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 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 missense mutations (Val23M, Arg45Q) 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-Pretend 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; FDL, familial defective apolipoprotein B-100; FDB, familial defective apolipoprotein B-100; SNR, single nucleotide polymorphism; TC, total cholesterol; LDL-C, LDL cholesterol; TG, triglycerides; SNR, single nucleotide polymorphism
90th centiles as computed on the basis of the data from the Swiss population (Swiss MONICA study\(^7\)). 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\(^19\) or by single strand conformation polymorphism (SSCP) analysis\(^20\) and Southern blotting.\(^20\) FDB and FDL were diagnosed by direct \(′500Q\) mutation in the apolipoprotein \(E\) gene were excluded using restriction enzyme digestion assays as described by Hansen\(^22\) and Hixson\(^21\) et al\(^\) with modifications.\(^21\) 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 Central Laboratory, University Hospital, Basel. TC concentrations were determined by enzymatic colorimetric cholesterol estimation\(^23\) from LDL cholesterol (LDLc), and triglyceride concentrations (TG) were determined by the Trinder 4-aminophenazone method (Roche Diagnostics, Basel, Switzerland). TG concentrations were determined by the enzymatic colorimetric cholesterol estimation\(^23\) from LDL c, and triglyceride concentrations (TG) were determined by the Trinder 4-aminophenazone method (Roche Diagnostics, Basel, Switzerland).

### 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\(^3\) and a total of 100 ng of genomic DNA, isolated using the salting out procedure with modifications according to Miserez\(^3\) 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 5-16 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 amplicons

PCR amplicons 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, Kyoto, Japan) according to the manufacturer. Forty µl of competent DH5α cells (Gibco BRL, Paisley, UK) were transformed with a 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: PCR and SSCP conditions for the detection of sequence variations in exons 5-10 of the human SREBP-2 gene

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<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>
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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

| Subject | Exon | Amino acid change | Wild type codon | Mutated codon | Protein domain | Age | Gender | TC (mmol/l) | TC adj (mmol/l) | LDLC (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            | gtg            | aig           | 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; LDLC=low density lipoprotein cholesterol; HDLC=high density lipoprotein cholesterol; VLDLC=very low density lipoprotein cholesterol; TG=plasma triglycerides; CAD=coronary artery disease.

Mutations screening of subjects from a random control sample

A control sample consisting of 167 randomly selected subjects from the SPREAD study was directly screened for the sequence variations causing amino acid substitutions in SREBP-2. PCR amplification, restriction enzyme cleavage, and identification of small fragments by polyacrylamide gel electrophoresis (PRECISE) were performed. The R371K sequence variant was screened by digestion with Ddel, the V623M mutation by digestion with NalIII, and the R645Q mutation by digestion with 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 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 (FB), or apolipoprotein E defects (FDB) were excluded from the mutation screening of the SREBP-2 gene as they had an obvious reason for inherited hypercholesterolaemia.

PCR amplicons leading to bands with aberrant electrophoretic migration during SSCP were subcloned and analysed by dideoxy DNA sequencing. In six subjects, four different sequence variations were detected in the investigated coding region of the SREBP-2 gene. All these sequence variations were heterozygous single base pair substitutions. Apart from the translationally silent C to G transition in codon 433 (P433P), the detected sequence variations were amino acid substitutions, R371K, V623M, and R645Q. A summary of the mutations and the corresponding phenotypic data regarding the detected sequence variations were amino acid substitutions in SREBP-2 by direct mutation testing using the PRECISE technique. In addition to the identification of the R371K sequence variant in three out of 70 hypercholesterolaemic subjects (4.3%), the R371K sequence variant was also found in five out of 167 control subjects (3.0%). In the five R371K positive subjects from 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 SREBP-2 are located at conserved sites, a multiple sequence alignment with the human, mouse, and Syrian hamster SREBP-2 sequences was performed. The amino acids arginine and valine at positions 371 (R371K) and 623 (V623M) are conserved between 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 and apolipoprotein B-100, only a few sequence variants modifying plasma TC concentrations have been identified so far. FDB, FDB, and FDB 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, 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 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 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 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 SREBP-2, identified in three of 70 hypercholesterolaemic subjects (4.3%), is located at a distance of 28...
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 transcriptional 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 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. Considering the pivotal role of SCAP as an intramembrane cholesterol sensor regulating the first sterol sensitive cleavage step of SREBP-2, 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. 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 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 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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REFERENCES

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