Identification of loci conferring risk for premature CAD and heterozygous familial hyperlipidemia in the LDLR, APOB and PCSK9 genes

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A R T I C L E   I N F O

Article history:
Received 13 March 2009
Accepted 30 March 2009

Keywords:
Early CAD onset
Heterozygous familial hyperlipidemia
Low density lipoprotein receptor
Apolipoprotein B
Protein convertase subtilisin/kexin type 9
Haplotype
Autosomal dominant hypercholesterolemia
Low HDL-cholesterol levels

A B S T R A C T

Background: Heterogeneous familial hypercholesterolemia (FH) partly underlies polymorphic changes in the low-density lipoprotein receptor (LDLR), apolipoprotein B (APOB) and protein convertase subtilisin/kexin type 9 (PCSK9), exhibiting intra-ethnical variations in its clinical features.

Methods: We employed the Affymetrix whole genome scan 250 sty1 array to characterize possible genomic linkage to heterozygous familial hypercholesterolemia (FH) and sequencing techniques to identify related mutations in the above three genes in a Saudi family of 11 individuals harbouring clinical features of FH. The propositus had early onset of coronary artery disease (CAD) and very significantly elevated cholesterol (Chol) level of 10.1 mmol/L and LDL-cholesterol (LDL-C) of 7.9 mmol/L as well as low HDL-C level of 0.51 mmol/L, while 4 siblings were affected with FH.

Results: Whole genome scan for the autosomal dominant model showed high homology for the affected individuals in several regions including chromosomes (chr) 1 and 2 which harbour PCSK9 and APOB, respectively. Subsequent sequencing of the coding regions of these two and LDLR identified 11 single nucleotide polymorphisms (SNPs) in the LDLR, 8 in the APOB and 6 in the PCSK9 genes. The propositus uniquely carried the homoygus mutant genotypes (haplotype) for all 11 LDLR SNPs, in direct contrast to the only normolipidemic sibling and a control who carried the homoygous wild type genotypes at these loci. Another set of 7 SNPs in the APOB also isolated with FH. Interestingly, all family members were heterozygous for all except the rs2228671 C>T of this gene, to which the mother shared the C/C genotype with the propositus, two other affected off-springs and a control, all of whom exhibited low HDL-C levels. A confirmation experiment involving 70 individuals harbouring low HDL-C revealed 74.3% of them as C/C carriers.

Conclusions: Our study identified a haplotype in the LDLR as a marker for early onset of CAD, and rs2228671 C>T in the LDLR in association with a reduction in HDL-C concentrations in FH. The results also substantiate the notion of genetic heterogeneity in FH, underlining the essence of recognizing ethn-specific gene variability as a potential basis for appropriate management of FH.

1. Introduction

The low-density lipoprotein (LDL) receptor (LDLR) is the key component in the maintenance of cholesterol (Chol) homeostasis in the body, as a pivotal regulator of the hepatic catabolism of LDL-cholesterol (LDL-C) [1–3]. Familial hypercholesterolemia (FH) is triggered by defective catabolism of these lipoproteins, eventually progressing to premature atherosclerosis and early coronary artery disease (CAD). It is an autosomal dominant genetic disorder caused by, more often than not, a partial or total lack of functional LDLRs [1]. Both homozygous and heterozygous phenotypes of the disease have been described, whereby the homozygous form is rare and the heterozygous is common [2,3]. However, the later remains under-diagnosed worldwide. The clinical phenotype of heterozygous FH (HHF) is characterized by increased plasma levels of total Chol and LDL-C, tendinous xanthomata, and symptoms of premature CAD. It is inherited as an autosomal dominant hypercholesterolemia (ADH), a frequent monogenic disorder characterized by isolated elevation of LDL-C inducing premature cardiovascular disease, with homozygotes having a more severe phenotype than the heterozygotes. The disease is commonly known to result from mutations in two primary genes: the LDLR [3–14] and its natural ligand, the apolipoprotein...
system, Minneapolis, MN, USA), and for the genome-wide scanning performed using the Affymetrix Gene Chip 250 sty1 mapping array (Affymetrix, Inc., Santa Clara, CA, USA). Briefly, 250 ng of genomic DNA was digested with the restriction endonuclease StyI and mixed with Sty1 adaptors and ligated with T4 DNA ligase. The mixture was then added to four separate PCR reactions, amplified, pooled and purified to remove the unincorporated dNTPs. The PCR product was then fragmented, end-labelled with biotin, and hybridized to the 250 sty1 array for 18 h, washed, stained and scanned as recommended by the manufacturer. SNP genotypes, linear chromosomal locations and marker ordering were detected with the Affymetrix GeneChip® Genotyping Analysis Software (GTYPE Version 4.0).

