL-C >4.0 mmol/l if <16 years
(b) Tendon xanthoma (TX) in patient or in first or second degree relative
(c) Family history of MI <60 years in first degree relative or family history of MI <50 years in second degree relative
(d) Family history of total cholesterol >7.5 mmol/l in first or second degree relative

Diagnosis was classified into two classes. For a diagnosis of 'definite' FH both a+b must be present but for 'possible' FH both a+c or a+d must be observed.[13] FH is one of the few hyperlipidaemias to express from birth, so if a child from a family with 'possible' FH has hypercholesterolaemia then there is a high probability that the patient has FH. For this reason, any child who was suspected of having FH but who did not meet the criteria in every way was still analysed for LDLR and APOB mutations. In adults, if either the 'definite' or 'possible' criteria were fulfilled, a 5–10-ml EDTA blood sample was collected from the proband and any first degree relatives. Samples and referral forms were sent by standard post.

Molecular analysis

Genomic DNA was isolated from frozen whole blood or buccal samples using standard methods.[34,35] LDLR was screened by SSCP analysis.[36] Potentially FH-causing SSCP band shifts were subsequently sequenced using dRhodamine Bigdye fluorescent terminator sequencing, according to Perkin Elmer Applied Biosystems on an ABI DNA 377 Sequencer. LDLR major rearrangements were screened by analysing exons 3, 5, 8, 14, 17 by universal primer

quantitative fluorescent multiplex PCR (UPQFM-PCR).[37] The R3500Q and R3531C mutations were screened by direct PCR assays.[7,38] The R3500W mutation was only tested in patients of Asian background, by a PCR-NlaIII assay.[8] To confirm SSCPs due to polymorphisms, restriction enzyme assays or 'forced site' assays were used.[39,40]

Results

Referrals

Samples were received from 13 UK lipid/genetic clinics, although most came from the paediatric clinic at GOSH (22%) and the adult lipid clinic at Charing Cross Hospital, London (69%). Three hundred and sixty-eight samples, 227 probands and 141 family members, were collected over 4 years.

Molecular characterisation

All patients were screened for the APOB R3500Q mutation by two direct assays which avoid the possibility of false positive and negatives. The coding region and splice sites of the LDLR were screened by SSCP analysis. In one microtitre tray 23 PCR reactions were carried out in four patients and then loaded onto a double gel. Any failures were repeated. SSCP patterns due to known polymorphisms were excluded using direct assays[39,40] and any remaining SSCP band shifts were characterised by sequencing. Sequence alterations were confirmed by a direct assay if available or by repeating the sequencing reaction on a different PCR product.

Mutation spectrum

Mutations designated as pathogenic were identified in 76 probands (Table 1), 67 (88%) in LDLR and nine (12%) in APOB. LDLR mutations included 41 missense (63%), six nonsense (9%), four splice junction (6%), six small deletions (9%), four small insertions (4%) and four major rearrangements (6%). Twenty-three mutations were previously undescribed and two unreported non-pathogenic changes were observed, 2025C>T and 2390-16G>A. The greatest number of LDLR mutations were found in exons 3 (10%), exon 4 (28%), exon 10 (10%) and exons 14 (21%) (Figure 1); 46% of LDLR mutations were found in the ligand binding domain (exons 3–6) and 46% were found in the EGF precursor-like domain (exons 7–14). In paediatric cases, mutations were only found in nine exons, 2, 3, 4, 5, 8, 10, 12, 13 and 14 (Figure 1), which encode two domains of the LDL-receptor protein, and account for 84% of cases, the ligand binding domain (46%) and the EGF precursor homology domain (38%). Exons 4 and 14 accounted for 53% of these mutations and three mutations, D206E, E207X and P664L accounted for these high figures.

Mutation detection

To evaluate detection rates, adults and paediatric cases were analysed independently. The adult (n=170) and paediatric (n=57) detection rates were 28 and 53% respectively which was significantly different ($P<0.01$). Adults referred by one clinician were further divided into 'definite' (TX+) or 'possible' FH (TX−). The group with a diagnosis of 'definite' FH (n=122) had an overall detection rate significantly higher than in the group with a 'possible' FH diagnosis (n=48) (32 *vs* 14%, $P<0.01$). In 41 paediatric/adolescent FH probands referred from GOSH and Middlesex Hospital (London) the detection rate was 59%.

