

## SHORT REPORT

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

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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 NH<sub>2</sub>-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 (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.

Human cells tightly regulate their cholesterol content by controlling their low density lipoprotein (LDL) receptor mediated uptake of extracellular cholesterol containing particles and their intracellular cholesterol biosynthesis.<sup>1</sup> Mutations in the gene encoding the LDL receptor cause familial hypercholesterolaemia (FH).<sup>2</sup> Mutations in the genes encoding the ligands of the LDL receptor, apolipoprotein B-100 and apolipoprotein E, cause familial defective apolipoprotein B-100 (FDB)<sup>3</sup> and familial dysbetalipoproteinaemia (FDL),<sup>4</sup> respectively. All these disorders are characterised by the accumulation of cholesterol rich lipoproteins in the plasma and are associated with a markedly increased risk of coronary artery disease (CAD). The promoters of the LDL receptor gene and of key genes involved in cholesterol and triglyceride homeostasis contain specific nucleotide sequences, so-called sterol regulatory elements (SREs).<sup>1,5</sup> SRE binding proteins (SREBP)-1 and -2 bind to the SREs of the respective genes when activated by sterol regulated cleavage.<sup>6,7</sup> SREBP-1 and -2 are members of the dimeric basic helix-loop-helix leucine zipper (bHLH-Zip) transcription

factor family. They are embedded in the membranes of the endoplasmic reticulum (ER) in a hairpin-like shape; their NH<sub>2</sub>- and COOH-terminal domains are located on the cytosolic surface of the membrane.<sup>8</sup> The NH<sub>2</sub>-terminal domain, which is the mature and, thus, active form of the SREBPs, is released from the membrane by a two step proteolysis. First, the site 1 protease (S1P) cleaves SREBP within the luminal loop between the two membrane spanning segments.<sup>9</sup> Sterols suppress site 1 cleavage by interacting with a polytopic membrane protein designated SREBP cleavage-activating protein (SCAP). SREBP and SCAP interact in the ER membranes by their cytoplasmic COOH-terminal domains.<sup>10</sup> In the second cleavage step, the site 2 protease (S2P) cleaves SREBP within the first transmembrane segment and releases the NH<sub>2</sub>-terminal fragment of the SREBP precursor which enters the nucleus.<sup>11</sup> The binding of the NH<sub>2</sub>-terminal domain of SREBP to specific promoter elements results in an increase in the LDL receptor mediated uptake of LDL particles from the plasma and in an increase in endogenous cholesterol biosynthesis.

As SREBPs play a central role in the regulation of lipoprotein metabolism, mutations in the genes encoding the SREBPs may result in alterations in plasma lipoprotein concentrations. We recently cloned and characterised the genomic structure and the promoter of *SREBP-2* as well as the promoters of the alternatively spliced forms of *SREBP-1*, *SREBP-1a* and *-1c*.<sup>12</sup> Since *SREBP-2*, rather than *SREBP-1a* and *1c*,<sup>13</sup> plays a key role in cholesterol homeostasis<sup>14,15</sup> particularly mutations in *SREBP-2*, may have an influence on plasma cholesterol concentrations.<sup>16</sup> However, no naturally occurring genetic variants in the gene encoding human *SREBP-2* have been described so far. To determine whether genetic variants in *SREBP-2* may influence plasma cholesterol concentrations, we screened the exons that encode the crucial functional domains, namely exons 5 to 10, for sequence variations in hypercholesterolaemic subjects and in randomly selected control subjects.

## 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 elements; ER, endoplasmic reticulum; S1P, site 1 protease; S2P, site 2 protease; SCAP, SREBP cleavage activating protein; TC, total cholesterol; LDLC, 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

Exon	Amplicon size in base pairs	Intronic oligonucleotide primers with <i>EcoRI</i> cleavage sites at the 5' end (5'-3')	[MgCl <sub>2</sub> ] conc (mmol/l)	PCR thermal cycling profile	Conditions of SSCP electrophoresis
5	278	F: cgggaattctattctagaccctgggtgggc R: cgggaattcgtaccacccttggcgctctgt	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: cgggaattctgggtcactctggtttcactcctc R: cgggaattcgcagggtctgacacagccttttctca	3.5	Denaturing: 94°C/45 sec Annealing: 56°C/30 sec Extension: 72°C/1 min Cycles: 32	65 V, 14 h
7	313	F: cgggaattcaaaagcagatcatttaccagggtgggg R: cgggaattctgtgacctcccagctatctttaggggaa	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: cgggaattccacctcgaggccctttttggagggtcaa R: cgggaattccatagtgggtccagccatgcccagg	2.5	Denaturing: 94°C/45 sec Annealing: 60°C/30 sec Extension: 72°C/1 min Cycles: 30	72 V, 14 h
9	268	F: cgggaattctccaagagactctgctgagacga R: cgggaattcgggggaggatgggggaagggcgagaca	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: cgggaattcgcagtgaccattaacaccttttga R: cgggaattctgcagcagccagctatcagcagct	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

