A novel 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) splice variant with an alternative exon 1 potentially encoding an extended N-terminus

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Abstract

Background: The major rate-limiting enzyme for de novo cholesterol synthesis is 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR). HMGCR is sterically inhibited by statins, the most commonly prescribed drugs for the prevention of cardiovascular events. Alternative splicing of HMGCR has been implicated in the control of cholesterol homeostasis. The aim of this study was to identify novel alternatively spliced variants of HMGCR with potential physiological importance.

Results: Bioinformatic analyses predicted three novel HMGCR transcripts containing an alternative exon 1 (HMGCR-1b, -1c, -1d) compared with the canonical transcript (HMGCR-1a). The open reading frame of the HMGCR-1b transcript potentially encodes 20 additional amino acids at the N-terminus, compared with HMGCR-1a. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used to examine the mRNA levels of HMGCR in different tissues; HMGCR-1a was the most highly expressed variant in most tissues, with the exception of the skin, esophagus, and uterine cervix, in which HMGCR-1b was the most highly expressed transcript. Atorvastatin treatment of HepG2 cells resulted in increased HMGCR-1b mRNA levels, but unaltered proximal promoter activity compared to untreated cells. In contrast, HMGCR-1c showed a more restricted transcription pattern, but was also induced by atorvastatin treatment.

Conclusions: The gene encoding HMGCR uses alternative, mutually exclusive exon 1 sequences. This contributes to an increased complexity of HMGCR transcripts. Further studies are needed to investigate whether HMGCR splice variants identified in this study are physiologically functional.

Keywords: 3-hydroxy-3-methylglutaryl-coenzyme A reductase, HMG-CoA, Transcription, Alternative splicing, Statin, Cholesterol

Background

Human 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGCR) is the rate-limiting enzyme in the isoprenoid/cholesterol biosynthesis pathway. The enzyme converts HMG-CoA to mevalonate and is the main target of commonly used lipid-lowering drugs (i.e., statins) [1]. Some of the major intermediates in the pathway are used to synthesise ubiquinone, dolichol, heme A and isoprenoid lipids [2]. The end-product, cholesterol, is the precursor for the synthesis of crucial compounds, such as steroid hormones, bile acid and vitamin D.

HMGCR is encoded by a single gene on chromosome 5 (5q13.3-q14). Two transcript variants are annotated in the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) database; the canonical full-length transcript composed of 20 exons (NM_000859.2) and an alternatively spliced variant lacking exon 13 (NM_001130996.1). Additionally, exon junction microarray experiments suggest the presence of at least three more HMGCR splice variants lacking exon 8 (AY429542), exon 18 (AY429543) and exons 17 and 18 (AY429544) [3].
Expression of HMGCR is strictly regulated at the transcriptional and post-transcriptional level. Cholesterol homeostasis is maintained by HMGCR transcriptional inhibition and enzyme modifications via negative feedback mechanisms (reviewed in [4]). Under sterol-depleted conditions, such as statin treatment, HMGCR transcription is co-ordinately activated with transcription of the low density lipoprotein receptor and other genes in the cholesterol biosynthetic pathway [5].

Genomic diversity is greatly expanded by the process of alternative splicing, by which introns are removed and exons are ligated together to form various mature mRNA transcripts. Alternative splicing has been suggested as another mechanism underlying HMGCR regulation and cholesterol homeostasis [6]. An HMGCR splice variant lacking exon 13 (denoted here as HMGCRΔexon13) was reported to have decreased enzymatic activity and to be less sensitive to statin inhibition [3,7,8]. Exon 13 was reported to have decreased enzymatic activity and to be less sensitive to statin inhibition [3,7,8]. Exon 13 was found to be associated with skipping of exon 13 [7]. The relative abundance of HMGCRΔexon13 mRNA has also been reported to be associated with interindividual variation in statin-mediated lipid lowering responses [8,11]. In the present study, nucleotide sequence database queries identified several novel HMGCR splice variants. One of these, HMGCR-1b, shows alternative exon 1 usage, with a novel HMGCR N-terminus predicted. We report the mRNA levels of novel HMGCR splice variants in a variety of healthy human tissues. Furthermore, we present the regulatory responses of these variants in HepG2 cells upon statin treatment.

