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 SREBP precursors 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 5 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 nonsense mutations (Y623M, 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-Predict 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 dysbetaetaproteinemia; CAD, coronary artery disease; SRE, sterol regulatory elements; 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
Table 1: PCR and SSCP conditions for the detection of sequence variations in exons 5-10 of the human SREBP-2 gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>Amplicon size in base pairs</th>
<th>Intronic oligonucleotide primers with EcoRI cleavage sites at the 5′ end (5′-3′)</th>
<th>[MgCl₂]conc (mmol/l)</th>
<th>PCR thermal cycling profile</th>
<th>Conditions of SSCP electrophoresis</th>
</tr>
</thead>
</table>
| 5    | 278                         | F: cggaattctctagagcccttgaggggc  
                          R: cggaattctgccacccggtggagtg | 3.5               | Denaturing: 94°C/45 sec  
                          Annealing: 58°C/30 sec  
                          Extension: 72°C/1 min  
                          Cycles: 30             | 75 V, 14 h            |
| 6    | 202                         | F: cggaattctggtcactgttttcactcctc  
                          R: cggaattctgccacgcgtacagcctcttc | 3.5               | Denaturing: 94°C/45 sec  
                          Annealing: 56°C/30 sec  
                          Extension: 72°C/1 min  
                          Cycles: 30             | 65 V, 14 h            |
| 7    | 313                         | F: cggaattctagagcaggtccaccttggaggggg  
                          R: cggaattctgccacgcgtacagcctcttc | 5.0               | Denaturing: 94°C/45 sec  
                          Annealing: 57°C/30 sec  
                          Extension: 72°C/1 min  
                          Cycles: 32             | 74 V, 14 h            |
| 8    | 284                         | F: cggaattctcggggggagggtagggccgaag  
                          R: cggaattctgccacgcgtacagcctcttc | 2.5               | Denaturing: 94°C/45 sec  
                          Annealing: 59°C/30 sec  
                          Extension: 72°C/1 min  
                          Cycles: 30             | 72 V, 14 h            |
| 9    | 268                         | F: cggaattctcggggggagggtagggccgaag  
                          R: cggaattctgccacgcgtacagcctcttc | 3.5               | Denaturing: 94°C/45 sec  
                          Annealing: 57°C/30 sec  
                          Extension: 72°C/1 min  
                          Cycles: 30             | 74 V, 14 h            |
| 10   | 363                         | F: cggaattctcggggggagggtagggccgaag  
                          R: cggaattctgccacgcgtacagcctcttc | 1.8               | Denaturing: 94°C/45 sec  
                          Annealing: 59°C/30 sec  
                          Extension: 72°C/1 min  
                          Cycles: 30             | 80 V, 14 h            |

SSCP, single strand confirmation polymorphism; F, forward; R, reverse

Biochemical analysis of plasma lipoproteins
Plasma TC, LDL cholesterol (LDLC), and triglyceride concentrations (TG) were determined at the Department Central Laboratory, University Hospital, Basel. TC concentrations were determined by the Wahlefeld Trinder 4-aminophenazone method (Roche Diagnostics). HDL cholesterol (HDLc) concentrations were determined by a direct enzymatic colorimetric cholesterol assay (Wahlefeld et al). Applying the data of the Swiss population, the 90th centile corresponds to 200 mg/dl for male and 150 mg/dl for female subjects aged 50 years. Exclusion criteria were FH, FDB, and FDL. FH resulting from 500Q mutation in the apolipoprotein E gene were excluded using restriction enzyme digestion assays as described by Hansen et al.

PCR amplification of exons 5 to 10 of the human SREBP-2 gene
A total of 100 ng of genomic DNA, isolated using the salting out procedure with modifications according to Miserez et al., was PCR amplified on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). PCR amplification was carried out in a final volume of 11 µl using the Taq PCR Core Kit (Qiagen, Hilden, Germany) and intronic primers designed on the basis of previously unpublished genomic DNA sequences (table 1).

Gel electrophoresis for non-radioactive SSCP analysis
Twenty-five µl of denaturing loading buffer (97% (v/v) formamide, 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) run with 30 mmol/l TAE buffer (30 mmol/l Tris-HCl, 15 mmol/l acetic acid, 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.
These subjects were screened for the R371K, V623M, and R645Q mutations. Apart from the R371K mutation, four different amino acid substitutions were detected in the investigated subjects. The putative role of the sequence variant was screened by digestion with \( \text{NlaIII} \) and the R645Q mutation by digestion with \( \text{MspI} \). All restriction enzymes were obtained from New England Biolabs.