90th centiles as computed on the basis of the data from the Swiss population (Swiss MONICA study<sup>17</sup>). 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<sup>18</sup> or by single strand conformation polymorphism (SSCP) analysis<sup>19</sup> and Southern blotting.<sup>20-21</sup> FDB and FDL were diagnosed by direct mutation testing. The R3'500Q 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*<sup>22</sup> and Hixson *et al*<sup>23</sup> with modifications.<sup>24</sup> 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 the enzymatic colorimetric cholesterol 4-aminophenazone method (Roche Diagnostics, Basel, Switzerland), TG concentrations were determined by the Wahlefeld/Trinder 4-aminophenazone method (Roche Diagnostics).

#### 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*,<sup>25</sup> 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  $\mu$ l 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  $\mu$ l of denaturing loading buffer (97% (v/v) formamide, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, 10  $\mu$ mol/l NaOH) were added to the PCR product. After denaturing (95°C) for five minutes and immediate chilling on

ice for 10 minutes, 7  $\mu$ l 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 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, Irvines, 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  $\mu$ l of competent DH5 $\alpha$  cells (Gibco BRL, Paisley, UK) were transformed with a 10  $\mu$ l 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  $\mu$ l were plated on LB agar plates (Bio 101) containing 100  $\mu$ g/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  $\mu$ g/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 2** Mutations in the human *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	Translationally silent	37	M	8.58	9.20	5.07	1.50	–	4.42	3/4	Negative
B0022	10	V623M	gtg	atg	Regulatory	65	F	9.03	8.27	7.33	1.41	0.29	2.36	3/3	Negative
B0083	10	R645Q	cgg	cag	Regulatory	49	F	10.04	10.32	8.28	1.41	0.35	1.35	3/3	Negative

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.

### Mutation screening of subjects from a random control sample

A control sample consisting of 167 randomly selected subjects from the SPREAD study<sup>23</sup> 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 *DdeI*, the V623M mutation by digestion with *NlaIII*, 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 (FBD), or apolipoprotein E defects (FDL) 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 blood lipid and lipoprotein concentrations of the mutation carriers is listed in table 2. An interesting clinical feature was that plasma TG concentrations were raised in the patients carrying the P433P and the V623M mutations, although the inclusion criterion was increased TC levels only. The R371K amino acid substitution was identified in three out of 70 unrelated hypercholesterolaemic subjects (4.3%); the other mutations (P433P, V623M, R645Q) were each found in one subject only (1.4%). The putative role of the sequence variations possibly involved in the development of hypercholesterolaemia in the investigated subjects was assessed by screening a control sample of 167 randomly selected subjects. These subjects were screened for the R371K, V623M, and R645Q 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.<sup>25</sup> 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.

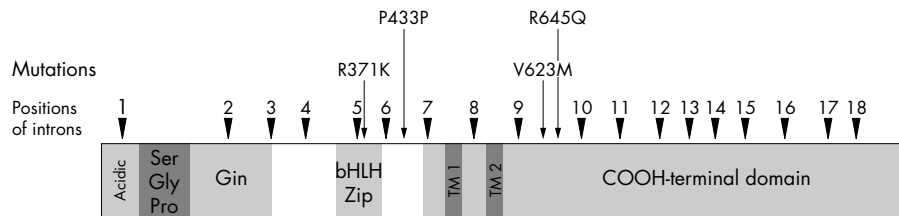
### DISCUSSION

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.<sup>18–24</sup> In line with these findings, studies from others reported the presence of further genomic loci suggested to be responsible for hypercholesterolaemia.<sup>27–29</sup> Moreover, besides polymorphisms in the genes encoding apolipoprotein E<sup>30–31</sup> or apolipoprotein B-100,<sup>32–33</sup> 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.<sup>18–34</sup> 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<sup>1</sup> (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



**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.

amino acids from the residues directly interacting with the nucleotides of the SRE promoter elements.<sup>7</sup> 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.<sup>1-5</sup> 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.<sup>25</sup> 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.<sup>10</sup> Considering the pivotal role of SCAP as an intramembrane cholesterol sensor regulating the first sterol sensitive cleavage step of SREBP-2,<sup>9, 10</sup> an impaired interaction between SREBP-2 and SCAP would result in a decreased cleavage rate of SREBP-2. Consequently, less transcriptionally active NH<sub>2</sub>-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.<sup>35-37</sup> 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.<sup>38</sup> 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.<sup>15</sup> 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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