**RP1-13D10.2 Is a Novel Modulator of Statin-Induced Changes in Cholesterol**

Katrina Mitchel, PhD; Elizabeth Theusch, PhD; Celia Cubitt, BS; Andréa C. Dosé, PhD; Kristen Stevens, BS; Devesh Naidoo, BS; Marisa W. Medina, PhD

**Background**—Numerous genetic contributors to cardiovascular disease risk have been identified through genome-wide association studies; however, identifying the molecular mechanism underlying these associations is not straightforward. The Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) trial of rosuvastatin users identified a sub–genome-wide association of rs6924995, a single-nucleotide polymorphism ≈10 kb downstream of myosin regulatory light chain interacting protein (MYLIP, aka IDOL and inducible degrader of low-density lipoprotein receptor [LDLR]), with LDL cholesterol statin response. Interestingly, although this signal was initially attributed to MYLIP, rs6924995 lies within RP1-13D10.2, an uncharacterized long noncoding RNA.

**Methods and Results**—Using simvastatin and sham incubated lymphoblastoid cell lines from participants of the Cholesterol and Pharmacogenetics (CAP) simvastatin clinical trial, we found that statin-induced change in RP1-13D10.2 levels differed between cell lines from the tails of the white and black low-density lipoprotein cholesterol response distributions, whereas no difference in MYLIP was observed. RP1-13D10.2 overexpression in Huh7 and HepG2 increased LDLR transcript levels, increased LDL uptake, and decreased media levels of apolipoprotein B. In addition, we found a trend of slight differences in the effects of RP1-13D10.2 overexpression on LDLR transcript levels between hepatoma cells transfected with the rs6924995 A versus G allele and a suggestion of an association between rs6924995 and RP1-10D13.2 expression levels in the CAP lymphoblastoid cell lines. Finally, RP1-13D10.2 expression levels seem to be sterol regulated, consistent with its potential role as a novel lipid regulator.

**Conclusions**—RP1-13D10.2 is a long noncoding RNA that regulates LDLR and may contribute to low-density lipoprotein cholesterol response to statin treatment. These findings highlight the potential role of noncoding RNAs as determinants of interindividual variation in drug response. (Circ Cardiovasc Genet. 2016;9:223-230. DOI: 10.1161/CIRCGENETICS.115.001274.)

**Key Words:** cholesterol ■ genome-wide association studies ■ long noncoding RNA ■ low-density lipoprotein cholesterol ■ simvastatin

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Elevated plasma low-density lipoprotein cholesterol (LDL-C) is a significant risk factor for cardiovascular disease, the leading cause of death in the world. Statins are the most widely prescribed class of drugs used to lower blood LDL levels and reduce cardiovascular disease risk. Specifically, statins competitively inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), the rate-limiting enzyme in the cholesterol biosynthesis pathway, and thus stimulate hepatic uptake of LDL through upregulation of the low-density lipoprotein receptor (LDLR). Although statin efficacy for reducing cardiovascular disease mortality has been well established, there is still substantial residual risk on treatment, and interindividual response with regard to statin effects on cholesterol lowering remains a concern.

Although factors such as smoking status, race, and age have been reported to affect statin efficacy, the pharmacogenomics of statin response is an area of active study. Both clinical trial and population-based cohorts have identified variants in genes such as LPA, APOE, SORT1, HMGCR, and LDLR that were associated with statin effects on LDL lowering. To date, the largest genome-wide association study performed in a single statin clinical trial was reported in 7000 participants of the Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) clinical trial. Among the gene variants identified from this analysis, Chasman et al. reported a sub–genome-wide significant association (P<1×10^{-6}) between rs6924995 and statin-induced change in plasma LDL-C. This particular association was notable because it was attributed to myosin regulatory light chain interacting protein (MYLIP, aka IDOL and inducible degrader of LDLR), an E3 ubiquitin ligase that regulates LDLR stability.

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and thus activity, in response to changes in intracellular cholesterol levels. Although rs6924995 is located 10 kb downstream of MYLIP, to date, there is no evidence that rs6924995 affects MYLIP expression levels or function.

Interestingly, rs6924995 is located within RP1-13D10.2, a processed pseudogene. Although RP1-13D10.2 has no known function, many pseudogenes have potential biological functions as noncoding RNAs. In addition, long noncoding RNAs (lncRNAs) have been implicated in cardiovascular disease. For example, the relationship between genetic variation at chromosome 9p21 associated with atherosclerotic risk has been attributed to expression changes in an antisense noncoding RNA. Recently, the lncRNA RP5-833A20.1 was shown to modulate cholesterol homeostasis; however, the potential involvement of lncRNAs in statin response has not yet been assessed. Thus, here we sought to determine if RP1-13D10.2 acts as a novel lncRNA regulating cellular cholesterol metabolism, specifically hypothesizing that RP1-13D10.2 may mediate the association between rs6924995 and statin-induced change in LDLC.