Methods

Bioinformatic analysis
Inspection of displayed mRNA, expressed sequence tag (EST) and RNA sequencing (RNA-seq) tracks at the HMGCR gene locus was performed using the Genome Browser Database provided by the University of California, Santa Cruz (UCSC) on human genome assembly GRCh37/hg19 [12]. The other bioinformatic tools used were Primer3 software [13], Finch TV1.4.0 (Geospiza, Seattle, USA), DNASTAR Lasergene 7.2 (Madison, WI, USA) and the NCBI database of SNPs.

Cell culture and statin treatment
The HepG2 cell line (American Type Culture Collection, Manassas, VA, USA) was grown in modified Eagle’s minimal essential medium (MEM; ATCC) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1× penicillin-streptomycin-glutamine (P/S/G) mixture (Sigma-Aldrich, St Louis, MO, USA) in collagen I-coated tissue culture flasks (BD Biosciences, San Jose, CA, USA). Cells were seeded at 2 × 10^5 cells/mL in 12-well collagen I-coated plates (BD Biosciences), and the statin treatment was carried out in triplicate on the following day. HepG2 cells were cultivated for 24 hrs in MEM with 1× P/S/G containing 3 mg/mL of human lipoprotein deficient serum (LPDS; Millipore, Billerica, MA, USA), with or without 10 μM of (3 S, 5 S)-Atorvastatin Sodium Salt (Toronto Research Chemicals North York, Ontario, Canada) dissolved in water in a total volume of 1 mL. Two lymphoblastoid cell lines (GM17752 and GM20518; Coriell Institute for Medical Research, Camden, NJ, USA) were cultured according to the manufacturer’s instructions. Sequencing confirmed the CC and AA genotypes of the rs3761740 polymorphism in GM17752 and GM20518 cell lines, respectively. Statin treatment was carried out as described above, except that 5 × 10^5 cells/mL of each cell line were seeded into 24-well plates and treated the same day. Cell cultures were maintained at 37°C/5% CO2.

Purification of total RNA from cells
Total RNA from HepG2 cells was isolated using an RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) by following the protocol provided by the manufacturer. DNase digestion with an RNase-Free DNase Set (Qiagen) was used to ensure DNA-free RNA samples. The RNA was eluted in 30 μL of RNase/DNase-free water and stored at −80°C until required. RNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was assessed by microfluidic capillary electrophoresis using an Agilent 2100 Bioanalyzer and the RNA 6000 Nano Chip kit (Agilent Technologies, Santa Clara, CA, USA). RNA samples were denatured for 2 min at 70°C prior to cDNA synthesis.

Total RNA from human tissues
High-quality pooled RNA samples from the Human Total Master panel II and the Human Adult Normal Tissue panel were purchased from Clontech (Mountain View, CA, USA) and BioCat GmbH (Heidelberg, Germany), respectively. Ten random RNA samples were assessed for concentration and integrity as described above. All samples were from anonymous donors and commercially available.

Synthesis of cDNA
The levels of mRNA expression were analysed using total RNA from tissues (1000 ng) and cells (200 ng) in a 20 μL cDNA synthesis reaction using an Omniscript RT Kit (Qiagen) and a mixture of 2.5 μM oligo-dT and 6.25 μM random hexamers (Applied Biosystems, Foster City, CA, USA). Reverse transcription (RT) was performed
at 37°C for 60 min, followed by 95°C for 5 min. The cDNA samples were diluted 1:5 in RNase/DNase-free water and stored at −20°C prior to use as templates for PCR amplification. A pooled cDNA sample was obtained by mixing an aliquot of each of the 44 undiluted cDNA samples and a 1:10 dilution in RNase/DNase-free water.

**PCR**

Polymerase chain reaction (PCR) amplification of the different first exons of *HMGCR* in a cDNA pool derived from 44 human tissues (as described above) was performed with HotStarTaq DNA Polymerase (Qiagen) following the manufacturer’s instructions. Forward primers (exon1a, 5′-GAG GTG TCG TAA GGT GAG G-3′; exon1b, 5′-GAG AGC AGA AGG AAC GCA CA-3′; exon1c, 5′-GAC ATG GTC CTG CAG ATG CG-3′; and exon1d, 5′-GCA GCA TTG CAT AAA TAC TGT CA-3′) were used in combination with a reverse primer in exon 3 (exon3, 5′-TGT CAG AAT TAT AAT GTC ACT GCT CAA-3′). The cycling conditions were 15 min at 95°C, followed by 37 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C and a final extension step at 72°C for 10 min. PCR products were separated on 1–3% (w/v) agarose gels. Images were captured using a Kodak Image Station 440CF and visualised using Kodak Molecular Imaging Software, version 4.5 (Eastman Kodak Co., New Haven, CT, USA). The PCR products of interest were excised and purified using the QIAquick Gel Extraction Kit (Qiagen) and confirmed by sequencing.