Discussion

Although some of the conclusions from the experience of the first 4 years of this clinical diagnostic service for FH apply only to the UK, many of the results are of direct relevance to the establishment of such a service in any country with a wide mutation spectrum for FH.

Mutation detection rates

Mutations were identified in 72 out of 227 probands. Compared to other disorders, the mutation detection rate in this study is low, but it is in line with results from other FH studies.[31,41] The mutation detection rate was significantly lower in adults (whether TX− or TX+) than in children, probably reflecting that FH is one of the few hyperlipidaemias expressed from birth rather than post puberty. A child who has hypercholesterolaemia is very likely to have monogenic FH whereas in adults, hypercholesterolaemia may be due to many other factors, eg environmental or polygenic causes. These data suggest that to reduce the number of costly genetic tests, only patients who fulfil specific clinical criteria should be included.

All mutation screening methods used are known to be less than 100% sensitive. SSCP analysis is routinely reported to have a sensitivity of 75–85%[42] suggesting that a mutation may have been missed for technical reasons in possibly 25–15% of patients. Thus the low detection rate is unlikely to be due entirely to lack of sensitivity of SSCP. Other detection systems such as Denaturing Gradient Gel Electrophoresis (DGGE) have been used in mutation screening of the LDLR[43–45] without achieving detection of mutations in all patients. The detection rate in one study using DGGE was 81%[45] although the sample size was small (n=32) and the clinical criteria were very strict, for example total cholesterol >9.5 mmol/l. This confirms the importance of the clinical diagnosis with respect to the detection rate. In a study of 42 adults from the UK, extensive mutation searching of the LDLR using SSCP, Southern blotting, DNA sequencing and RNA analysis only identified 28 mutations (detection rate 66%).[41] Additionally mutations may not be present in the LDLR or APOB but occur in the unidentified FH3 or FH4 genes.[10–12]

Pathogenicity of detailed sequence changes

As with any genetic diagnostic service, it was important to distinguish pathogenic and non-pathogenic sequence altera-

Table 1 Characterisation of LDLR and APOB mutations

LDLR exon or APOB	*Nucleotide*	*Amino acid*	*No. of probands (C/A)*
2	118delA*	FsI18*	1A
2	139G>A*	D26N*	1C
3	259T>G	W66G	1A, 1C
3	267C>G*	C68W*	1A
3	269A>G	D69G	1A
3	301G>A	E80K	1A, 1C
3	312T>G*	C83Y*	1C
4	324del 2(GT), ins2(TC)*	C88R*	1A
4	326G>A*	C88Y*	1C
4	353delA*	FsD97*	1A
4	501C>A	C146X	1A
4	530C>T	S156L	1A
4	551G>A	C163Y	1A, 1C
4	652del3(GGT)	dG197	2A, 1C
4	662A>G	D200G	1A
4	681C>G	D206E	1A, 2C
4	682G>T	E207X	1A, 3C
4	691T>G	C210G	1A
5	772G>T*	E237X*	1C
6	912C>G	D283E	1A
6	932del2(AA)*	FsK290*	1A
7	979C>T*	H306Y*	1A
7	1022C>G*	P320R*	1A
8	1121ins4(GGGT)	FsG353	2A
10	1374ins4(CAGA)*	FsA438*	1A
10	1436T>C*	L458P*	1A
10	1444G>C	D461H	1A, 1C
10	1474G>C	D471H	1A
10	1537C>T	P505S	1A
11	1694G>C*	G544A*	1A
12	1715del5(GTGGC),insA*	FsS552*	1A
12	1823C>T*	P587L*	1C
12	1833G>T*	L590F*	1A
13	1860T>C	W599C	1A
13	1944insC*	FsP628*	1A
14	2000G>T	C646F	1C
14	2029T>C	C656R	1A
14	2041T>A	C660S	1A, 1C
14	2054C>T	P664L	2A, 5C
14	2114ins7*	FsR685*	1C
Intron 14	2140+1 G>C		1A
Intron 14	2140+5 G>A		1A
Intron 16	2389+1 G>A*		1A
Min∇ex3		Min∇ex3	1C
∇ex7-18		4k~20kb ∇ex7-3′UTR*	1C
MinΔex3-5/∇ex8-17		MinΔex3-5/∇ex8-17*	1A
∇ex8-10		∇ex8-10*	1C
APOB	10708G>A	R3500Q	7A, 1C
APOB	10800C>T	R3531C	1C

