Functional analysis of 5-lipoxygenase promoter repeat variants

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Variants of a hexanucleotide repeat polymorphism in the promoter of the 5-lipoxygenase (5-LO) gene have been associated with cardiovascular disease traits in humans, which may be due, at least in part, to differential expression of the at-risk alleles. To more fully characterize these variants, we carried out gene expression and DNA methylation studies in primary leukocytes from healthy individuals carrying various 5-LO promoter alleles. Regardless of genotype, 5-LO and 5-LO-activating protein (FLAP) gene expression was higher in granulocytes compared with monocytes and lymphocytes, whereas leukotriene A₄ hydrolase (LTA4H) expression was higher in monocytes. In all three leukocyte populations, 5-LO mRNA levels were positively correlated with those of FLAP and LTA4H, with the highest correlation observed in granulocytes. In lymphocytes, individuals homozygous for the shorter 3 and 4 repeat alleles had between 20–35% higher 5-LO, FLAP and LTA4H expression compared with homozygous carriers of the wild-type 5 repeat allele (P < 0.03–0.0001). DNA methylation analysis of four CpG islands in a 1500 bp region encompassing the 5-LO promoter and the first 100 bp of intron 1 revealed relatively low overall DNA methylation across all genotypes and leukocyte populations. However, analysis of the promoter repeats themselves demonstrated that, regardless of cell population, the 4 allele was methylated approximately twice as much as the 3 allele (P < 0.0001). Our results demonstrate that, in lymphocytes, the shorter repeat alleles of the 5-LO promoter lead to higher gene expression, which may be regulated through differential DNA methylation of the CpGs located within these repeats.

INTRODUCTION

The 5-lipoxygenase (5-LO) pathway, which generates leukotrienes (LTs) from arachidonic acid, has recently garnered a great deal of attention for its potential role in cardiovascular disease (CVD)-related traits. This stems from a series of biochemical, genetic and pharmacological studies over the last few years, which collectively have provided strong evidence for the pro-atherogenic role of LTs (1–3). For example, deCode Genetics reported an association between FLAP haplotypes and myocardial infarction (MI)/stroke (4), which has been replicated in some populations (5) but not others (6–8). Haplotypes of the LTA4H gene have also been associated with MI in Caucasians and African Americans, with a more pronounced effect in the latter group (9), and LTC4 synthase variants have been associated with increased coronary artery calcification and carotid atherosclerosis (10). As part of our genetic studies in humans, we previously reported that individuals carrying certain shorter alleles of a repeat polymorphism in the 5-LO promoter had significantly increased carotid intima-media thickness (11) and an elevated risk of MI in the context of high-dietary arachidonic acid (12), the primary 5-LO substrate that leads to pro-inflammatory LTs. More recently, Crosslin et al. (13) also reported association of several 5-LO SNPs with CVD traits in a Caucasian sample.

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The core promoter region of the 5-LO gene contains 5 tandem GC-rich hexanucleotide repeats (GGGCCGG) located \(\sim200\) bp upstream of the ATG start codon, which function as overlapping Sp1 and early growth-response protein 1 consensus-binding sites (14,15). We and several other groups have previously reported that the number of Sp1-binding sites in the general human population varies from 3–9 repeats (16,17), with the most common allele consisting of 5 repeats (herein referred to as the wild-type allele), with alleles composed of 3, 4, 6–9 repeats occurring at lower frequencies (14). Importantly, the 3 and 4 alleles had previously been genetically and pharmacogenetically associated with asthma in certain populations (16,18,19), suggesting that, in asthma, the promoter polymorphism is functional. In addition, in vitro experiments with promoter–reporter constructs suggested a positive correlation between the number of repeat units and transcriptional activity (14,15). However, these results have not been replicated in experiments performed with buffy-coat-derived leukocytes from individuals with different genotypes (12), suggesting that, in vivo, these polymorphisms either do not influence gene transcription to an appreciable degree or that the regulation of 5-LO may involve other mechanisms. Given these uncertainties, the aim of the present study was to more fully characterize the functionality of the 5-LO repeat polymorphism in specific primary leukocyte populations from healthy individuals.

## RESULTS

### Expression of 5-LO, FLAP and LTA4H in leukocytes

We first determined real-time expression profiles for the 5-LO, FLAP and LTA4H genes in three primary leukocyte populations using all samples independent of 5-LO promoter genotype. As shown in Figure 1, the mRNA levels of 5-LO and FLAP were several fold higher in granulocytes compared with lymphocytes and monocytes, which expressed relatively low levels of these two genes. In comparison, differences in LTA4H expression were not as dramatically different between the three leukocyte populations (Fig. 1), with mRNA levels being the highest in monocytes. We next carried out a regression analysis to determine the relationship between the expression of 5-LO, FLAP and LTA4H. The mRNA levels of 5-LO and FLAP were highly positively correlated \((r^2 = 0.61; P < 0.0001)\) in granulocytes and less correlated in monocytes and lymphocytes (Fig. 2A). A similar pattern was also observed between the expression of 5-LO and LTA4H, with the strongest correlation in granulocytes \((r^2 = 0.54; P < 0.0001)\) relative to monocytes and lymphocytes (Fig. 2B). Given that gene expression levels of two granulocyte samples were much higher than the others, we re-performed the regression after removing these outliers from the analyses. Exclusion of these samples decreased the correlation between 5-LO and FLAP \((r^2 = 0.37)\) and between 5-LO and LTA4H \((r^2 = 0.28)\), but the results still remained statistically significant \((P < 0.0001)\) for both regression analyses.