**Molecular cloning**

The full-length *HMGCR*-1b transcript was PCR amplified using a ProofStart DNA polymerase kit (Qiagen) and a cDNA template synthesised from skin (BioCat GmbH). The PCR mix contained 1× proof start buffer, 1× Q-solution, 0.25 U ProofStart, 5 U HotStarTaq plus DNA polymerase, 350 mM dNTP mix, 2 μL of cDNA and 0.4 μM forward primer (exon1b, 5′-GAG AGA AGG AGA GGC ACA TTT CAG-3′) and reverse primer (exon2, 5′-GGA GGG GCT TCT TCT TGG TGC AAG C-3′) in a total volume of 50 μL. The cycling conditions were 5 min at 95°C, followed by 37 cycles of 30 s at 94°C, 1 min at 55°C, 3 min at 72°C and a final extension step at 72°C for 30 min. Purified PCR products were cloned using the CT-GFP Fusion TOPO TA Expression Kit (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer’s instructions. Positive clones were confirmed by sequencing.

**Quantitative PCR (qPCR)**

All qPCRs were performed on an ABI 7900HT Real-Time PCR System in a standard 96-well format. TaqMan hydrolysis probes (Custom TaqMan Gene Expression Assay from Applied Biosystems) were designed for specific gene quantification of *HMGCR*-1a, *HMGCR*-1b and *HMGCR*-1c. The following TaqMan primer and probe sets were used: 5′-CCA TGC ATT CGA AAA AGT CTT GAC A-3′ (forward primer), 5′-GTT TCG GTG GCC TCT AGT G-3′ (reverse primer) and the FAM-reporter probe 5′-CTG GAT CCT CCA GAT CT-3′ for targeting the *HMGCR* exon 1a-exon 2 boundary; 5′-GAT GGA TGT CGC ACA CAA GAG A-3′ (forward primer), 5′-CCA TGC ATT CGA AAA AGT CTT GAC A-3′ (reverse primer), and the FAM-reporter probe 5′-CTT GGA TCC TGC GTC TCT-3′ for targeting the *HMGCR* exon 1b-exon 2 boundary; and 5′-TTT CTG GGC TAC TAT ACT AAT GCA TGC-3′ (forward primer), 5′-CCA TGC ATT CGA AAA AGT CTT GAC A-3′ (reverse primer) and the FAM-reporter probe 5′-ACA AGG ATCCAA GGA TTC GAT A-3′ (forward primer) and the FAM-reporter probe 5′-ACA AGG ATCCAA GGA TTC GAT A-3′ for targeting the *HMGCR* exon 1c-exon 2 boundary. Each qPCR mix comprised 10 μL of TaqMan Universal PCR Master Mix, 7 μL of RNase-free water, 1 μL of TaqMan hydrolysis probe and 2 μL of cDNA. The cycling steps were 10 min at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Non-template controls were included in each run. All qPCR experiments were performed at least in duplicate. Samples exhibiting more than a 0.5 quantification cycle (Cq) difference between the duplicates were excluded from the relative quantification analysis. Samples showing Cq > 35 or undetermined were set to Cq = 38 and were considered not detectable.

**Reference gene selection**

The stability of 12 reference genes from Applied Biosystems (see [14] for assay IDs) was checked to confirm the reliability of gene expression results across the tissue samples tested. Five (GAPDH, PGK1, SDHA, CTBP1, GOLGA1) were found by geNorm [15,16] to give the most suitable normalisation factors for quantification of relative expression levels. For the atorvastatin treatment experiments in HepG2 cells, the PPIA, TBP, PGK1, ACTB, GAPDH, LUC, PPIB, and SDHA reference genes were tested. Two reference genes, PPIA and TBP, were found by geNorm to be the most stable and therefore were suitable for normalisation. The mean expression value was used for the normalisation, according to the “ΔΔCq method” for comparing relative expression results between treatments [17]:

\[
\text{ΔΔCq} = 2^{-(\Delta Cq_{\text{treated}} - \Delta Cq_{\text{non-treated}})}
\]