### 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 \( \text{SREBP-2} \) 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 5/0. In the random control sample, the mean plasma TC concentration, age and gender adjusted, was 4.56 (SD 0.34) mmol/l, the median age was 20.6 years, and the male/female ratio 5/0. In the 162 R371K negative control subjects, the mean plasma TC concentration, age and gender adjusted, was 5.26 (SD 0.99) mmol/l, the median age was 20.5 years, and the male/female ratio 162/0. Neither the V623M nor the R645Q mutation was detected in the controls.

In order to investigate whether the detected amino acid exchanges in \( \text{SREBP-2} \) are located at conserved sites, a multiple sequence alignment with the human, mouse, and Syrian hamster \( \text{SREBP-2} \) sequences was performed. The amino acids arginine and valine at positions 371 (R371K) and 623 (V623M) are conserved across all three mammalian species. The amino acid arginine at position 645 (R645Q), however, is not conserved.

### 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 E\(^{18,19}\) or apolipoprotein B-100,\(^{20}\) only a few sequence variants modifying plasma TC concentrations have been identified so far. FH, FDB, and FDL 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.

Owing to its pivotal function in the regulation of cholesterol homeostasis, \( \text{SREBP-2} \) is an important candidate gene for hypercholesterolaemia. In the present study, its crucial functional domains, represented at the DNA level by exons 5 to 10, were screened for the presence of sequence variations in 70 hypercholesterolaemic subjects. Exons 5 to 10 of the \( \text{SREBP-2} \) gene encode the DNA binding domain of the bHLH-Zip, the two transmembrane domains and the intervening intra-luminal loop, the two cleavage sites, and the adjacent part of the COOH-terminal regulatory domain interacting with \( \text{SCAP} \) (fig 1).

The four novel sequence variations identified in exons 6, 7, and 10 can be assigned to three categories on the basis of the \( \text{SREBP-2} \) protein domain in which the amino acid exchange occurs: (1) one amino acid exchange in the bHLH-Zip domain, (2) two amino acid exchanges in the COOH-terminal regulatory domain, and (3) one translationally silent transition in exon 7.

The R371K amino acid exchange in the bHLH-Zip DNA binding domain of \( \text{SREBP-2} \), identified in three of 70 hypercholesterolaemic subjects (4.3%), is located at a distance of 28

### Table 2 Mutations in the \( \text{SREBP-2} \) 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 \( \text{SREBP-2} \) gene in a total of 70 hypercholesterolaemic subjects.

| Subject | Exon | Amino acid change | Wild type codon | Mutated codon | Protein domain | Age | Gender | TC (mmol/l) | TC adj (mmol/l) | HDLC (mmol/l) | VLDLC (mmol/l) | TG (mmol/l) | ApoE | CAD |
|---------|------|------------------|-----------------|--------------|---------------|-----|--------|---------|-------------|-------------|-------------|------------|---------|-----|-----|
| B0211   | 7    | P433P            | ccc             | cct          | Transitionally silent | 37  | M      | 8.58   | 9.20        | 5.07        | 1.50        | –          | 4.42    | 3/4  |
| B0022   | 10   | V623M            | gtt             | gtt          | Regulatory     | 65  | F      | 9.03   | 8.27        | 7.33        | 1.41        | 0.29       | 2.36    | 3/3  |
| B0083   | 10   | R645Q            | cgg             | cag          | Regulatory     | 49  | F      | 10.04  | 10.32       | 8.28        | 1.41        | 0.35       | 1.35    | 3/3  |

TC:total plasma cholesterol; TC adj:age and gender adjusted total plasma cholesterol; HDLC:low density lipoprotein cholesterol; VLDLC:very low density lipoprotein cholesterol; TG:plasma triglycerides; CAD:coronary artery disease.
amino acids from the residues directly interacting with the nucleotides of the SRE promoter elements. Any decrease in the affinity of the mutated mature SREBP-2, either to its dimeric counterpart or to the SRE promoter elements, would reduce the transcripational activation of genes containing SREs in their promoters. One of the most important genes containing SREs is that encoding the LDL receptor. A reduced activation of the LDL receptor gene would lead to a decreased clearance of LDL particles and, thus, might cause raised plasma LDL cholesterol concentrations. Although more frequent in hypercholesterolaemic subjects (4.3%), the R371K amino acid exchange was also identified in five of 167 (3.0%) randomly selected control subjects. This difference is not statistically significant. All five control subjects had plasma cholesterol concentrations clearly below the age and gender specific 90th centiles. This sequence variant may therefore be considered as a single nucleotide polymorphism (SNP) that causes an amino acid exchange. Nevertheless, extensive population based studies are required to determine whether this SNP modifies plasma TC concentrations.

The V632M 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 concentrations.

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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Another aspect is the question to what extent SREBP-1, the human paralogue 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. This is in contrast to the observation in SREBP-1 gene deficient mice, in which a disruption of the SREBP-1 gene is only partially lethal. 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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