*Indicates mutations not previously described.

tions. All identified nucleotide alterations had to meet certain criteria to be classified as pathogenic and the criteria used[46] were similar to that previously described.[47] In addition, care was taken, for example, in the determination of which mutation was pathogenic when two mutations were identified in an individual. In this study the T705I and 1061-8T>C variants were identified on the same haplotype in one individual as reported by others[41,48] but both have now been confirmed to be non-pathogenic variants.[49] In addition to these criteria, strict measures were taken to assure that the nucleotide change was a true result by confirming the sequence change in a second PCR product and if possible a second sample from the patient, although the latter was not strictly necessary since tests were carried out under EQA guidelines, where sample transfer and genetic test set up must be observed by a second scientist (http://www.cmgs.org). A number of polymorphisms are known to exist in LDLR (FH website) and although most resulted in recognisable SSCP band shift patterns, in a clinical genetic diagnosis setting a confirmation assay[39,40] was always used to

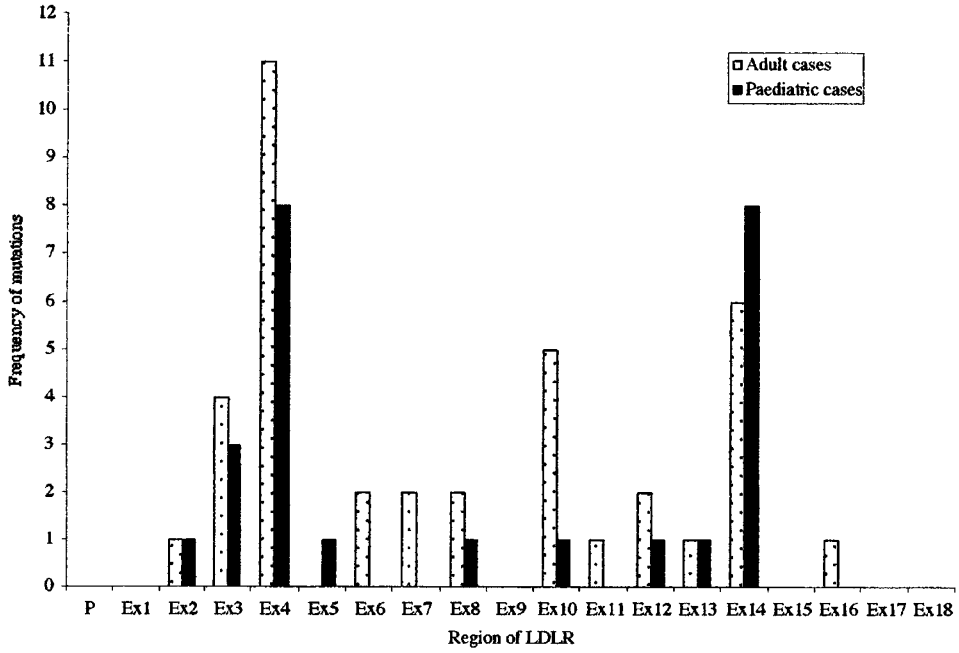

Figure 1 Spectrum of LDLR mutations identified in paediatric and adult FH probands.

ensure that the band shifts were due to the polymorphism and not from FH-causing sequence changes.

Mutation spectrum

The position of the identified mutations were concentrated in exons 3 (7/67=10%), 4 (19/67=285) and exon 14 (14/67=21%), as previously reported.[3,4] Mutations were also not evenly distributed among the ligand-binding domain. As reported previously,[3,4] repeat 5 (encoded in exon 4) contains three times as many mutations as any of the other repeat, probably because repeat 5 occurs at a crucial structural position so that any alteration in its sequence interferes with folding and is thus pathogenic. The high detection rate for this repeat may also reflect patient referral selection bias, as repeat 5 is the only cysteine-rich repeat which binds apoE and apoB[1] resulting in a more severe FH phenotype,[50] and a higher probability of a patient carrying such a mutation being identified and referred.

The other region where a high number of mutations were identified in paediatric probands was exon 14 (23%), but this was also observed in adult probands (21%). Exon 14 encodes the third growth factor repeat, repeat C, in the EGF precursor-like homology domain, and the high frequency of mutations in this region is probably also due to the functional significance of this domain. The P664L mutation accounted for five of the eight exon 14 mutations in the paediatric cases and this mutation is one of the more 'common' LDLR mutations world-wide (FH website).

