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The role of HMGCR alternative splicing in statin efficacy

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Abstract

Statins, or 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) inhibitors, are widely prescribed to lower plasma cholesterol levels and reduce cardiovascular disease (CVD) risk. Despite the well documented efficacy of statins, there is large inter-individual variation in response. Using a panel of immortalized lymphocyte cell lines incubated with simvastatin, we recently found that the magnitude of expression of an alternatively spliced HMGCR transcript lacking exon 13 was inversely correlated with in vivo reductions of total cholesterol, LDL cholesterol, apoB, and triglycerides following statin treatment of the individuals from whom the cells were derived. This review will discuss the potential significance of alternative splicing as a mechanism contributing to variation in statin efficacy as well as the utility of immortalized lymphocyte cell lines for identifying pharmacogenetically relevant polymorphisms and molecular mechanisms.

Introduction

Statins are the most commonly prescribed drugs for CVD prevention and management. Reduced CVD risk results primarily from statin-induced decreases of plasma LDL-cholesterol, although recent evidence suggests that lower levels of C-reactive protein, a marker of inflammation, are also associated with statin's benefit on CVD (Ridker et al. 2009). Despite the considerable efficacy of statins, a substantial number of CVD events occur in statin-treated individuals, indicating a persistence of underlying pathophysiology despite therapy. Variation in LDL-cholesterol response to statin treatment has been attributed to both genetic and non-genetic factors and include single nucleotide polymorphisms (SNPs) and haplotypes in genes encoding key regulators of cholesterol metabolism including *HMGCR*, *APOE*, *PCSK9*, *ACE*, *LDLR* and *ABC B1* (Chasman et al. 2004, Donnelly et al. 2008, Krauss et al. 2008, Thompson et al. 2009), as well as phenotypic predictors such as race, age, and smoking status (Simon et al. 2006). However, the extent of variation in statin efficacy explained by these polymorphisms is small. Moreover, whole genome approaches have not identified statistically significant SNPs associated with statin response (Thompson et al. 2009). We here describe recent findings from studies of HMGCR that indicate the potential value of cellular phenotyping for augmenting pharmacogenetic information derived from SNP analysis.

HMGCR alternative splicing is likely a determinant of statin efficacy

HMGCR catalyzes the rate-limiting step of cholesterol biosynthesis and is the target of statin inhibition (Goldstein and Brown 1990). We and others have reported that the minor alleles of haplotypes within *HMGCR* are associated with reduced LDLC response to statin (Chasman et

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al. 2004, Krauss et al. 2008). One of these, the H7 haplotype, is defined by three intronic SNPs, rs17244841, rs3846662, and rs17238540, none of which is located in a region of obvious functionality, (e.g. splice site), nor is in linkage disequilibrium with exonic SNPs. To identify the molecular mechanism underlying the H7 phenotype, we utilized immortalized lymphocyte cell lines derived from 944 genotyped individuals in the Cholesterol and Pharmacogenetics (CAP) study, a six week trial of simvastatin 40 mg/day (Krauss et al. 2008, Medina et al. 2008). Following *in vitro* incubation of the cells with simvastatin vs. placebo, we quantified both the full length *HMGCR* transcript, *HMGCR13(+)*, and an alternatively spliced transcript lacking exon 13, *HMGCR13(−)*. Rs3846662, one of the three H7 SNPs, is located in intron 13 and was found to be associated with magnitude of statin-induced expression of *HMGCR13 (−)* (Medina et al. 2008). Rs3846662 regulation of *HMGCR* exon 13 skipping has been independently confirmed (Burkhardt et al. 2008). Notably, the extent of statin-induced *HMGCR13(−)* measured in the lymphocyte cell lines was found to be inversely correlated with the magnitude of statin response *in vivo*, as assessed by reductions of total cholesterol, LDL-cholesterol, apoB and triglycerides ($p<0.0001$). This relationship was seen in cell lines derived from both African American and Caucasian American populations. Together with x-ray crystallographic evidence for a polar interaction between simvastatin and the region of HMGCR encoded by exon 13 (Istvan and Deisenhofer 2001), these results suggest that the HMGCR13(−) isoform has reduced statin sensitivity compared to the HMGCR13(+) catalytic domain. Consistent with this hypothesis, we demonstrated reduced statin inhibition of HMGCR enzymatic activity in human kidney 293 cells following selective small interfering RNA (siRNA) knockdown of the full-length *HMGCR* transcript (Medina et al. 2008).