Methods

Cell Culture

Lymphoblastoid cell lines (LCLs) from donors of the cholesterol and pharmacogenetics (CAP) population (ClinicalTrials.gov ID: NCT00451828) with rs6924995 genotypes previously imputed were grown in RPMI Medium 1640 supplemented with 10% fetal bovine serum (Hyclone), 500 U/mL penicillin/streptomycin and 2 nmol/L GlutaMAX. Cells were exposed to 2 µmol/L activated simvastatin or milrinone, and transcript levels were quantified using a quantitative PCR SYBR Green assay with the following primers: RP1-13D10.2 (TGTGGCTCTATCACCCTCAA and AAGATGATTGCGAACACAGC), MYLIP (TCTCTTCTGCCACCTTGAC and TCTATGCGCCACCATCT), RP1-13D10.3 (TGGAAAACAAAACARTGCTCA and TGGATTGCGAAA CACTCAATT), and RP1-13D10.4 (CAGGAAGTTAGCCCTGCTACC and TGGTTGTTAAGGATGTTTGT). HMGC, LDLR, HMGCS1, and PCSK9 were quantified by quantitative PCR with assays as previously reported. All reactions were performed in triplicate on an ABI PRISM 7900 Sequence Detection System. Given the lack of introns in RP1-13D10.2, RP1-13D10.3, and RP1-13D10.4, no reverse transcriptase controls were prepared for each sample, and transcript levels of these lncRNAs were calculated as the difference of the reverse transcriptase versus no reverse transcriptase to prevent detection of residual genomic DNA. All values were normalized to CLTPM1 as a loading control as previously described.

RNA-seq Library Preparation and Data Analyses

RNA-seq analyses were performed on 75 sham- and statin-exposed CAP LCLs chosen from the tails of the plasma LDLC statin response distribution (Table; Figure I in the Data Supplement) that survived quality control criteria described below. These RNA-seq data form a subset of the RNA-seq data deposited in dbGaP under accession phs000481.v2.p1. Starting with 500 ng of total RNA, LabCorp (formerly Covance, Seattle, WA) made indexed, strand-specific, paired-end Illumina sequencing libraries, which were then sequenced at the University of Washington on Illumina HiSeq 2000 machines with a 101 bp read length. Paired-end fragments were aligned to the human (hg19) and EBV (NC_007605) genomes with Bowtie. Suspected polymerase chain reaction (PCR) duplicates were removed using Picard MarkDuplicates, and RNA genotypes in well transcribed (read depth >30) regions (that were also directly genotyped in the corresponding individuals’ genomic DNA) were estimated using allelic ratios derived from the SAMtools pileup command and compared with genonomic genotypes as an identity check. Samples of unknown identity, sex mismatches, sample mixes, samples that were 5’ to 3’ bias outliers (evaluated using Picard Collect RNaseqMetrics), and LCLs that did not have paired control and statin RNA-seq data were excluded from analyses. Aligned fragments in known Ensembl v67 genes were counted using HTSeq-count, adjusted for library size, and variance stabilized using DESeq. Statin-induced changes in gene expression (gene expression δ) were calculated as statin-sham variance stabilized expression levels. Statistically significant differences between statin-induced changes in gene expression between LDLC response groups (ie, black high versus low or white high versus low) was calculated from the variance stabilized δ using a 2-tailed t test.

Table. Clinical Characteristics of Study Participants Split by Race and LDLC Statin Response

<table>
<thead>
<tr>
<th></th>
<th>White</th>
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<tr>
<td></td>
<td>High</td>
<td>Low</td>
<td>Low</td>
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<td>n</td>
<td>23</td>
<td>21</td>
<td>13</td>
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<tr>
<td>BMI</td>
<td>27.9±6.3</td>
<td>27.5±4.5</td>
<td>32.1±6.6</td>
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<td></td>
<td>48%</td>
<td>62%</td>
<td>85%</td>
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<tr>
<td>Age, y</td>
<td>50.8±12.1</td>
<td>52.7±9.7</td>
<td>50.8±10.5</td>
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<tr>
<td></td>
<td>55.1±15.0</td>
<td>62±9.6</td>
<td>55.1±15.0</td>
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<tr>
<td>Before treatment LDLC level, mg/dL</td>
<td>136±34</td>
<td>132±28</td>
<td>141±31</td>
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<td></td>
<td>122±42</td>
<td>122±42</td>
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<tr>
<td>LDLC percent change after statin treatment, %</td>
<td>−58.7±4.1</td>
<td>−22.0±7.6</td>
<td>−53.9±5.7</td>
</tr>
<tr>
<td></td>
<td>−62.7±7.3</td>
<td></td>
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<tr>
<td>LDLC level change after statin treatment, mg/dL</td>
<td>−80.3±22.4</td>
<td>−29.3±12.4</td>
<td>−76.0±18.3</td>
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<td>−33.6±16.4</td>
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Data are presented as numbers, percentages, or means±SDs. None of these participants were smokers. BMI indicates body mass index; and LDLC, low-density lipoprotein cholesterol.
according to the manufacturer’s protocol. Twenty-four hours before transfection, HepG2 cells were seeded into 6-well plates at a concentration of 7.5×10^4 cells/well, and Huh7 cells were seeded into 6-well plates at a concentration of 5.0×10^6 cells/well. Cells were transfected for a total of 48 hours with either the pCMV6-EV or either allele of the RP1-13D10.2 overexpression plasmids, and cellular phenotypes were measured after 48 hours.