**Reporter vector construction**

Different regions of the human *HMGCR* promoter covering −1626 to +12 (relative to the annotated *HMGCR* transcription start) were amplified by PCR. Genomic DNA from the Human Variation Panels (NA07055;
Coriell Institute for Medical Research) heterozygous for the HMGCR promoter SNP C>A (rs3761740) were used as PCR templates. The PCR primers were: 5’-AGC GGT ACC GCC ATT TAC ACT AAT GGG TAA AT-3’ (forward primer) and 5’-AGT AAG CTG GCC ATT TAC ACT AAT GGG TAA AT-3’ (reverse primer) for construction of the reporter vector 1 (~559 to +12); 5’-AGC GGT ACC AAT GGG TAG GCA TAT CCA AGG CT-3’ (forward primer) and 5’-AGT AAG CTG GCC ATT TAC ACT AAT GGG TAA AT-3’ (reverse primer) for construction of reporter vectors 2 and 3 (~1009 to ~615); and 5’-AGC GGT ACC TTG ACA AGG CTG CTA AGA GCC ATT TAC ACT AAT GGG TAA AT-3’ (forward primer) for construction of vector-reporter vectors 4 and 5 (~1626 to ~615) in combination with the same reverse primer used for reporter vectors 2 and 3. Restriction sites for KpnI and HindIII are underlined in the forward and reverse primer sequences, respectively. The PCR was performed using a Phusion High-Fidelity PCR kit (Finzymes Oy, Espoo, Finland) according to the manufacturer’s instructions. Cycling conditions were as follows: 30 s at 98°C, followed by 35 cycles of 10 s at 98°C, 30 s at 63°C, 1 min at 72°C and a final extension step of 10 min at 72°C. The PCR-amplified promoter regions were cloned into the KpnI and HindIII restriction sites of the promoterless firefly luciferase vector pGL4.10 (Promega, Madison, WI, USA).

Transfection and reporter gene assay
HepG2 cells were seeded on the day prior to transfection at 1.25 x 10^5 cells/mL into a 24-well collagen I-coated plate (BD Biosciences) in 0.5 mL of culture medium. For each transfection, 485 ng of the firefly luciferase empty control vector (pGL4.10) or a test reporter vector was co-transfected with 15 ng of renilla luciferase internal control vector (pGL4.73) to normalise for transfection efficiency. Transfection was carried out in triplicate using 1.25 μL of FuGENE HD (Roche Diagnostics Corporation, Indianapolis, IN, USA) reagent per well. Cells were washed with 1 mL 1x phosphate-buffered saline at 24 h post-transfection and then treated with 100 μL of Passive Lysate Buffer supplied in the Dual-Glo luciferase assay kit (Promega). After gentle rocking (15 min), the lysates were assayed for luciferase activity with the Dual-Glo luciferase assay system according to the manufacturer’s instructions. Firefly luciferase and *renilla* luciferase activities were measured using the Wallac 1420 Victor™ luminometer in a 96-well Wallac B&W Isolipple™ (Perkin Elmer, Turku, Finland). Delay and measurement times were set to 2 s and 10 s, respectively. The linear range of light detection was determined using a dilution series of QuantiLum Recombinant Luciferase (Promega). Firefly luciferase activity was normalised to *renilla* luciferase expression. Luciferase (Luc) activity induction was calculated as fold-change according to the following equation:

\[ \text{Fold} = \frac{\text{Mean Firefly vector}}{\text{Mean Firefly empty control vector}} \]