These results have identified a mechanism for the association of rs3846662 with reduced LDL-cholesterol response to statins (Figure 1). Indeed, carriers of H7 in conjunction with a second haplotype containing rs3846662 (H2) had even further attenuation of LDL-cholesterol response to simvastatin compared to those who only carried one copy of the allele (Krauss et al. 2008). The potential importance of *HMGCR* alternative splicing as a determinant of statin response is highlighted by the fact that variation in statin-induced *HMGCR13(−)* expression explained between 6–15% of the variation in LDL-cholesterol response to statins, compared to less than 2% explained by *HMGCR* genotypes (Krauss et al. 2008). However the strength of this relationship is not sufficient to justify measurement of *HMGCR13(-)* expression in clinical practice.

Role and frequency of alternative splicing between races

Although the relationship between *HMGCR13(−)* expression and statin response was seen in cell lines derived from both African American and European donors, the allele frequency of rs3846662 differs dramatically between these groups. The “A” allele is found in 16.9–17.4% of the African American population, but is much more prevalent in both European and Asian populations, 54.2–60.9% and 43.0–50.0% respectively. Thus, in our CAP population, lower expression of *HMGCR13(−)* in cell lines from African American vs. Caucasian individuals can be fully explained by the difference in rs3846662 allele frequency.

Functional implications of HMGCR alternative splicing

Burkhardt *et al*. (2008) recently reported that expression of the HMGCR13(−) catalytic domain was unable to rescue growth of UT2 cells, a cell line with *HMGCR* mutations that requires mevalonate supplementation for growth. This result suggests that the HMGCR13(−) catalytic domain is not enzymatically active, and thus its expression would not be expected to directly influence enzyme sensitivity to statins. However, catalytically active HMGCR is found as a tetramer comprised of two dimers (Istvan et al. 2000), and it is possible that these tetramers or dimers may contain a combination of 13(+) and 13(−) monomers (Figure 2). Thus, although

expression of the HMGCR13(−) catalytic domain alone may not be catalytically active, it is possible that its expression in combination with the 13(+) domain may still attenuate overall HMGCR enzymatic activity and statin sensitivity. Verification of this hypothesis requires further testing.

HMGCR is highly regulated by multiple mechanisms, including transcription, mRNA stability, protein degradation and phosphorylation (Goldstein and Brown 1990); and, as described above, there is genetic evidence for the regulation of *HMGCR* alternative splicing as well. However, rs3846662 explains only a fraction of the inter-individual variation in *HMGCR* alternative splicing, suggesting that this process is influenced by other factors. For example, *HMGCR* is transcriptionally regulated by the SREBP/SCAP pathway in response to intracellular cholesterol content (Goldstein and Brown 1990). However, it remains unknown if the mechanism of alternative splicing itself is sterol regulated, or if the resulting 13(−) isoform is subject to post-transcriptional regulation.