To measure LDL uptake, cells transfected with the EV-negative control or either allele of the RP1-13D10.2 expression constructs (rs6924995 A or G) were incubated with 10 μg/mL of DiI-LDL (Biomedical Technologies Inc, BT-904) in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum for 2 hours. Cells were washed twice in PBS and scraped from wells. Levels of DiI-LDL uptake were quantified by fluorescence-activated cell sorting on a BD FACS Calibur Flow Cytometer, and values were calculated using the average of 10,000 gated events. Experiments were performed 4×. Effects of RP1-13D10.2 overexpression on media levels of apolipoprotein B were identified by ELISA as previously described. To measure the effect of RP1-13D10.2 overexpression on LDLR transcript stability, Huh7 cells were transfected for 48 hours with either the pCMV6-EV or the RP1-13D10.2 overexpression plasmids, treated with 1 μg/mL actinomycin D and harvested >6 hours. Transcript half-life was calculated as described previously.

SREBF1 and SREBF2 knockdown was achieved by 48-hour transfection of 1.5×10^5 Huh7 cells/well in 6-well plates using siPORT NeoFX transfection agent (Ambion) with either Silencer Select siRNA (Ambion) or nontargeting control, according to the manufacturer’s protocol. HepG2 cells were incubated with 1 μmol/L GW3965 (Sigma-Aldrich) dissolved in dimethyl sulfoxide for 24 hours before collection for RNA isolation.

**Cycloheximide Treatment**

Huh7 cells were transfected with RP1-13D10.2 G or A plasmid using GenJet (SigmaGen Laboratories) according to manufacturer’s protocol. Cells were incubated with cycloheximide (20 μg/mL) for 24 hours. To quantify total cellular protein, cells were dissolved in Celllytic M (Sigma-Aldrich) and centrifuged at 16,000g. Supernatant was quantitated using the Bio-Rad Protein Assay kit (Bio Rad) and measured on a Synergy H1 microplate reader (Biotek).

**Statistical Analyses**

Differences between EV and RP1-13D10.2 overexpression (either A or G plasmid) on cellular phenotypes including gene expression, LDL uptake, and media apolipoprotein B were identified using 2-tailed t tests in which samples within an experimental batch were paired. One-way ANOVA with post hoc 2-tailed paired t tests was used to identify differences in gene expression levels after cellular incubation under various conditions. Unless otherwise stated, all statistical analyses were performed in GraphPad Prism 6.0.

**Results**

**Statin-Induced Change in RP1-13D10.2 Expression Differs Between High Versus Low LDLC Statin Responders**

We first sought to determine if either RP1-13D10.2 or MYLIP gene expression was related to LDLC statin response using simvastatin and sham exposed LCLs from participants of the CAP clinical trial with either high or low LDLC response (Table). Statin-induced change in RP1-13D10.2 expression levels significantly differed between high and low LDLC responders in cell lines from both white (23 high responders versus 21 low responders) and black (13 high responders versus 18 low responders) donors (Figure 1A). The statin-induced change in RP1-13D10.2 expression in the high responders of both populations was greater than the low responders.

![Figure 1](http://circgenetics.ahajournals.org/)

**Figure 1.** A, Statin-induced change in RP1-13D10.2 expression levels differs between high and low low-density lipoprotein cholesterol (LDLC) responders to statin treatment. CAP lymphoblastoid cell lines from the tails of the white and black LDLC distribution were incubated with 2 μmol/L simvastatin or sham buffer for 24 hours, after which RP1-13D10.2 and myosin regulatory light chain interacting protein (MYLIP) expression levels were quantified by RNA-seq. Because variance stabilization is approximately like a log2 transformation, the approximate fold change was estimated as 2(variance stabilized sham-variance stabilized statin). Although fold changes (mean±SE) are displayed for ease of interpretation, P values were calculated from t tests on the variance stabilized deltas. Transcripts on the + strand are indicated in blue and transcripts on the − strand are indicated in green. B, RP1-13D10.2 is located on chromosome 6 to 10 kb downstream of MYLIP and is proximal to 2 additional processed, uncharacterized pseudogenes, RP1-13D10.3 and RP1-13D10.4.
RP1-13D10.2 Transcript Structure

RP1-13D10.2 is annotated to the + strand of chromosome 6. As shown in Figure 1B, it is located ≈10 kb downstream of MYLIP, and it is adjacent to 2 processed pseudogenes, RP1-13D10.3 (annotated on the − strand) and MRPL42P2 (aka RP1-13D10.4, annotated on the + strand). On closer inspection of our RNA-seq data, we found evidence of a splice junction joining the annotated 3′ end of RP1-13D10.2 to another exon that partially overlaps RP1-13D10.3 in the antisense direction (Figure II in the Data Supplement).

