Identification of mutations in the gene encoding sterol regulatory element binding protein (SREBP)-2 in hypercholesterolaemic subjects

P Y Muller, A R Miserez

Human cells maintain their cholesterol homeostasis by regulated cleavage of membrane bound transcription factors, so-called sterol regulatory element binding proteins (SREBPs). If cells are deprived of cholesterol, SREBPs are cleaved by two proteolytic steps. The NH2-terminal domain of the SREBPs is released from the membranes of the endoplasmic reticulum and transported into the nucleus, where it binds to specific nucleotide sequences in the promoters of the low density lipoprotein receptor gene and of key genes involved in cholesterol and triglyceride homeostasis. Given the central role of SREBPs in the regulation of cholesterol metabolism, we investigated whether subjects with inherited forms of high plasma cholesterol carry specific sequence variations in SREBP-2 that might be involved in the development of hypercholesterolaemia. Exons 3 to 10, encoding the DNA binding and the regulatory domains of SREBP-2, were screened for sequence variations in a cohort of 70 hypercholesterolaemic subjects. Two missense mutations (V623M, R645Q) in the regulatory domain, one single nucleotide polymorphism (R371K) in the DNA binding domain, and one translationally silent mutation (P433P) were identified in SREBP-2. However, none of the mutations found in the regulatory domain could be detected in 167 subjects of a random control sample. A potential causative mechanism of these mutations for high plasma cholesterol concentrations is discussed. In summary, this is the first report of mutations in the human SREBP-2 gene to suggest that these and/or other mutations in this key regulator of cholesterol metabolism are associated with hypercholesterolaemia.

METHODS
Subjects enrolled in the study
Screening for sequence variations was performed in 70 unrelated hypercholesterolaemic subjects from the Swiss Make Early Diagnosis-Prevent Early Deaths (MED-PED) programme, Basel, Switzerland. Inclusion criteria were pretreatment plasma total cholesterol (TC) levels from overnight fasting blood samples exceeding the age and gender specific...

Abbreviations: SREBP, sterol regulatory element binding protein; LDL, low density lipoprotein; FH, familial hypercholesterolaemia; FDB, familial defective apolipoprotein B-100; FDL, familial dysbetalipoproteinaemia; CAD, coronary artery disease; SRE, sterol regulatory element; ER, endoplasmic reticulum; S1P, site 1 protease; S2P, site 2 protease; SCAP, SREBP cleavage activating protein; TC, total cholesterol; LDL-C, LDL cholesterol; TG, triglycerides; SNP, single nucleotide polymorphism.
90th centiles as computed on the basis of the data from the Swiss population (Swiss MONICA study\(^1\)). Applying the data of the Swiss population, the 90th centile corresponds to 8.25 mmol/l in male and 7.93 mmol/l in female subjects aged 50 years. Exclusion criteria were FH, FDB, and FDL. FH resulting from LDL receptor defects was diagnosed either by cosegregation analysis with intragenic restriction fragment length polymorphism (RFLP) markers as previously described\(^{2,3}\) or by single strand confirmation polymorphism (SSCP) analysis\(^{2,3}\) and Southern blotting.\(^{2,3}\) FDB and FDL were diagnosed by direct mutation testing. The R3\(^+\)50Q mutation in the apolipoprotein B-100 gene and the C112R and R158C mutations in the apolipoprotein E gene were excluded using restriction enzyme digestion assays as described by Hansen et al\(^{4}\) and Hixson et al\(^{5}\) with modifications.\(^{6}\) Informed consent for mutation screening was obtained from all subjects investigated.