Multipoint parametric linkage analysis was performed using the GeneHunter Easy Linkage analysis software 4.0 (Affymetrix, Inc., Santa Clara, CA, USA) for estimating the LOD scores. Both autosomal recessive and dominant models of inheritance were used to search chromosomal linkage regions, using Asian SNP allele frequencies. Copy Number Analyzer for GeneChip® Ver. 3.0 (CNAG) (Affymetrix, Inc., Santa Clara, CA, USA) was employed to check the shared chromosomal regions for homozygosity.

2.3. Screening for mutations

All exons and intron–exon junctions of the genes of interest were genotyped by sequencing using the MegaBACE DNA analysis system (Amersham Biosciences, Sunnyvale, CA, USA). Briefly, 5 µl of PCR product were treated with 2 µl of ExoSAP-IT (USB Corporation, Cleveland, OH, USA) at 37 °C for 30 min to allow the hydrolytic removal of excess primers and dNTPs by exonuclease 1 and shrimp alkaline phosphatase. The enzymes were inactivated at 80 °C for 15 min, and the sequencing reaction was initiated by mixing 2 µl DNA, 1 µl of 5 µmol primer, 8 µl of DyeNamIC ET Dye Terminator (Amersham Biosciences, Buckinghamshire, UK) and 9 µl of distilled water. The mixture was thermally cycled 40× at 95 °C for 20 s, 50 °C for 15 s, and 60 °C for 1 min. Unincorporated dye-labelled terminators were removed by gel-filtration through the DyEx 96 plate (Qiagen, GmbH, Hilden Germany). The eluent was
vacuum-dried and dissolved in 10 µl of loading solution (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK) for sequencing. Data was analyzed for SNPs by both Chromaspro program (Technelysium Pvt. Ltd., Tewantin, Qld, Australia) and Laser-gene software (DNASTAR, Inc. Madison, WI, USA).

2.4. Statistical analysis

All statistical analyses were performed using the SPSS software version 14 (SPSS Inc., Chicago, USA). A two-tailed p value <0.05 was considered statistically significant.

3. Results

The first step of this study was to identify chromosomal loci linked to HFH using both the autosomal recessive and dominant models by whole genome scanning approach. Scanning for homozygosity in the affected family members with the Affymetrix 250 sty1 chip yielded several peaks in different chromosomes (Chr) including chr1, 2, 5, … isolating at least three of affected siblings (S4, S6 and D3) for both models (Supplementary Table 1) using the autosomal dominant model. Interestingly, all models placing the propositus together with other members of the family almost always yielded no results. However, CNAG analysis showed peaks on Chr 12 and 13 isolating him individually and on Chr 15 together with two other affected siblings. It is also noteworthy that the dominant model did not isolate chr19, which harbours the LDLR gene, either as a potential locus for HFH or homozygous for the propositus and any other family members.

Based on currently available data however, we elected to first sequence all the coding areas including the intro–exon junctions of the LDLR gene (ENSG00000130164) as the most likely candidate for the early onset of CAD. Eleven single nucleotide polymorphisms (SNPs) were found in the LDLR in the cohorts. Two of these SNPs signified novel mutations consisting of a T > C change at nt43237 and C > A change at nt43279 in the untranslated region of the gene (Table 2). Five others were familiar SNPs residing in the 3’-untranslated region. Most remarkably, the index case carried the homozygous mutant alleles for all SNPs. In direct contrast, the only normolipidemic sibling (all three lipid levels in the normal range) carried the homozygous wildtype alleles for all, except for the rs2228671C > T (p.Cys27Thr) and ns4380 C > A for which he was heterozygous. Thus, a diametrically contrasting pattern in the LDLR SNPs was delineable at nine positions between the propositus and the disease-free sibling. Furthermore, both parents were heterozygous for all SNPs, except rs2228671 for which the mother was a C/C carrier (p.Cys/Cys). Interestingly also, the other carriers of this genotype comprising three affected (S4, S6 and D2) and one unaffected off-spring (S1) also harboured low-to-borderline HDL-C levels like the mother. Even more significant in this regard, was the observation that a control subject carrying the C/C genotype also exhibited low HDL-C, thus pointing to the isolation of this genotype with the reduction in HDL-C level, in general. In order to test the general applicability of this finding, we genotyped 70 individuals with clinical manifestation of low HDL-C levels randomly selected from our CAD registry. Interestingly, the study demonstrated that 52(74.3%) were homozygous for the C allele, 17(24.3%) were C/T carriers and only 1(1.4%) carried the T/T genotype, unequivocally linking the p.Cys/Cys to the presence of low HDL-C levels.