RP1-13D10.2 Increases LDLR Expression and Stimulates LDL Uptake

To determine if RP1-13D10.2 affects genes involved in cholesterol metabolism, we transiently transfected Huh7 cells with a plasmid overexpressing the RP1-13D10.2 gene containing either the rs6924995 A or G allele, or pCMV6-entry, a control plasmid that contained the empty vector backbone. Although we identified a novel splice variant of RP1-13D10.2, we chose to focus our functional investigation on the canonical RP1-13D10.2 transcript. After 48 hours, overexpression of RP1-13D10.2 was confirmed by quantitative PCR (Figure IVA in the Data Supplement), and genes involved in cholesterol biosynthesis (HMGCR and HMGCS1) and cholesterol uptake (LDLR, MYLIP, and PCSK9) were quantified (Figure 2A). Overexpression of the RP1-13D10.2 containing the rs6924995 A allele of increased LDLR transcripts by 2.35±0.51-fold, P=0.002, whereas overexpression of the G allele increased LDLR transcripts by 2.04±0.57-fold, P=0.03. Notably, these effects seemed to be specific for LDLR because there were no consistent expression differences in any other genes, including MYLIP. Similar effects of RP1-13D10.2 overexpression were also observed in a second human hepatoma cell line, HepG2 (Figure IV in the Data Supplement). To verify the functional impact of RP1-13D10.2 overexpression, we tested the effect of overexpression on uptake of DiI-labeled LDL. Consistent with increased expression levels of LDLR, we found that RP1-13D10.2 overexpression increased DiI-LDL uptake with either the ‘A’ allele (1.85±0.08 fold, P<0.0001) or ‘G’ allele (1.66±0.13 fold, P=0.0002; Figure 2B), as well as

Figure 2. RP1-13D10.2 overexpression increases low-density lipoprotein receptor (LDLR) expression and activity. Huh7 cells were transiently transfected with 1 of 2 different constructs expressing RP1-13D10.2 containing either the rs6924995 A (RP1-A) or G (RP1-G) allele, or an empty vector (EV) control for 48 hours. **A**, Gene expression levels were quantified by quantitative polymerase chain reaction (qPCR) and normalized to CLPTM1; n=12. **B**, Cells were incubated with DiI-LDL for 3 hours, and the mean fluorescence of 10,000 gated events was quantified to measure LDL uptake; n=8. **C**, Media apolipoprotein B levels were measured by ELISA; n=5 to 12. **D**, Cells were incubated with 1 µg/mL actinomycin D after which aliquots of cells were harvested >6 hours, and LDLR transcript levels were quantified by qPCR; n=4. For **A** and **B**, values shown are mean±SE. *P<0.05, **P<0.01, ***P<0.001 by 2-tailed paired t test.
reduced media levels of apolipoprotein B (Figure 2C). Finally, as LDLR transcript levels are known to be regulated at the level of transcription as well as mRNA stability, we tested if RP1-13D10.2 altered LDLR stability using incubation with actinomycin D, but found no evidence of an effect (Figure 2D).

**RP1-13D10.2 Affects LDLR Transcript in the Absence of Protein Synthesis**

RP1-13D10.2 is currently annotated as a processed pseudogene by Gencode. However, recent findings have reported that some noncoding RNAs are translated and function as micropeptides, rather than as noncoding RNAs.24 RP1-13D10.2 does have an open reading frame that, if translated, would encode a protein of 97 amino acids with the rs6924995 G allele (Figure V in the Data Supplement). Notably, this putative open reading frame would be disrupted by the rs6924995 A allele, TGG (tryptophan) to TAG (stop), resulting in a protein of 82 amino acids. Thus, to test the possibility that RP1-13D10.2 functions as a protein, we tested if overexpression in the presence of cycloheximide, an inhibitor of protein synthesis, was able to stimulate increased expression levels of LDLR. Although cycloheximide treatment reduced total cellular protein by \( \approx \) 50%, it had no effect on RP1-13D10.2 induction of LDLR expression levels (Figure 3).

To further evaluate the possibility that RP1-13D10.2 is translated, we used 3 in silico prediction programs, the coding–noncoding index,25 the coding potential assessment tool,26 and the coding potential calculator.27 Both the coding–noncoding index and the coding potential assessment tool analyses indicated that the RP1-13D10.2 transcript with either the A or G rs6924995 alleles was noncoding, whereas the coding potential calculator analysis indicated there was weak evidence that both A and G allele containing transcripts were coding.

**Effects of rs6924995 on RP1-13D10.2 Regulation of LDLR**

We observed a consistent trend of slightly larger effect sizes of the A allele versus G allele overexpression on both LDLR expression and LDL uptake; however, this difference was not statistically significant (\( P = 0.10 \) for LDL uptake). Because this trend was observed in 3 different hepatoma cell lines (Figure IV in the Data Supplement), we hypothesized that our model of extreme RP1-13D10.2 overexpression (\( \approx 10^{10} \)-fold increase) may obscure the potential differences between the 2 alleles. With reduced levels of RP1-13D10.2 overexpression (\( \approx 10^7 \) fold) we continued to observe a similar trend of smaller increases in LDLR upregulation with the G allele versus A allele; however, this difference did not achieve statistical significance (\( P = 0.06 \), Figure IVD and IVE in the Data Supplement). Further reductions in the degree of RP1-13D10.2 overexpression failed to produce consistent stimulation of LDLR transcript with either allele.