Alternative splicing, cholesterol metabolism, and CVD risk

92–94% of human genes undergo alternative splicing with a minor isoform frequency of 15% or more (Clark et al. 2007, Nembaware et al. 2004, Wang et al. 2008b), thus the impact of alternative splicing on CVD risk may extend beyond its role in determining HMGCR statin sensitivity. For example, rs3846662, the *HMGCR* SNP that regulates exon 13 skipping, is in tight linkage disequilibrium ($r^2 > 0.8$) with another intronic *HMGCR* SNP, rs3846663, associated with lower plasma LDL-cholesterol by genome wide association study in multiple independent populations (Burkhardt et al. 2008, Kathiresan et al. 2008). A panel including this SNP and 11 others associated with levels of either LDL-cholesterol or HDL-cholesterol was reported to be predictive of CVD risk independently of lipid levels (Kathiresan et al. 2008). Alternative splicing has also been associated with a number of mutations of the *LDLR* gene that cause familial hypercholesterolemia (Holla et al. 2009). Moreover, a common polymorphism in *LDLR* associated with increased LDL-cholesterol (rs688) results in decreased exon 12 splicing efficiency (Zhu et al. 2007), suggesting that *LDLR* alternative splicing may contribute to variation in plasma LDL-cholesterol (Tveten et al. 2006). Lastly, a group of seven tightly linked non-coding SNPs within the lectin-like oxidized low density lipoprotein receptor-1 (*LOX-1*) associated with elevated risk of myocardial infarction, have also been shown to regulate exon 5 skipping (Mango et al. 2005).

The potential impact of alternative splicing on CVD risk factors is further suggested by evidence for alternative splicing of other genes within the cholesterol biosynthetic pathway such as *HMG-CoA synthase* and *mevalonate kinase* (Gil et al. 1987, Houten et al. 2001). Furthermore, since statins are known to inhibit not only cholesterol biosynthesis, but also the production of isoprenoids, which are thought to mediate the anti-inflammatory properties of statin inhibition (Wang et al. 2008a), it is possible that alternative splicing may be an important regulatory mechanism of the pleiotropic effects of statins.

Immortalized lymphocyte cell lines as a model system

Our work has demonstrated the value of immortalized lymphocyte cell lines obtained by Epstein-Barr virus transformation as a model system for examining genetically regulated pathways and mechanisms related to statin response (Medina et al. 2008). Although transcriptional profiles in immortalized cell lines are altered by the transformation process, these cells still provide a meaningful model in which transcript expression can be related to DNA variation (Cheung et al. 2003, Cheung et al. 2005, Morley et al. 2004), and differences in cellular drug response between ethnic populations are retained (Price et al. 2008, Spielman et al. 2007). In addition, the cell lines can be exposed to a wide range of drugs and treatment conditions for the identification of genetic influences and molecular pathways underlying drug

response (Choy et al. 2008, Shukla and Dolan 2005, Watters et al. 2004), a particularly powerful method since cellular phenotypes may be more directly related to underlying genetic and biochemical pathways than the clinically assessed symptoms of disease (Huang et al. 2008, Shukla et al. 2008). Despite concerns that genetic influences on statin-responsive cholesterol metabolism pathways in lymphocytes may not faithfully reflect statin effects on the liver, both freshly isolated and immortalized lymphocyte cell lines have been successfully utilized to study genetically regulated variation in cholesterol metabolism (Anderson et al. 1998, Fasano et al. 2009, Haas et al. 2007, Kelley et al. 2002, Leikin et al. 1982). This system becomes particularly powerful when repositories of immortalized lymphocyte cell lines are derived from clinical trials, since *in vitro* cellular phenotypes may be correlated with phenotypes measured *in vivo* in the donor individuals.

Conclusion

There is a wide range of inter-individual variation in statin response, which has been attributed to both genetic and non-genetic factors. *HMGCR* exon 13 alternative splicing is a genetically regulated mechanism that contributes to differences in LDL-cholesterol response by reducing the statin sensitivity of the resulting HMGCR enzyme. While alternative splicing has been linked to a variety of pathologic conditions, including CVD, this finding suggests that regulation of alternative splicing also may be a general mechanism underlying variation in drug response. The results also highlight the utility of immortalized lymphocyte cell lines for functional studies of pharmacogenetically relevant SNPs, and for identifying novel pharmacogenetic mechanisms. Thus, it would be particularly powerful to apply genomic tools such as whole transcriptome sequencing (aka RNA-seq) to assess the regulation of alternative splicing in conjunction with the generation of cellular phenotypes resulting from systematic variation in drug exposure. Such studies could lead to the identification of new pathways that influence cholesterol homeostasis and statin response, and ultimately to the development of new pharmacological approaches for enhancing CVD risk reduction.