Two of the other loci picked individually by the chips on Chr 1 and 2 contain the PCSK9 and APOB genes, respectively, both of which are also candidate genes for FH. We were keen to further screen for possible association of these genes with HFH in our family. In the APOB (ENSG00000084674), we identified 8 SNPs altogether, two of which (ns28G > T, ns405 G > A) were novel

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**Table 2**

**Low-density lipoprotein receptor genotyping in family with heterozygote familial hyperlipidemia.**

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<thead>
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<th>SNP</th>
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The table shows the SNPs identified in the coding region of LDLR in the family. FT, father; MT, mother; Male off-springs are denoted as S1–S6, females as D1 and D2 and controls as C1–C5. Novel mutations are denoted with ns (+nucleotide position relative to the gene). AA, amino acid; UTR, untranslated region.

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**Table 3**

**Apolipoprotein B genotyping in family with heterozygote familial hyperlipidemia.**

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<tr>
<th>SNP</th>
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<th>MT</th>
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</table>

The table shows the SNPs identified in the coding area of the apolipoprotein B in the studied family. FT, father; MT, mother; Male off-springs are denoted as S1–S6, females as D1 and D2 and controls as C1–C5. AA, amino acid; Novel mutations are denoted with ns (+nucleotide position relative to the gene).
In the present study, we first employed the whole genome linkage strategy to identify chromosomal loci exhibiting homozygosity for HFH in a family with evidence of FH. This approach pointed to a number of putative regions of the genome as potential candidates. However, these tests could not identify loci shared by the proband with any of the affected members of the family, pointing to the likelihood of unique sequences being responsible for early onset of CAD in this individual. It is also noteworthy that heterozygous and complex disorders such as HFH or CAD often fail to demonstrate delinable linkage on currently available platforms. Hence, our approach for searching the potential candidate genes was based on available data which implicate particularly three genes, the LDLR, APOB and PCSK9, as the most likely candidates. Sequencing of the coding regions of these genes resulted in the identification of several novel as well as familiar point mutations in all three genes in the studied family. Of the three genes, the LDLR exhibited the highest level of polymorphic changes. We identified homozygosity for the minor allele of seven of the SNPs (haplotype) in the propositus in direct opposition to the homozygosity for the minor alleles of these SNPs, the rs676210C > T represents a change to homozygous mutant allele leading to Leu2739Leu, of which both parents carried the heterozygote Pro2739Leu. Furthermore, three of these SNPs were common among the heterozygote carriers, and one each was shared with either of the parents. This finding pointed to a high level of heterogeneity of the APOB mutant genotypes in the family, possibly depicting an autosomal dominant nature of the disease in the family.

We then embarked on further searching for the possibility that the PCSK9 gene may also isolate with some of the phenotypic features of the present family. Of the 6 SNPs discovered in the PCSK9 (ENSG00000169174), two (nt28G > T, nt405G > A) were novel mutations (Table 4). Notably, all of the off-springs harbouring low HDL-C levels were heterozygous for the nt28G > T mutation, possibly linking it with reduction in HDL-C in general. Other unique features included the sharing of Lys39Lys primarily by the affected off-springs at which the parents carried the Gln39Lys and that of the Val474Ile by three of these individuals with the mother, also possibly pertaining to the autosomal dominance and heterogeneity of the disease.

### 4. Discussion

4.1. Comparison with Literature

The table shows the coding SNPs identified in the protein convertase subtilisin/kexin type 9 genotype in family with heterozygous familial hyperlipidemia. (Table 3). Notably, the proband and two other affected siblings (S4 and D2) shared homozygosity for 7 of the variants. Among these SNPs, the rs676210C > T represents a change to homozygous mutant allele leading to Leu2739Leu, of which both parents carried the heterozygote Pro2739Leu. Furthermore, three of these SNPs were common among the heterozygote carriers, and one each was shared with either of the parents. This finding pointed to a high level of heterogeneity of the APOB mutant genotypes in the family, possibly depicting an autosomal dominant nature of the disease in the family.