![Figure 3. RP1-13D10.2 stimulation of LDLR does not require protein synthesis. Huh7 cells (n=3) were transfected with a construct containing either RP1-13D10.2 rs6924995 G alleles or an empty vector (EV) control in duplicate, after which 1 aliquot was treated with 20 \( \mu \)g/mL cycloheximide for 24 hours, and LDLR transcript was quantified by quantitative polymerase chain reaction. Values shown are means±SE.](image)

![Figure 4. RP1-13D10.2 expression levels are sterol regulated. A, RP1-13D10.2 transcript levels were quantified in HepG2 cells were exposed to 4 culture conditions: fetal bovine serum (FBS, control), 48-hour incubation with 2-\( \mu \)mol/L simvastatin+10% lipoprotein-deficient serum (LPDS), 24-hour incubation with statin+LPDS after which low-density lipoprotein cholesterol (LDLC) or 25HC was added back and incubated for an additional 24 hours. The experiment was repeated 6×, with means±SE shown. Statistically significant differences in gene expression were assessed using 1-way ANOVA (\( P = 0.01 \)), with 2-tailed paired t tests used to identify differences between groups. B, RP1-13D10.2 transcript levels were quantified after SREBF1 and SREBF2 knockdown in Huh7 cells. C, RP1-13D10.2 and myosin regulatory light chain interacting protein (MYLIP) transcript levels were quantified in HepG2 cells after 24-hour incubation with 1 \( \mu \)mol/L GW3965 (n=6).](image)
RP1-13D10.2 Expression and rs6924995 Genotype

Given the fact that rs6924995 is contained within RP1-13D10.2, we next sought to determine if this single-nucleotide polymorphism was associated with expression levels of the pseudogene. We observed a suggestive association between rs6924995 genotype and RP1-13D10.2 expression in both the statin and the sham-treated cells, with trends toward greater RP1-13D10.2 expression observed in the GG homozygotes (Figure VI in the Data Supplement). In contrast, there was no relationship between rs6924995 and MYLIP expression levels (Figure VI in the Data Supplement). Closer examination of genomic region between MYLIP and RP1-13D10.2 found that rs6924995 is within a small block of linkage disequilibrium that contains RP1-13D10.2, but not MYLIP (Figure VII in the Data Supplement).

RP1-13D10.2 Expression Levels Are Sterol Regulated

Many genes that affect cholesterol metabolism are themselves subject to sterol regulation. To test if RP1-13D10.2 transcript levels were affected by changes in intracellular sterol content, we exposed HepG2 cells to conditions of extreme sterol depletion (2 μmol/L simvastatin + 10% lipoprotein–deficient serum) for 24 hours, after which we added-back LDL. Sterol depletion reduced RP1-13D10.2 expression levels 0.43±0.12-fold (Figure 4A). LDLC add-back not only reversed this effect but also highly induced RP1-13D10.2 expression.

Sterol response element–binding proteins and LXR are well-known transcription factors that mediate cellular response to changes in intracellular sterols. RP1-13D10.2 expression levels were significantly reduced after SREBF2 knockdown in Huh7 cells (0.5±0.11-fold, SREBF2 knockdown in levels were significantly reduced after expression of the GG homozygotes (Figure VI in the Data Supplement). In contrast, there was no relationship between rs6924995 and MYLIP expression levels (Figure VI in the Data Supplement). Closer examination of genomic region between MYLIP and RP1-13D10.2 found that rs6924995 is within a small block of linkage disequilibrium that contains RP1-13D10.2, but not MYLIP (Figure VII in the Data Supplement).

Discussion

We have examined RP1-13D10.2, an lncRNA containing a single-nucleotide polymorphism, rs6924995, reported to have a sub–genome-wide association with LDLC response to rosuvastatin.3 Here, we report that RP1-13D10.2 overexpression increases transcript levels and activity of LDLR, which encodes the major receptor for uptake of plasma LDLC. RP1-13D10.2 expression seems to be sterol regulated, and notably we observed a relationship between interindividual variation in the magnitude of this regulation with statin-induced changes in LDLC from a panel of LCLs derived from participants of a statin clinical trial. In particular, statin incubation increased RP1-13D10.2 expression levels in cell lines from both whites and blacks with high LDLC response to statin treatment, whereas either no change or reduced RP1-13D10.2 expression was detected in cell lines from donors with low LDLC response. Together, these findings support the identification of RP1-13D10.2 as a novel marker, and possibly determinant, of variation in statin efficacy for plasma LDLC lowering. Although lncRNAs have been well established to play a role in cardiovascular biology and disease,28 to our knowledge, RP1-13D10.2 is the first lncRNA that has been identified to play a role in statin response.