Compared to other regions of the gene, the number of mutations in exons 3, 4 and 14 was (non-significantly) higher in paediatric probands compared to adults (63 *vs* 46%, P=0.16). These regions may be more deleterious for receptor function so the children attending the lipid clinics may represent the more severely affected individuals. During adulthood, cholesterol levels increase in carriers of mutations in other exons and this is brought to the attention of GPs and lipidologists, who are thus referred for molecular diagnosis. Although the frequency of LDLR mutations was higher in paediatric cases than adults, the frequency of APOB R3500Q mutations was (non-significantly) lower in adults than in paediatric cases (15 *vs* 3% $P<0.5$). Several studies have shown that FDB individuals have slightly lower cholesterol than FH individuals[6] and that cholesterol levels in FDB children become more elevated in their twenties[51]. Thus the different spectrum of mutations detected in the paediatric and adult cases is likely to be due to selection criteria.

No mutations were identified in the promoter, or exons 1, 9, 15, 17 or 18, and only one mutation was identified in exons 5, 11 and 16 in the paediatric and adult cases. Not only are the number of unique mutations lower in these exons world-wide (FH website) but also the frequency of these mutations is lower. Explanations for the lack of reported mutations in particular exons could be because of the failure to detect mutations present in these exons because of technical reasons, or that less mutations occur because the sequence contains less CpG dinucleotides. It may also be because these regions are less functionally important so that variations that occur are non-pathogenic and patients carrying such variations are not then referred.

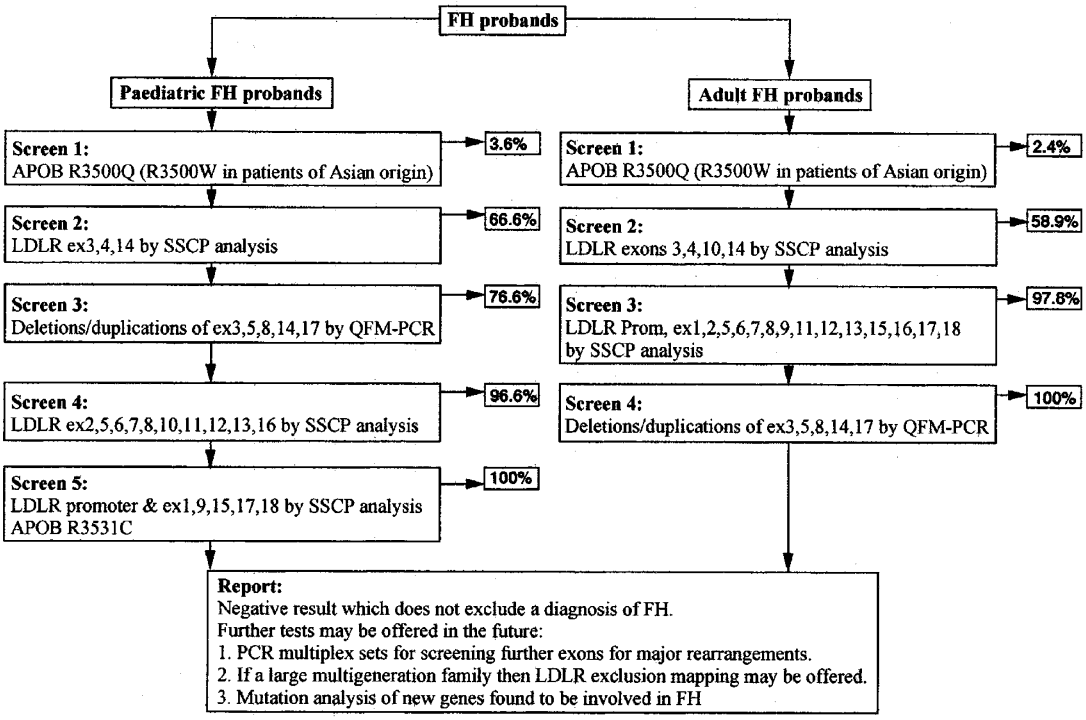

Figure 2 Proposed screening cascades for FH genetic testing in paediatric and adult probands.