4.2. Heterogeneity of the Disease

The present study, therefore, provides evidence for the involvement of the heterozygous LDLR as contributing to the disease in this family. Moreover, as mentioned previously, several other LDLR mutations have also been associated with FH in different populations [5,6], and severity of HFH was partly attributed to homozygosity in this gene [11,14,16]. Among the alterations discovered in the LDLR gene, only the rs2228671C > T in exon 1 yields a change in the protein sequence. Particularly remarkable in this regard was the observation that the homozygous C/C genotype of this SNP was not only present in the proband, but also shared with the mother and three kindreds who likewise harboured low DHL-C levels. Since this genotype was dominant in the affected individuals, its involvement in HFH cannot be completely ruled out. Specifically, this may explain the importance of heterozygosity in the LDLR as contributing to the disease in this family. More importantly however, was the observation that the only feature shared by the affected off-springs with two of their non-affected siblings carrying the p.Cys277Cys was the harbouring of low HDL-C levels. This observation strongly implicates this phenotype in a causative fashion for the reduction in HDL-C concentrations. Furthermore, the fact that an unrelated individual carrying the p.Cys277Cys variant also had low DHL-C concentrations pointed to a general applicability of this phenomenon in the general population. Indeed, our confirmatory study to test the notion of this constituting a general phenomenon related to low HDL-C levels, unequivocally isolated this mutation as contributing to events leading to the reduction in HDL-C levels in our population in general. Interestingly, one study has associated the T allele of this SNP (p.Met278Thr) with reduction in LDL-C levels [17], while the Cys277Cys in combination with other SNPs has been linked with an elevation in Chol levels [15]. Put together however, these findings appear to further link homozygosity at this gene locus with various forms of events leading to perturbations in cholesterol homeostasis. Possible implications are therefore that this motif is involved in yet unidentified mechanism(s) directly regulating Chol metabolic pathways. Hence, the variations in the findings among different ethnic groups can be viewed as reflective of the heterogeneity in the underlying genetic causes of the disease.

The other gene of interest, the APOB, also exhibited several point mutations in our study family, which included two novel findings. Among the interesting features of these changes was the sharing of
several variants by three of the affected individuals. While the precise role of these combinations of mutations needs to be elucidated, it can be speculated that these mutations exert an impact on the manifestation of HFH, in a fashion that is probably dependent on the prevalence of other factors. The rs676210C>T (p.P2739L) has been associated with variability in Chol level in the general population [18] while the rs679899, rs1042034, rs1042031 and 10699G > A (p.R3500Q), have been discussed with respect to variations in HDL-C and LDL-C levels [15,19]. Probably the most well studied of these is the R3500Q (currently denoted as R3527Q), which has been associated with FH [20–24] as well as other forms of hypercholesterolemia [25–27] in various ethnic groups. Its role in dyslipidemic disorders appears to be well characterized. In the present study, however, this mutation was not detected, dissociating it from the phenotypes in the family. To our knowledge, there is hardly any other data pertaining to a possible role of the other SNPs in FH. It is nonetheless noteworthy that combinations of mutations in the APOB were associated with the decrease of HDL-C levels in the same individuals that harboured the Cys27Cys in the LDLR as well as high Chol and LDL-C levels. The fact that mutations in two separate genes were simultaneously related to a reduction in HDL-C levels combined with the observation that unrelated individuals carrying the same Cys27Cys had a similar phenotype pointed to a general phenomenon linked to the reduction in HDL-C concentrations.

Like the APOB, the PCSK9 gene displayed heterogeneity in the distribution of mutations among the study cohorts. In contrast to APOB, however, no particular delineation of the trends in the relationship of the SNPs could be established with the disease. Moreover, since none of the individuals presented with isolated elevated LDL-C, the presence of ADH in this family might be precluded. Besides, none of the family members appeared to have hypocholesterolemia, and the type and severity of hyperlipidemia varied among the subjects. On the other hand, three of the PCSK9 SNPs described here (rs28385701, rs540796 and rs562556) have also been previously discussed as risk factors for hyperlipidemia [28]. Our study does not necessarily preclude these SNP as potential candidates for FH in this family. Moreover, there are other mutations in the PCSK9 gene absent in our case study that have also been linked with changes in LDL-C levels [8,12] and the severity of CAD [19] in different ethnic groups. It appears therefore that mutations in the PCSK9 may exert different effects on Chol handling in hyperlipidemic disorders.