rs6924995 was first reported to be associated with LDLC response to statin in the JUPITER placebo-controlled trial of rosuvastatin (20 mg/d) response. The A allele was associated with both greater absolute (β=4.1, P=5.3E-07) and fractional (β=3.8, P=1.4E-06) LDLC reduction in individuals with genetically confirmed European ancestry. The Heart Protection Study, a five-year trial of 3895 self-reported Caucasians prescribed 40 mg simvastatin/d, failed to replicate this association with LDLC statin response.29 In addition, the largest genome-wide meta-analysis of LDLC response to statin treatment published to date, comprising 18,596 subjects from clinical trial and population-based cohorts, did not identify this single-nucleotide polymorphism30; however, the report did not include a direct test for replication of this locus. This lack of replication may be because of the unique nature of the JUPITER study population. Statins are traditionally prescribed to individuals with hypercholesterolemia; in contrast JUPITER was composed of participants with relatively normal levels of LDLC (<130 mg/dL), but who had high measures of inflammation (C-reactive protein ≥2 mg/dL).

The association between rs6924995 and LDLC response to statin treatment was originally attributed to MYLIP (aka IDOL and inducible degrader of LDLR),3 a known regulator of LDLR protein levels.31 Here, we failed to identify a relationship between rs6924995 genotype and MYLIP transcript levels in LCLs. In contrast, we found suggestions of an association between RP1-13D10.2, an lncRNA that contains rs6924995 and is located 10 kb downstream from MYLIP, expression levels with rs6924995 genotype. In addition, although we were unable to verify a genotype difference in RP1-13D10.2 effects on LDLR, the trend of slightly greater stimulation of LDLR transcript with the RP1 A allele constructs versus the G allele constructs is consistent with the genome-wide association studies association between the rs6924995 A allele and greater LDLC lowering on statin treatment. Thus, additional study is necessary to ascertain the true relationship between rs6924995 and RP1-13D10.2 transcript levels, transcript structure, and activity.

We observed no direct effects of RP1-13D10.2 overexpression on MYLIP transcript levels. Although we did not test for an effect of RP1-13D10.2 on MYLIP protein levels, MYLIP is an E3-dependent ubiquitin ligase complex that mediates sterol-dependent degradation of LDLR protein,30 thus it is unlikely that the effects of RP1-13D10.2 on LDLR transcript levels are mediated by MYLIP. Although further study will be required to absolutely discount a relationship of either rs6924995 or RP1-13D10.2 to MYLIP, these findings suggest the intriguing possibility that RP1-13D10.2 and MYLIP may be mechanistically independent regulators of LDLR activity that happen to be in close proximity to one another, similar to other known clusters of lipid-related genes (ie, APOC3 and APOA5, SREBF2 and mir-33a).

We found that RP1-13D10.2 expression levels were increased with statin treatment in LCLs from donors with high LDLC response to statin treatment. Sterol response element–binding protein 2 (gene name SREBF2) is a well-known
transcription factor that is activated by conditions of sterol depletion, such as in vitro statin exposure. In human hepatoma cell lines, we found that SREBF2 knockdown reduced RP1-13D10.2 expression levels, consistent with the likelihood that RP1-13D10.2 may be an SREBF2 target gene. In addition, RP1-13D10.2 expression levels were also increased after incubation with an LXR agonist, suggesting that it may also be an LXR target gene. Notably, MYLIP is a well-known LXR target gene, and thus the close proximity between RP1-13D10.2 and MYLIP may allow for shared transcription factor regulatory sequences. Paradoxically, these 2 genes would be expected to oppose one another, as LXR-induced expression of RP1-13D10.2 would increase LDLR activity, whereas LXR-induced expression of MYLIP would stimulate LDLR decay. However, this is similar to the well-known phenomena in which SREBF2 both induces LDLR transcription, whereas stimulating expression of a factor, PCSK9, that promotes LDLR protein decay.31

A question that remains is the precise mechanism by which RP1-13D10.2 specifically increases transcript levels of LDLR. Our observation that RP1-13D10.2 upregulates LDLR after cycloheximide incubation supports the likelihood that RP1-13D10.2 functions as an IncRNA; however, as the cells were treated with cycloheximide 24 hours after transfection with the RP1 plasmid, these findings cannot exclude the possibility that RP1-13D10.2 acts as a highly stable protein that persists after inhibition of protein synthesis. However, when combined with our in silico analysis that does not support the coding potential of RP1-13D10.2, our findings strongly support the likelihood that RP1-13D10.2 functions as a noncoding RNA.