### Biochemical analysis of plasma lipoproteins

Plasma TC, LDL cholesterol (LDLc), and triglyceride concentrations (TG) were determined at the Department of Clinical Biochemistry of the University Hospital, Basel. TC concentrations were determined by the enzymatic colorimetric cholesterol (Roche Diagnostics, Basel, Switzerland) method, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, 10 µmol/l NaOH) were added to the PCR product. After denaturing (95°C) for five minutes and immediate chilling on ice for 10 minutes, 7 µl were loaded on an Elchrom Gene Mutation Analysis (GMA) gel (Elchrom Scientific Inc, Cham, Switzerland) and run with 30 mmol/l TAE buffer (30 mmol/l Tris-HCl, 15 mmol/l acetate, 0.75 mmol/l EDTA). Electrophoresis was performed at 5-12 V/cm for 10-16 hours (table 1) in an Elchrom SEA 2000 submarine electrophoresis apparatus (Elchrom Scientific). The buffer temperature was equilibrated to 9°C during the entire run. The gel was stained with 50 ml SYBR Gold (Molecular Probes, Eugene, OR, USA) dissolved in 10 mmol/l TAE buffer according to the recommendations of the manufacturer. After destaining in 100 ml of water for 40 minutes, the gel was analysed and digitally recorded at 302 nm UV transillumination using the Gel Doc 2000 system (Bio-Rad, Irvine, CA, USA). On every gel, a non-denatured amplification sample, amplified from control DNA, was loaded in order to distinguish double stranded DNA bands from putative heteroduplex bands.

### Subcloning and sequencing of the PCR amplons

PCR amplions exhibiting aberrant migration patterns during SSCP analysis were digested with 60 U EcoRI (New England Biolabs, Beverly, MA, USA) and ligated into the dephosphorylated pcDNA3 vector (Invitrogen, San Diego, CA, USA) using the DNA Ligation Kit version 1 (Takara, Tokyo, Japan) according to the manufacturer. Forty µl of competent DH5\(\alpha\) cells (Gibco BRL, Paisley, UK) were transformed with 10 µl aliquot of the ligation mixture. After chilling on ice for 20 minutes and a heat shock procedure for 45 seconds at 42°C, 0.5 ml of L-Broth medium (Bio 101, Carlsbad, CA, USA) was added, followed by an incubation at 37°C for 30 minutes. Of this mixture, 100 µl were plated on LB agar plates (Bio 101) containing 100 µg/ml of ampicillin followed by an overnight incubation at 37°C. Five individual bacterial colonies were selected and expanded at 37°C overnight in 3 ml of LB medium (Bio 101) containing 100 µg/ml of ampicillin. From 1.5 ml of the culture, plasmid DNA was purified using the Plasmid Mini Kit (Qiagen). Dideoxy DNA sequencing of the inserts was carried out (Microsynth Inc, Balgach, Switzerland) using the T7 primer and the ABI-377 automatic DNA sequencer (Applied Biosystems). All sequence variations reported were identified in plasmid DNA from at least two independently transformed bacterial colonies.

### Table 1

<table>
<thead>
<tr>
<th>Exon</th>
<th>Amplicon size in base pairs</th>
<th>Intronic oligonucleotide primers with restriction enzyme cleavage sites at the 5’ end (5′-3′)</th>
<th>[MgCl(_2)] conc (mmol/l)</th>
<th>PCR thermal cycling profile</th>
<th>Conditions of SSCP electrophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>278</td>
<td>F: cggaattctctctggaacctca 1.8 Denaturing: 94°C/45 sec 80 V, 14 h</td>
<td>3.5</td>
<td>Denaturing: 94°C/45 sec</td>
<td>75 V, 14 h</td>
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<tr>
<td>6</td>
<td>202</td>
<td>F: cggaattcgggttcagctcagctcctgctta 3.5 Denaturing: 94°C/45 sec 74 V, 14 h</td>
<td>3.5</td>
<td>Denaturing: 94°C/45 sec</td>
<td>65 V, 14 h</td>
</tr>
<tr>
<td>7</td>
<td>313</td>
<td>F: cggaattcttaacactgagaaacactgagctcagctcagctcctgctta 5.0 Denaturing: 94°C/45 sec 74 V, 14 h</td>
<td>5.0</td>
<td>Denaturing: 94°C/45 sec</td>
<td>74 V, 14 h</td>
</tr>
<tr>
<td>8</td>
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<td>72 V, 14 h</td>
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<td>9</td>
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<td>3.5</td>
<td>Denaturing: 94°C/45 sec</td>
<td>74 V, 14 h</td>
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<tr>
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<td>363</td>
<td>F: cggaattctctctggaacctca 1.8 Denaturing: 94°C/45 sec 74 V, 14 h</td>
<td>1.8</td>
<td>Denaturing: 94°C/45 sec</td>
<td>80 V, 14 h</td>
</tr>
</tbody>
</table>