![Gene expression differences as a function of 5-LO promoter genotype](image)

**Figure 1.** Real-time quantitation of 5-LO, FLAP and LTA4H mRNA levels in leukocytes. 5-LO and FLAP are expressed to a much higher degree in granulocytes compared with monocytes and lymphocytes, whereas LTA4H is expressed the highest in monocytes. Total RNA was isolated from the three cell populations and reverse-transcribed into cDNA. Real-time PCR was carried out in triplicate and expression levels were normalized to beta-glucuronidase as an endogenous control. Pairwise comparisons of gene expression across all three genes and cell types are significantly different \((P < 0.0001)\) with the exception of those between 5-LO and FLAP in granulocytes. The number of samples analyzed for each gene is given in parentheses. Data are shown as mean \(\pm\) SE in relative units (ru).

Gene expression differences as a function of 5-LO promoter genotype

We next determined whether 5-LO, FLAP and LTA4H gene expression differed as a function of 5-LO promoter genotype in the three cell populations. On the basis of previous studies (11,16), carriers of the short alleles with 3 and 4 Sp1 repeats (i.e. genotypes of 33, 34 or 44) were categorized as the homozygous ‘deleted’ group, those carrying one short and one wild-type allele (i.e. 35 or 45 genotypes) were designated as heterozygotes and individuals with two copies of the 5 allele were considered homozygous wild-type. In granulocytes, these analyses revealed significantly higher FLAP expression in the 33/34/44 and 35/45 groups compared with 55 homozygotes, whereas there was no difference in 5-LO or LTA4H mRNA levels (Fig. 3A–C). In monocytes, there were no significant differences in 5-LO, FLAP or LTA4H expression across the three genotype groups (Fig. 4A–C). In comparison, 5-LO mRNA levels in lymphocytes were significantly higher in the 33/34/44 and 35/45 groups by \(\sim30\)% compared with the homozygous 55 group \((P = 0.01\) and \(P = 0.01,\) respectively; Fig. 5A). Similarly, FLAP gene expression (Fig. 5B) was significantly higher between 20 and 35% in the 33/34/44 and 35/45 groups compared with the 55 homozygotes \((P < 0.0001\) and \(P = 0.03,\) respectively), with heterozygote carriers having intermediate mRNA levels. Consistent with these observations, LTA4H mRNA levels (Fig. 5C) were also significantly higher in the 33/34/44 individuals compared with the 55 group \((P = 0.01)\). Importantly, the gene expression differences we observed in lymphocytes were not being driven by any one particular genotype since the 33, 34, 44, 35 and 45 genotypes individually also showed increased mRNA levels (Supplementary Material, Fig. S1A–C). Confounders such as age, gender or BMI also did not affect the differences in gene expression (data not shown).
DNA methylation analysis of the 5-LO gene

Given previous studies in cell lines indicating DNA methylation as a potential mechanism by which 5-LO gene expression is regulated (20), we analyzed the extent of DNA methylation in the three leukocyte populations and across all 5-LO promoter genotypes. Analysis of the genomic sequence

Figure 2. Correlation between gene expression of 5-LO, FLAP and LTA4H in leukocytes. (A) 5-LO and FLAP mRNA levels are highly correlated in granulocytes and moderately correlated in lymphocytes and monocytes, suggesting that the expression of these genes is coordinately regulated. (B) 5-LO and LTA4H mRNA levels are highly correlated in granulocytes and modestly correlated in lymphocytes and monocytes, similar to what is observed for 5-LO and FLAP. Total RNA was isolated from the three cell populations and reverse-transcribed into cDNA. Real-time PCR was carried out in triplicate and expression levels were normalized to beta-glucuronidase as an endogenous control. The number of samples analyzed for each cell population is given in parentheses.