There are 4 major described functions of IncRNAs (recently reviewed by Uchida and Dimmeler28): (1) imprinting—the IncRNA directly inhibits expression of a proximal locus; (2) guide molecules—the IncRNA recruits functional proteins, often epigenetic or transcription factors, in a transacting manner; (3) enhancer activation—the IncRNAs is transcribed from the site of an enhancer element and aids in enhancer activity; (4) molecular sponges—the IncRNA binds miRNAs, disrupting miRNA inhibition of mRNAs. Endogenous levels of RP1-13D10.2 are low compared with LDLR, thus it is unlikely that RP1-13D10.2 functions as a molecular sponge, which often requires similar stoichiometry of the effector and target molecules.29 In addition, we found that RP1-13D10.2 overexpression increases LDLR transcript levels without affecting LDLR transcript stability, suggesting that RP1-13D10.2 enhances LDLR transcription. However, RP1-13D10.2 is located on chromosome 6, whereas LDLR is located on chromosome 19, thus RP1-13D10.2 does not likely affect LDLR through either imprinting or changes in LDLR enhancer activity. Thus, the most probable function of RP1-13D10.2 is as a guide molecule. Notably, neither moderate nor high expression is the most probable function of RP1-13D10.2 is as a guide molecule, our findings strongly support the potential of RP1-13D10.2, our findings strongly support the likelihood that RP1-13D10.2 functions as a noncoding RNA.

One of the major findings of the large transcriptomic projects of the past decade is the widespread transcription of the human genome, and recent estimates using RNA- seq data suggest that ≈80% of the genome is transcribed, with many of these transcribed sequences representing ncRNAs.12,23 Using a combination of gene expression and functional studies, here we identify the IncRNA RP1-13D10.2 as a novel marker, and possible determinant, of LDLR response to statin treatment that regulates LDLR. Thus, our findings illustrate the potential of noncoding regulatory RNA as a determinant of variability in drug response. In addition, as genome-wide association studies identified single-nucleotide polymorphisms are most often annotated based on the their proximity to protein coding genes, these results demonstrate the importance of functional validation studies not only as an alternative approach for validating pharmacogenetic associations, but also for ensuring the correct annotation of genome-wide association study findings.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Statins are among the most prescribed drugs in the United States, used to decrease low-density lipoprotein cholesterol (LDLc) levels for the prevention and treatment of cardiovascular disease. However, response to statins is variable, and many patients are left with insufficient LDLc-lowering, despite treatment. Genome-wide association studies have identified a number of DNA variants that are associated with this interindividual variation. Although simply identifying variants may be sufficient for the development of diagnostics, understanding the molecular mechanisms underlying these associations is essential for fully leveraging these findings into new biology that may inform advances in biomedical research. Previously, a sub–genome-wide association was observed between rs6924995 and rosuvastatin response in the Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) clinical trial. Located downstream of myosin regulatory light chain interacting protein, a gene known to regulate the major LDL receptor, rs6924995 was originally annotated as the myosin regulatory light chain interacting protein locus. However, rs6924995 is located within the uncharacterized long noncoding RNA, *RP1-13D10.2*, thus here we explored the contribution of this long noncoding RNA to LDLc statin response. Using cell lines established from participants of a statin clinical trial with either high or low LDLc response, we found significant differences in statin-induced change in *RP1-13D10.2* expression between the two groups, while no change was observed with myosin regulatory light chain interacting protein expression levels. Furthermore, *RP1-13D10.2* overexpression in hepatoma cell lines up-regulated the major LDL receptor and increased uptake of LDL. Our data support the hypothesis that *RP1-13D10.2* is a novel marker, and possible determinant, of LDLc response to statin treatment, and highlight the importance of functional studies for annotation of genome-wide association studies identified loci.
RP1-13D10.2 Is a Novel Modulator of Statin-Induced Changes in Cholesterol
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**Figure S1. Distribution of percent change LDLC in CAP.** Distributions are shown separately for Caucasians (n=609) and African Americans (n=335), with the LDLC statin responses of high and low plasma LDLC responders highlighted in dark green. The LDL-C statin response was calculated as the residuals of Log(Statin LDL-C)-Log(Baseline LDL-C) adjusted for age, race, and smoking status.
Figure S2. Detection of a novel *RP1-13D10.2* splice variant in LCLs. A novel splice junction connecting the 3’ end of the annotated *RP1-13D10.2* transcript to a novel exon antisense and partially overlapping the annotated *RP1-13D10.3* transcript was detected in RNA-seq libraries from lymphoblastoid cell lines. By piecing together partially overlapping RNA-seq sequence reads, we assembled the partial sequence of a transcript containing this novel splice junction as shown above. We verified this novel transcript by qPCR using primers targeted to the underlined regions.