SSCP, single strand confirmation polymorphism; F, forward; R, reverse
Mutations in the SREBP-2 gene identified in 70 hypercholesterolaemic subjects. Three different single base pair substitutions were detected in the coding region of exons 5–10 of the human SREBP-2 gene in a total of 70 hypercholesterolaemic subjects.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Exon</th>
<th>Amino acid change</th>
<th>Wild type codon</th>
<th>Mutated codon</th>
<th>Protein domain</th>
<th>Age</th>
<th>Gender</th>
<th>TC (mmol/l)</th>
<th>TC adj (mmol/l)</th>
<th>LDLC (mmol/l)</th>
<th>HDLC (mmol/l)</th>
<th>VLDLC (mmol/l)</th>
<th>TG (mmol/l)</th>
<th>ApoE</th>
<th>CAD</th>
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<tbody>
<tr>
<td>B0211</td>
<td>7</td>
<td>P433P</td>
<td>ccc</td>
<td>CCT</td>
<td>Transitionally silent</td>
<td>37</td>
<td>M</td>
<td>8.58</td>
<td>9.20</td>
<td>5.07</td>
<td>1.50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B0022</td>
<td>10</td>
<td>V623M</td>
<td>gti</td>
<td>cta</td>
<td>Regulatory</td>
<td>65</td>
<td>F</td>
<td>9.03</td>
<td>8.27</td>
<td>7.33</td>
<td>1.41</td>
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<td>B0083</td>
<td>10</td>
<td>R645Q</td>
<td>cgg</td>
<td>cag</td>
<td>Regulatory</td>
<td>49</td>
<td>F</td>
<td>10.04</td>
<td>10.32</td>
<td>8.28</td>
<td>1.41</td>
<td>0.35</td>
<td>1.35</td>
<td>3/3</td>
<td></td>
</tr>
</tbody>
</table>

TC = total plasma cholesterol; TC adj = age and gender adjusted total plasma cholesterol; LDLC = low density lipoprotein cholesterol; HDLC = high density lipoprotein cholesterol; VLDLC = very low density lipoprotein cholesterol; TG = plasma triglycerides; CAD = coronary artery disease.

RESULTS

A total of 70 subjects with clinically determined forms of inherited hypercholesterolaemia were screened for sequence variations in exons 5 to 10 and the adjacent intron/exon junctions of the human SREBP-2 gene using a novel non-radioactive SSCP method. The mean plasma TC concentration of these subjects, adjusted for age and gender, was 11.08 (SD 2.32) mmol/l, the median age was 48.4 years, and the male/female ratio 37/33. All subjects enrolled in this study were selected on the basis of their family histories (at least two further family members with hypercholesterolaemia) and plasma TC levels exceeding the age and gender specific 90th centiles of the Swiss population. Subjects with LDL receptor (FH), apolipoprotein B-100 (FDB), or apolipoprotein E (FDE) accounted for 18% of the study  population. In the present study, its crucial polymorphisms in the genes encoding apolipoprotein E loci. In line with these findings, studies from others reported the presence of further genomic loci suggested to be responsible for hypercholesterolaemia. Moreover, besides polymorphisms in the genes encoding apolipoprotein E2/3 or apolipoprotein B-100, only a few sequence variants modifying plasma TC concentrations have been identified so far. FH, FDB, and FDE account for TC concentrations above the 90th centiles in only 2-7% in most populations surveyed. Therefore, mutations in further genes are expected to explain at least some of the remaining cases with TC concentrations above the 90th centile.