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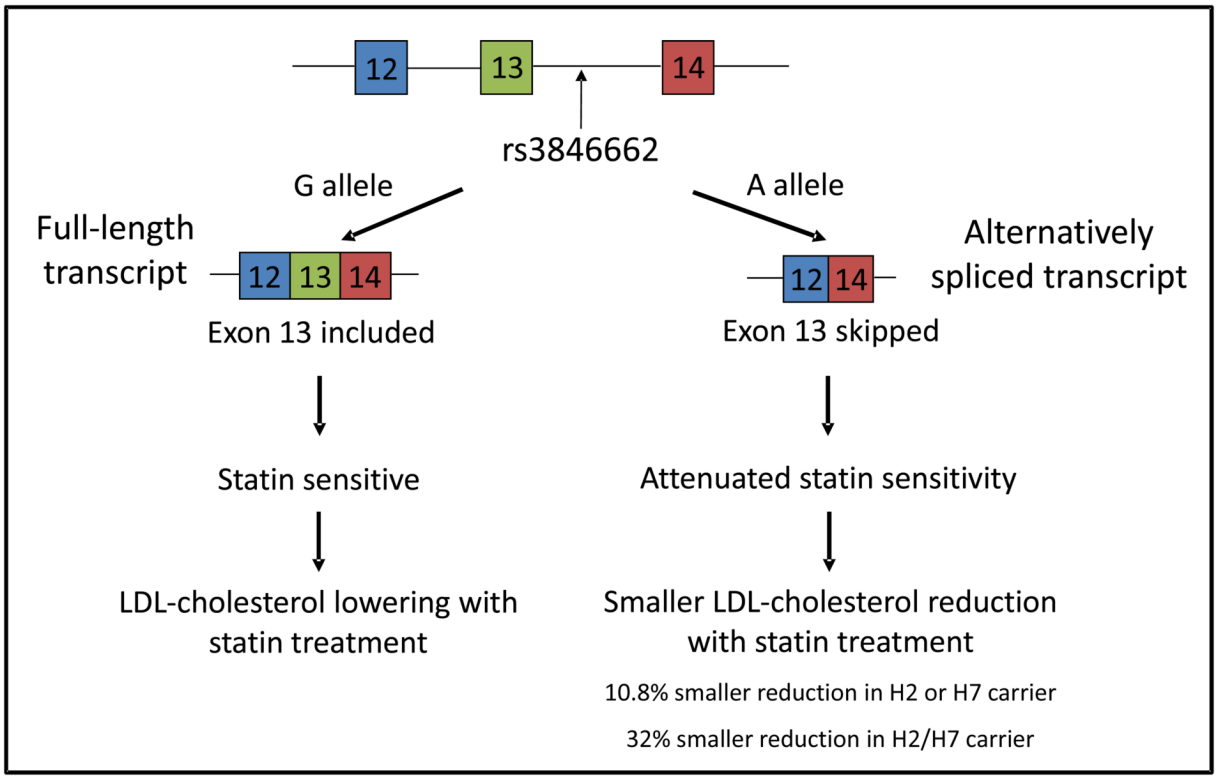