Overall, our results point to various SNPs in the studied genes individually or combined as being responsible for some of the forms of FH. Similar combinations of polymorphic changes have also been described previously in the three genes in individuals with FH [29–33]. For example, some rare missense mutations of PCSK9 are thought to negatively enhance the clinical phenotypes of patients carrying LDLR mutations [34–36]. Also, a meta-analysis identified a number of genes including LDLR and PCSK9 as presenting common risk for hyperlipidemia and CAD [37]. Therefore, our results strongly furnish support for a link for these changes with the mechanisms regulating Chol at various levels. The impact of PCSK9 on ADH or familial hypocholesterolemia, is thought to be a result of a gain [34] or loss [38,39] of function of the gene in eliciting the degradation of the LDLR protein [40]. To this effect, it should be noted that mechanistically, PCSK9 promotes the degradation of the LDLR in hepatocytes apparently both intracellularly and through its secretion and binding onto the receptor leading to its internalization [41,42]. A plethora of possible mechanisms has been hypothesized, and it is now accepted that PCSK9 regulates LDLR post-transcriptionally. One school of thought purports that PCSK9 may affect both the level of LDLR and that of circulating APOB-containing lipoproteins in an LDLR-dependent and -independent fashion [41]. In the latter case, the incidence of CAD is reduced, thereby demonstrating that low LDL-C levels from birth are highly beneficial [38]. Apparently, PCSK9 polymorphism may also influence Chol levels indirectly, related to an overproduction of APOB [23]. Accordingly, the PCSK9 variants linked with FH would influence the secretion of APOB-containing lipoproteins, possibly explaining the marked increase in circulating LDL in heterozygous carriers [43]. It has been further suggested that the LDLR is the dominant partner in regulating the cellular trafficking of PCSK9, whereby the later promotes the degradation of the LDLR by an endocytotic mechanism [41]. Based on this, it has been argued that PCSK9 mutations could be important factors that cumulatively influence plasma LDL-C levels in the general population [44].

However, several questions concerning its mode of action remain unanswered. In particular, it remains unknown whether it acts directly on the LDLR or activates another protein that in turn causes degradation of the receptor. Nonetheless, intuitively the presence of combinations of such mutations in these genes is indeed likely to be detrimental. Altogether therefore, the finding of common mutations in at least two genes shared by the individuals with FH and/or low HDL-C levels add to the continually growing body of data supporting the presence of hyperlipidemic risk at various chromosomal loci and furnish justification for further studies in these regions to facilitate the identification of the actual causative alleles that may be of clinical value. In particular, the possibility that an LDLR haplotype may signify manifestation of CAD in FH provides a plausible basis for early screening for premature CAD in patients with FH. Recently, efforts have been directed at identifying potential clinical value for some of the mutations found in the three genes as diagnostic tools for familial hypercholesterolemia [45–52]. It is also thought that patients homozygous or compound heterozygous for LDLR mutations or double heterozygous for LDLR and APOB mutations have higher LDL-C levels, more extensive xanthomatosus and more severe premature CAD than simple heterozygotes for mutations in either these genes or for missense mutations in PCSK9 gene.

In summary, we have identified several novel mutations that isolate with heterozygote hyperlipidemia in a Saudi family with high prevalence of hyperlipidemia. The results demonstrate a haplotype in the LDLR that is strongly associated with premature CAD as well as an independent SNP that may be related to the reduction in DHL-C in this family. It can be concluded that the manifestation of FH and early onset of CAD may be primarily due to the mutations in LDLR under these conditions. The differences between the profiles of mutations found in the present study and those discovered in other studies imply that there are variations in combinations of mutations responsible for FH in different ethnic groups. It is therefore important to identify the pattern of the SNPs for the individual genes in order to procure the correct clinical database for diagnostic and possibly therapeutic purposes for the individual population of interest.

Acknowledgement

This work was supported by the Royal Cardiovascular Research Grant No. 2010013 under the King Faisal Specialist Hospital and Research Centre. The authors express their gratitude for this financial support.

References