Results
Identification of HMGCR splice variants
Bioinformatic analysis and visual inspection of the region between HMGCR exon 2 and its adjacent, 5′-flanking gene in the UCSC genome browser indicated at least three alternative first exons, each spliced to the constitutive exon 2 (Acc. No: AK296499, DA809145, BX952828, designated exons 1b, 1c and 1d, respectively; Figure 1A). Tracks of published RNA-seq data indicated transcription of exon 1b, whereas no convincing evidence was found for transcription of exons 1c and 1d. Transcription of exons 1b and 1c was confirmed by RT-PCR (Figure 1B) of the cDNA pool derived from 44 different tissues. Expression of the predicted exon 1d could not be confirmed by RT-PCR in any of the investigated tissues. The open reading frame of the canonical HMGCR transcript (=888 amino acids) is maintained in exon 1c, but sequence analysis of exon 1b predicted an additional 20 amino acids at the N-terminus. We cloned and sequenced HMGCR-1b (exon 1b–exon 20) and confirmed that exons 1a and 1b were mutually exclusive. We also found that HMGCR-1b transcripts were subject to exon 13 skipping (HMGCR-1bΔ13) (Figure 1C). Exon 1b is potentially translated in certain primates as indicated by alignment of nucleotide sequences from different vertebrate species (Figure 1D).

Tissue distribution of HMGCR-1a, 1b and 1c
The RT-qPCR experiments showed variable levels of alternative first exons of HMGCR in 36 different tissues (Figure 2). The canonical HMGCR-1a transcript was ubiquitously expressed at the highest levels in the cerebellum, fetal brain, testis, skin and adrenal gland (Figure 2A). The highest expression of HMGCR-1b was detected in the skin, esophagus, uterine cervix and prostate (Figure 2B). HMGCR-1b mRNA levels were below detection levels (Cq > 35.0) in six tissues. The level of HMGCR-1a in liver (pooled from three different donors) was approximately two-fold higher than that of HMGCR-1b. We observed higher HMGCR-1a expression in most of the tissues analysed with the exception of the skin, esophagus, uterine cervix and colon, in which the levels of HMGCR-1b were higher (approximately 1.5–2.0-fold; Figure 2D). HMGCR-1c was detectable (Cq < 35) in only nine of the tissues analysed and was less abundant than HMGCR-1a. The highest expression of
HMGCR-1c was detected in the cerebellum, fetal brain, testis and adrenal gland (Figure 1C). HMGCR-1c could not be quantified in the skin or uterine cervix (Cq > 35), where HMGCR-1b was relatively abundant.

Expression analysis before and after atorvastatin treatment in HepG2 cells

HepG2 cells, a model system for cholesterol biosynthesis studies, were treated with atorvastatin for 24 h. The RT-qPCR experiments showed significant mRNA induction (p < 0.05) for HMGCR-1a (2.1 ± 0.2-fold (mean ± SE; Figure 3A), HMGCR-1b (1.6 ± 0.2-fold; Figure 3B) and HMGCR-1c (2.6 ± 0.3-fold; Figure 3C) in cells after atorvastatin treatment compared with untreated cells. The magnitude of induction was significantly different between HMGCR-1a and HMGCR-1b, and between HMGCR-1b and HMGCR-1c (p < 0.05), but not between HMGCR-1a and HMGCR-1c (p = 0.16). The HMGCR-1c and HMGCR-1b splice variants were less abundant (approximately 60-fold lower) in HepG2 cells compared with the canonical HMGCR-1a. The fact that exon 1b was found to be approximately two-fold lower than exon 1a in the liver suggests that there are major differences in the expression levels of these transcripts, either between different liver donors or because of cell immortalisation and/or an artificial cell culture environment for the HepG2 cells.