Screening strategies

The current method of FH genetic testing involves screening all 18 exons and the promoter. This required evaluating as to whether this method was the most practical or whether a cascade screening system would be more cost effective. In many disorders it is common practice to examine specific exons of a gene and sometimes in a sequential manner (eg CF, Marfanís syndrome) and to terminate mutation analysis when a pathogenic mutation is found. However, the actual pathogenic mutation may be missed when two mutations occur on a single allele since not all the coding sequence is analysed. For FH this is known to occur, for example the LDL-receptor was found to be defective when the N543H and 2393del9 mutations occurred together on the same allele, while receptor function was not greatly impaired when only one of the mutations was present.[52] If cascade screening were to be implemented then such double mutations on the same alleles might be missed, and thus additional care must be taken in predicting if a detected mutation is pathogenic (Figure 2).

We have previously reported that major rearrangements in the LDLR can be detected in roughly 5% of UK patients[53] and that in this sample of patients two such rearrangements were detected in the adults and two in the children using a limited screen of five exons.[35] Including these rearrangements, overall, 78% of the detected mutations would have been found if in addition only exons 3, 4, 10 and 14 of the LDLR, and the R3500Q mutation in APOB were analysed.

Since the frequency of the different mutation classes were different between paediatric and adult cases two cascade screens were examined. For adult FH cases this would involve firstly, SSCP analysis of LDLR exons 3, 4, 10 and 14 plus APOB R3500Q mutation by a direct assay, secondly, testing nine exons where a few mutations have been identified (exons 2,5,6,7,8,11,12,13,16) and, thirdly, screening for major rearrangements of LDLR by UPQFM-PCR. If no mutation were identified, then exons 1, 9, 15, 17, 18 and the promoter could be screened but this final stage is likely to detect less than 1.3% of mutations in this group, and only 2.1% of all cases reported in the UK. In paediatric FH cases, the first stage would involve testing for the R3500Q mutation, the second would involve screening exons 3, 4 and 14 of LDLR, and the third to screen for major rearrangements. If no mutation were identified then the regions where a few mutations had been identified could be screened, exons 2,5,6,7,8,11,12,13,16. Finally if no mutation had been identified, then exons 1, 9, 15, 17, 18 and the promoter could be screened, but again with only very few mutations expected.

In the UK sample several mutations occurred in more than one proband, notably P664L in seven, and E207X in four and D206E and dG197 in three probands each. Although direct restriction enzyme tests for these mutations exist or could be developed using 'forced'-site PCR none of these mutations were common enough to be included as a cost effective pre-screen to SSCP (ie in this sample no single LDLR mutation was as common as APOB R3500Q).

Reports

Reports were sent to the referring clinician within four months of referral. Each had a short summary of the clinical and genetic aspects of FH, reason for referral, a results table and details of the result, including: (1) Mutation details with nucleotide and amino acid characterisation; (2) extent of DNA analysed, specifying the region and extent of DNA analysed (particularly where only part of the gene was analysed); (3) mutation and phenotype segregation details; (4) if a missense mutation was identified, information was included as to whether or not a conserved amino acid was changed by the base change and also which species the conservation was compared with; (5) expression analysis was described if published data was available; and (6) results of the analysis of 94 normal individuals (if a previously unreported missense mutation). In cases where no sequence variant was found, the report stated that no mutation had been found in the coding region and splice sites of LDLR, and that the subject did not carry the R3500Q and R3531C mutations in APOB. It was stated that a negative result does not exclude the individual from having FH since only 32% of LDLR mutations in adults and 53% of mutations in paediatric cases are detected by current methodology. It was also stated that further analysis will be carried out when new tests become available and if positive, results will be reported. These include additional screening, further mutation screening using denaturing high-performance-liquid-chromatography (DHPLC), further deletion screening and tests for mutations in the FH3[11,12] and possible FH4[11] genes when identified.

As a result of the service, unequivocal negative results and reassurance, and unequivocal positive results and advice were given to many individuals, enabling patients to make informed decisions with regard to their risk reducing therapy and life-style. The identification and characterisation of the mutation may also have relevance in the decision of which treatment an individual should be given.[54,55] The further value of genetic testing in FH was shown in several cases (manuscript in preparation) where children who currently had cholesterol levels within the normal range for their age tested positive for a pathogenic mutation carried by an affected relative (ie a 'false-negative' diagnosis based on lipid levels alone). Whether there is any long-term detrimental psychological effects associated with a genetic diagnosis of FH is unknown, but identification of FH by non-genetic methods has been shown to be of low emotional impact.[56–58] A detailed evaluation of the clinical and psychological impact of such diagnoses is under way.

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