DISCUSSION

In previous studies, we found that in a considerable proportion of subjects with clinically defined familial forms of hypercholesterolaemia, the phenotypes did not cosegregate with the LDL receptor, apolipoprotein B-100, or apolipoprotein E loci. In line with these findings, studies from others reported the presence of further genomic loci suggested to be responsible for hypercholesterolaemia. Moreover, besides polymorphisms in the genes encoding apolipoprotein E2/3 or apolipoprotein B-100, only a few sequence variants modifying plasma TC concentrations have been identified so far. FH, FDB, and FDE account for TC concentrations above the 90th centiles in only 2-7% in most populations surveyed. Therefore, mutations in further genes are expected to explain at least some of the remaining cases with TC concentrations above the 90th centile.
The interaction of SREBP-2 with the WD repeats of SCAP. Therefore, these missense mutations might reduce or even distort the terminal domain of SREBP-2 that interacts with SCAP. Thereby, concentrations.

Nevertheless, extensive population based studies are required to determine whether this SNP modifies plasma TC concentrations. The V623M and R645Q mutations are located in the COOH-terminal domain of SREBP-2 that interacts with SCAP. Therefore, these missense mutations might reduce or even distort the interaction of SREBP-2 with the WD repeats of SCAP.63 Considering the pivotal role of SCAP as an intramembrane cholesterol sensor regulating the first sterol sensitive cleavage step of SREBP-2,63 an impaired interaction between SREBP-2 and SCAP would result in a decreased cleavage rate of SREBP-2. Consequently, less transcriptionally active NH2-terminal fragments of SREBP-2 would be released from the ER membranes and, thus, enter the nucleus. As a result, this would lead to a reduced transcriptional activation of SRE containing genes such as the LDL receptor gene. Furthermore, neither of the two novel missense mutations identified in the COOH-terminal domain of SREBP-2 was found in the 167 randomly selected control subjects. Therefore, there is evidence; although not functionally confirmed, that the V623M and the R645Q mutations are true pathogenic mutations leading to hypercholesterolaemia. Since for both of these mutations no large families were available for putative cosegregation analyses, this hypothesis remains to be proven.

Although translationally silent sequence alterations in the coding region, such as the P433P mutation, are usually not considered as pathogenic, recent publications suggest that such alterations might affect the mRNA stability or the function of exonic splicing enhancers.64-66 Hence, it cannot conclusively be ruled out that the translationally silent C to G transition in codon 433 impairs the function of SREBP-2.

Another aspect is the question to what extent SREBP-1, the human parologue of SREBP-2, may be able to partially take over SREBP-2 specific functions. This might be crucial with respect to subjects carrying mutations in the gene encoding SREBP-2 that might impair the function of the protein. A homozygous disruption of the SREBP-2 gene has been reported to be lethal in embryonic mice.67 This is in contrast to the observation in SREBP-1 deficient mice, in which a disruption of the SREBP-1 gene is only partially lethal.68 These findings suggest that a compensatory increase in the levels of nuclear SREBP-1 may not be able to compensate the loss of SREBP-2.

In summary, we identified the first genetic variants of human SREBP-2 suggesting that mutations in this central regulator of cholesterol metabolism might be associated with certain inherited forms of hypercholesterolaemia.

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Figure 1 Domain structure of human SREBP-2 and positions of the sequence variations found. Ser Gly Pro, serine-glycine-proline rich domain; bHLH Zip, basic helix-loop-helix leucine zipper domain (transcriptionally active domain); TM 1, first transmembrane domain; TM 2, second transmembrane domain.

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REFERENCES


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