HMGCR promoter analysis

Bioinformatics showed that exon 1b is located 35 bp upstream of the CpG island in the previously characterised HMGCR promoter. We performed luciferase reporter gene assays in HepG2 cells under various conditions to investigate the promoter activity driving the transcription of HMGCR-1b and the genetic influence of the promoter SNP rs3761740.
Figure 2 Expression of HMGCR-1a, -1b and -1c in various human tissues. HMGCR-1a, -1b and -1c mRNA levels were determined by RT-qPCR in various human tissues using exon 1-specific primers. (A) HMGCR-1a, (B) HMGCR-1b (C) HMGCR-1c mRNA levels were normalised to a tissue-dependent factor based on five reference genes and calculated as ratios relative to the highest mRNA level (reference), which was set to 1. (D) Ratios of HMGCR-1a and HMGCR-1b mRNA levels are given on a logarithmic scale (base 2). HMGCR-1b is in low abundance compared with the canonical HMGCR-1a in most of the analysed tissues (ratio >0), except in the skin, esophagus, uterine cervix and colon (ratio <0).
promoter region upstream of exon 1a (reporter vector 1, which was used as a positive control) was active under standard culture conditions with 10% FBS (Figure 4). The exon 1a promoter was further activated under sterol-depleted conditions with LPDS (1.7 ± 0.2-fold) and LPDS + atorvastatin (3.0 ± 0.6-fold) when compared with the activity of transfected cells cultured under standard conditions, containing 10% FBS. The reporter vectors 2,
3, 4 and 5, each containing genomic fragments upstream of exon 1b, exhibited low promoter activities that were not induced by sterol depletion (LPDS), or by higher degrees of sterol depletion (LPDS + atorvastatin). The minor A allele of the promoter SNP C > A (rs3761740) did not affect the promoter activity of exon 1b when compared with the major allele C reporter vector. The effect of the promoter SNP C > A (rs3761740) on HMGCR exons 1a and 1b mRNA levels was investigated after atorvastatin treatment in two different lymphoblastoid cell lines homozygous for either allele. The RT-qPCR experiments (n = 5) showed similar induction of HMGCR-1b mRNA levels after atorvastatin treatment in both lymphoblastoid cell lines, independent of the allelic status. HMGCR-1b was induced 1.2 ± 0.1-fold (mean ± SE) and 1.3 ± 0.1-fold in cells with the major CC and the minor AA genotype of rs3761740, respectively. The magnitude of induction was not significantly different between the two different cell lines. The canonical HMGCR-1a transcript was upregulated 1.7 ± 0.1-fold in both lymphoblastoid cell lines after atorvastatin treatment.

Discussion

HMGCR is the key enzyme in the cholesterol biosynthesis pathway and is the major target of cholesterol-lowering statin drugs. Here, we demonstrate the existence of alternative exon 1 usage in human HMGCR, resulting in several novel transcript variants. One of these, HMGCR-1b, encodes a novel N-terminus which potentially extends the membrane domain by 20 amino acids compared with the canonical sequence. The canonical HMGCR protein is composed of 888 amino acids with a regulatory, sterol-sensing transmembrane domain (residues 10 – 339), a flexible linker (residues 340 – 449) and a C-terminal catalytic region (residues 450 – 888) [18]. We studied HMGCR-1b at the genomic and transcriptional levels. Whether the translation machinery actually recognises the first AUG as a start codon to produce a protein with 20 additional amino acids in the membrane domain remains to be determined. Unfortunately, initial experiments using rabbit antibodies raised against the novel N-terminus of the predicted HMGCR-1b polypeptide exhibited non-specific binding (data not shown).

In mammalian genomes, only one gene encodes the HMGCR enzyme, which mainly resides in the membrane of the endoplasmic reticulum [19,20]. A minor fraction (approximately 5%) of the reductase activity has been demonstrated in rat liver [21-23] and mouse brain [24] to be localised in the matrix of peroxisomes. The signal that targets HMGCR to peroxisomes has not yet been determined. The HMGCR-1b transcript identified in this study is remarkably less abundant than the canonical HMGCR-1a in most of the tissues analysed, and it is tempting to speculate that exon 1b might encode the missing HMGCR peroxisomal targeting information [25]. However, bioinformatic analysis of the N-terminus of the hypothetical HMGCR-1b protein did not reveal any peroxisomal targeting signals (data not shown).

Our finding that HMGCR generates multiple 5’ end transcripts is not unusual in the human genome [26]. There are numerous other examples in the literature. The gene locus encoding the uridine diphosphateglucuronosyltransferase 1 family contains several different coding first exons, which generate at least nine different transcripts encoding various isoenzymes with unique N-termini and substrate specificities [12]. Another example is the CYP19A1 gene encoding aromatase, the rate-limiting enzyme of estrogen biosynthesis, which contains alternative non-coding first exons affecting RNA stability and aromatase levels in tissues. Differential use of non-coding first exons is suggested to be an important mechanism of post-transcriptional regulation of aromatase gene expression [27].

Whether an alternatively spliced variant is functional is considered in the context of tissue-specific expression and conservation in most studies [28]. The mRNA levels for the alternative HMGCR-1b splice variant were more abundant than the canonical HMGCR-1a levels in epithelial tissues, such as skin. Skin is one of the major sites for cholesterol production in the body [29], where HMGCR activity and cholesterol synthesis are crucial for hindering fluid loss from the body by maintaining the permeability barrier [30]. In addition, the production of non-cholesterol isoprenoid intermediates by HMGCR has been reported to be important during acute wound healing [31]. Sequence alignment of HMGCR-1b did not show conservation of the open reading frame in all terrestrial animals. Therefore, it remains to be determined whether the HMGCR-1b splice variant exerts an epithelial or skin-specific role in sustaining the epithelial barrier function in certain primates.