...AGTGACT<AG>GAGCCCTCTGCTCTGTTGCTGTGTCCGAATCATCCTCCTGTTGTAATACACCCCGTGAAA
TACACCCAGACCTTCAGCCTTCATGGCAAGCCTGCTGTCACATCTATTACTGACCTACAGCTTCTAGTGCT
TGCCCAACATTATATCTGACTGCTGCTGCTCTCTGCTGTGCTGCTGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
ACAACCTCAGACCA[AG]TTTCCTCTCTGGCTATTGGACACTAATTATATATATATATATATATATATATATATATAT
TCCCTCAGCTGCTAGCCTCTCCTGCTGGGCTTCCCATGACTTGGTTTGGTTTATCTGTCTGTAAGCTCTAGTCT
CTCTCTTTTGACATTATATATATATATATATATATATATCTGGAATTCTCTATAATCTCCTCCTCTTCTGGTCTGATCT
AATCAACATTTGCTC...
Figure S3. Detection of *RP1-13D10.2* expression in LCLs. LCLs from subjects of the CAP clinical trial were incubated with either 2 uM simvastatin or sham buffer for 24 hours. Although samples were subject to DNaseI treatment during the RNA extraction process to account for amplification from residual genomic DNA contamination, a no-reverse transcriptase (no RT) control was also created for each cDNA sample. *RP1-13D10.2, RP1-13D10.3,* and *RP1-13D10.4* were quantified by qPCR, and only *RP1-13D10.2* had detectable expression levels.
Figure S4. Huh7, HepG2 and Hepa 1-6 (a mouse hepatoma line) cells were transiently transfected with one of two different constructs containing RP1-13D10.2 with either the rs6924995 “A” (RP1-A) or “G” (RP1-G) allele or an empty vector (EV) control for 48 hours. A) Validation of RP1-13D10.2 overexpression in Huh7 cells (n=12). B) Change in candidate gene transcript levels after RP1-13D10.2 overexpression in HepG2 cells. Transcript levels were quantified by qPCR, normalized to CLPTM, and expressed as a fold change from EV treated cells. N=6 *p<0.05. C) Change in LDLR after RP1-13D10.2 overexpression in Huh7, HepG2 and Hepa1-6 cells shows slightly greater, non-statistically significant, effects with the “A” allele versus “G” allele construct (n=12 Huh7, N=6 HepG2, N=3 Hepa1-6). D) Huh7 cells transfected with reduced levels of the RP1-13D10.2 constructs have ~1/1000 lower RP1-13D10.2 transcript levels compared to the original incubation conditions, and E) demonstrate a suggestive difference in the magnitude of LDLR up-regulation between the “A” versus “G” allele (n=11). P-values were calculated using two-tailed paired t-tests.
**RP1-13D10.2 rs6924995 “G” allele**

291 nucleotides  
97 amino acids

1  ATGGCCTTTGACATC ATCATCACACGGCT GCCAGGGGCTGGTTG CAGCCGAGTGACAC ATCCACACATCCTCG
1  M A F D I I I I T P A G R G W L Q A S D H I H T S S

76  CCGGAGTGAGACGC TCCCCAACAGTTGCG CACCAGGTCTCTAG GGGGAGACATGGTG AAGAGGCGGTGCTAT
26  P E W R R S H K C G H Q G L M G G D M V K S G C Y

151  GCTCTCTGCTGGCTCTGCTATTCCATCCTCTGCTTCTTTGCCCTCTA TGGATCCCAAGCGCT
51  A P L W L Y H P Q D L F H P L L L C P L W I P S A

226  TGTCTTCGTAGGAGT GACTGGAGCTCTCTCG CTCTGGTTGCTGTGT TCCGAATCATCCTTC TGGTAA
76  C L R R S D W S L S L W L L C S E S S F W *

**RP1-13D10.2 rs6924995 “A” allele**

246 nucleotides  
82 amino acids

1  ATGGCCTTTGACATC ATCATCACACGGCT GCCAGGGGCTGGTTG CAGCCGAGTGACAC ATCCACACATCCTCG
1  M A F D I I I I T P A G R G W L Q A S D H I H T S S

76  CCGGAGTGAGACGC TCCCCAACAGTTGCG CACCAGGTCTCTAG GGGGAGACATGGTG AAGAGGCGGTGCTAT
26  P E W R R S H K C G H Q G L M G G D M V K S G C Y

151  GCTCTCTGCTGGCTCTGCTATTCCATCCTCTGCTTCTTTGCCCTCTA TGGATCCCAAGCGCT
51  A P L W L Y H P Q D L F H P L L L C P L W I P S A

226  TGTCTTCGTAGGAGT GACTGGAGCTCTCTCG CTCTGGTTGCTGTGT TCCGAATCATCCTTC TGGTAA
76  C L R R S D W S L S L W L L C S E S S F W *

**Figure S5.** The RP1-13D10.2 transcript contains an open reading frame that if translated, would redefine rs6924995 as a missense SNP versus an intergenic SNP with no predicted function.
Figure S6. *RP1-13D10.2* and *MYLIP* expression levels were quantified by qPCR in CAP LCLs and tested for association with rs6924995 genotype using an additive model (N=20 per genotype). Sham p=0.03; statin p=0.18. Samples with *RP1-13D10.2* expression levels less than the genomic control were set to 0.
Figure S7. Local linkage disequilibrium of rs6924995. Plot created in SNAP using the 1000 genomes CEU pilot population with an LD threshold of $r^2=0.8$. 
Figure S8. Degree of SREBF1 and SREBF2 knock-down. Huh7 cells (n=4 replicates/condition) were reverse transfected with Silence Select siRNAs targeting either SREBF1, SREBF2, both SREBF1 and SREBF2, or a non-targeting control (NTC). After 48 hr, SREBF1 and SREBF2 transcript levels were quantified by qPCR with values normalized to CLPTM1.