Figure 1.
Scheme of *HMGCR* alternative splicing

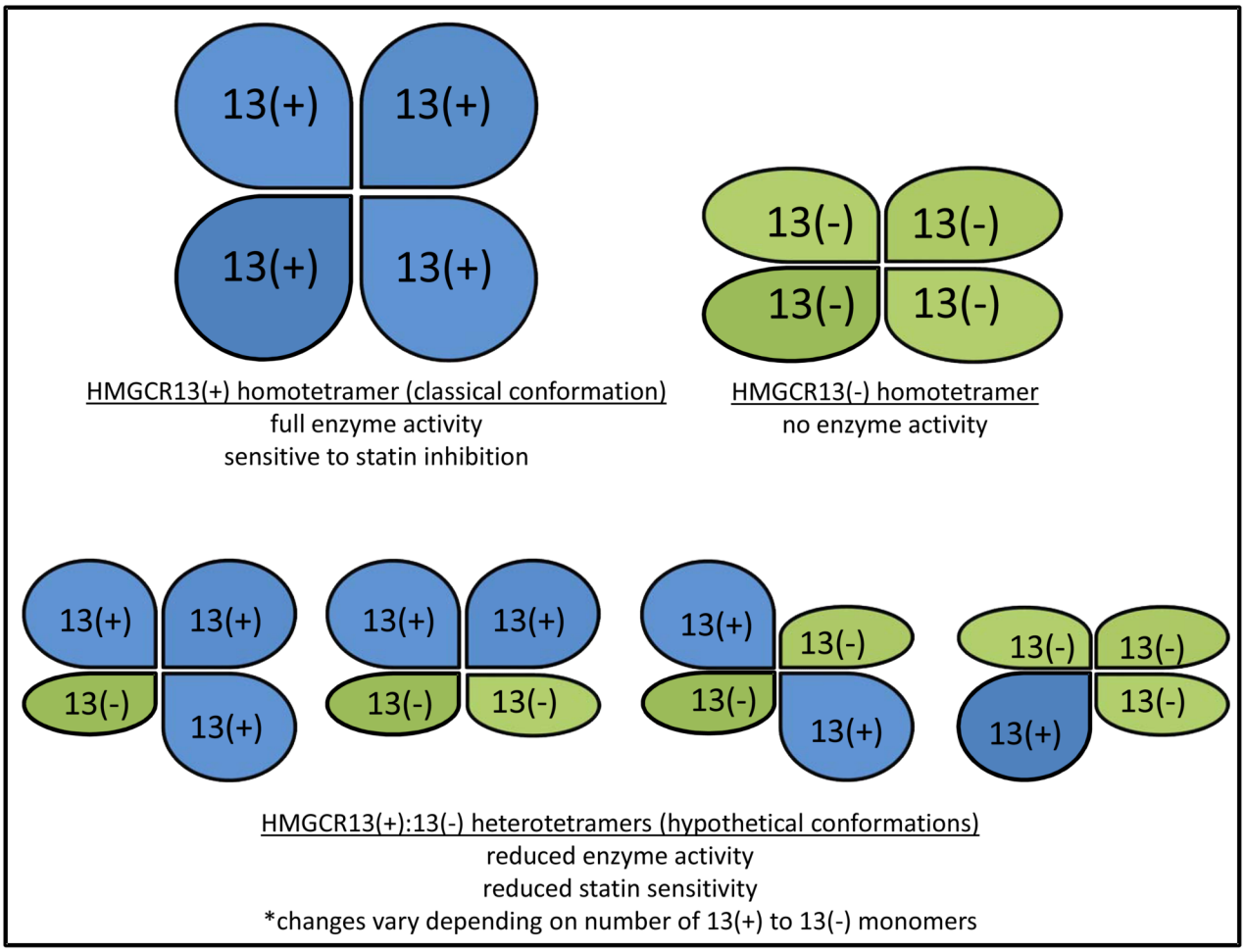

Figure 2.

Oligomerization of HMGCR catalytic domains. The catalytic domain of the classical HMGCR enzyme, HMGCR13(+), has been shown to exist as a tetramer comprised of two dimers (Istvan et al. 2000). This conformation is fully active and sensitive to competition inhibition by statins. Although evidence suggests that deletion of exon 13 abolishes HMGCR enzymatic activity (Burkhardt et al. 2008), these studies did not consider the possibility that the 13(+) and 13(−) monomers may form heterogenous dimers or tetramers comprised of varying compositions of the 13(+) and 13(−) monomers. In the context of these hypothetical conformations, 13(−) may attenuate HMGCR enzyme activity and statin sensitivity, consistent with our reports that modulating the relative levels of the *13(+)* to *13(−)* transcript alters the statin sensitivity of the resulting HMGCR enzyme.