Tissue-specific gene expression may be regulated via differential gene promoter activity [32]. The weak promoter activity of the genomic region upstream of exon 1b is in accordance with the rather low, basal mRNA expression level of exon 1b found in HepG2 cells. Unexpectedly, the alternative promoter activity in the reporter assay was not induced by sterol depletion, although there was an increase in the HMGCR-1b mRNA level in HepG2 cells. Based on these results, the increased HMGCR-1b mRNA level due to sterol depletion in HepG2 cells most likely results from post-transcriptional regulation and/or regulatory promoter DNA sequences not captured in our reporter vector constructs. Moreover, previous studies have identified a functional C > A
SNP (rs3761740) in the HMGCR promoter [23,33]. Keller et al. identified a sterol regulatory element (SRE) in this position. They found that the minor A allele was more responsive to the overexpression of sterol regulatory element-binding protein 2, compared with the major C allele [33]. In our experiments, this promoter SNP, which is close to the transcription start of exon 1b, did not contribute significantly to the proximal promoter activity or mRNA level of HMGCR-1b or to the transcription of the canonical HMGCR-1a variant upon sterol depletion.

Approximately half of human genes are predicted to have alternative promoters [34]. A comparative study of different promoter features in human and mouse genes reported a distinct class of “non-conserved” promoters in humans [35]. Alternative promoters differ from the major promoter in their responsiveness to a specific condition, resulting in different expression levels in tissues or developmental stages and thereby increasing the flexibility of gene expression. Given that the putative HMGCR-1b promoter identified in our study (i) exhibits a different response to statin treatment compared with the canonical HMGCR promoter, (ii) is of minor usage, (iii) is lacking CpG islands, and (iv) that the HMGCR-1b transcript shows an expression pattern distinct from that of HMGCR-1a, it seems likely that the HMGCR-1b promoter represents an alternative, non-conserved HMGCR promoter that contributes to the expression diversity of this gene.

With respect to HMGCR-1a, we also found that exon 13, which encodes amino acids in the catalytic region, is a cassette exon in the HMGCR-1b transcript, meaning that this exon is either included (HMGCR-1b) or skipped (HMGCR-1bA13). The catalytically active HMGCR is formed as a tetramer with two dimers [10]. Medina et al. have proposed that the inactive HMGCR lacking exon 13 modulates the HMGCR enzyme activity by being part of the catalytically active tetramer and thereby reducing its total enzyme activity [11]. Further studies are needed to clarify whether the delta-exon 13 variant of the HMGCR-1b transcript also contributes to HMGCR enzyme activity modulation and statin response.

Conclusions
We have shown that human HMGCR uses different first exons to produce multiple alternatively spliced variants, thereby contributing to increased transcriptional complexity. We have designated these splice variants HMGCR-1a, HMGCR-1b and HMGCR-1c. The novel HMGCR exon 1 (HMGCR-1b) we identified contains an in-frame ATG translational start codon predicting an extended N-terminus of HMGCR. Moreover, we showed that this transcript variant is differentially expressed in a wide variety of healthy human tissues, and that its mRNA levels are regulated upon statin treatment. Further studies are needed to elucidate potential physiological roles of differential first exon usage in HMGCR.

Abbreviations
HMGCR: 3-Hydroxy-3-Methylglutaryl-Coenzyme A reductase; HMG-CoA: 3-Hydroxy-3-Methylglutaryl-Coenzyme A; SNP: Single-Nucleotide Polymorphism; EST: Expressed Sequence Tag; RNA-seq: RNA sequencing; MEM: Modified Eagle’s Minimal essential medium; FBS: Fetal Bovine Serum; LPDS: Lipoprotein Deficient Serum; RT: Reverse Transcription; qPCR: Quantitative Polymerase Chain Reaction; Luc: Luciferase; Atv: Atorvastatin.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
CS carried out the experimental work and drafted the manuscript with assistance from RMG and M KK. AP, MKK and JPB participated in the study design, interpretation of data and revising the manuscript. All authors read and approved the final manuscript.

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