Figure 3. Differences in 5-LO, FLAP and LTA4H mRNA levels in granulocytes as a function of 5-LO promoter genotype group. The expression of 5-LO (A) and LTA4H (C) in granulocytes is not significantly different across different promoter repeat genotype groups, whereas FLAP (B) expression is significantly higher in subjects carrying the 33, 33 or 44 genotypes compared with homozygous 55 individuals. Total RNA was isolated from the three cell populations and reverse-transcribed into cDNA. Real-time PCR was carried out in triplicate and expression levels were normalized to beta-glucuronidase as an endogenous control. The number of samples analyzed for each genotype group is given in parentheses.
in the upstream regulatory region and the first two exons of the 5-LO gene predicted four CpG islands: three were located in the first 1000 bp of the proximal promoter region and the fourth spanned exon 1 and the first 100 bp of intron 1 (Fig. 6). Across granulocytes, monocytes and lymphocytes and the six 5-LO promoter repeat genotypes examined, quantitative analysis by the MassARRAY system showed very little (i.e., <5%) DNA methylation of the three CpG islands (regions 1, 2 and 3) located throughout the promoter (Fig. 6). In the CpG island spanning exon 1 and the beginning of intron 1 (region 4), there was slightly greater overall average DNA methylation (~10%), but this pattern also did not differ between the three cell types or 5-LO promoter genotype groups (Fig. 6). We next examined the extent of DNA methylation of the cytosine residues located within the 5-LO promoter repeats (GGGCGG_n), which are located within region 3. Since the size of the fragment generated by the 5 repeat allele was beyond the detection limit of our methodology, we were only able to perform this analysis with the 3 and 4 repeat alleles. As shown in Figure 7, the average DNA methylation of the 3 allele is ~2-fold less than the 4 allele in all three leukocyte populations (P < 0.0001). Bisulfite sequencing of the same PCR products (approximately 100 individual clones per sample) that were subjected to the mass spectrometry analysis using lymphocyte DNA of a 33, 44 and 55 subject yielded a trend in DNA methylation that was consistent with the MassARRAY results, although the overall percent DNA methylation values were quantitatively lower. We next carried out a regression between percent DNA methylation of the 3 and 4 repeats and 5-LO gene

Figure 4. Differences in 5-LO, FLAP and LTA4H mRNA levels in monocytes as a function of 5-LO promoter genotype. The expression of 5-LO (A), FLAP (B) and LTA4H (C) in monocytes is not significantly different in individuals carrying different promoter repeat alleles. Total RNA was isolated from the three cell populations and reverse-transcribed into cDNA. Real-time PCR was carried out in triplicate and expression levels were normalized to beta-glucuronidase as an endogenous control. The number of samples analyzed for each genotype group is given in parentheses.

Figure 5. Differences in 5-LO, FLAP and LTA4H mRNA levels in lymphocytes as a function of 5-LO promoter genotype. Compared with individuals with two copies of the 5 allele, subjects carrying the 33, 34 or 44 genotypes have significantly higher 5-LO (A), FLAP (B) and LTA4H (C) mRNA levels in lymphocytes. Heterozygous 35 and 45 subjects have significantly higher mRNA levels of 5-LO (A) and FLAP (B) but not LTA4H (C) compared with the 55 group. Total RNA was isolated from the three cell populations and reverse-transcribed into cDNA. Real-time PCR was carried out in triplicate and expression levels were normalized to beta-glucuronidase as an endogenous control. The number of samples analyzed for each genotype group is given in parentheses.
expression in the three leukocyte populations with samples for which both types of data were available. This analysis did not reveal a correlation between 5-LO mRNA levels and percent DNA methylation in granulocytes, monocytes or lymphocytes (Supplementary Material, Fig. S2).

**DISCUSSION**

In the present study, we provide evidence that a hexanucleotide repeat polymorphism in the 5-LO promoter is functional with respect to mRNA expression of three LT pathway genes. Specifically, carriers of the shorter 3 and 4 repeat alleles express higher levels of 5-LO, FLAP and LTA4H in lymphocytes and higher levels of FLAP in granulocytes. These results provide a functional basis for the observed genetic association of the shorter ‘deleted’ alleles with increased carotid atherosclerosis (11) and risk of MI, particularly in the context of high-dietary arachidonic acid (12),
which is the main substrate for the generation of pro-inflammatory LTs. Of note, genotypic differences in 5-LO, FLAP and LTA4H transcript levels were primarily observed in lymphocytes, a population of inflammatory cells that has been implicated in atherosclerosis. For example, T-cells infiltrating the subendothelial space can mount adaptive immune responses to local antigens and differentiate into Th1 effector cells, which release pro-inflammatory cytokines that can activate macrophages and propagate the atherosclerotic process (21). Interestingly, 5-LO deficiency in mice results in a shift of CD4\(^+\) T-cell differentiation and cytokine production towards a Th2-type profile (22), which can promote anti-atherosclerotic immune reactions (23). Moreover, a recent study demonstrated that a variety of peripheral T-cell populations, including CD4\(^+\), CD8\(^+\) and Th2 cells, express 5-LO at both the mRNA and protein levels and are capable of producing LTs when provided with exogenous arachidonic acid (24).

By comparison, 5-LO expression in atherosclerotic plaques has been reported to extensively co-localize with macrophages but not T-cells (25). One potential explanation for the discrepancy between this latter study and our gene expression results with respect to T-cells could be that we employed quantitative PCR for detecting mRNA expression, which is a more sensitive method than the immunohistochemical analysis used by Spanbroek et al. (25). Alternatively, the expression profile of peripheral leukocyte populations, and T-cells in particular, may change once the cells enter the microenvironment of the sub-endothelial space. Thus, although our *ex vivo* results are suggestive and consistent with previous reports that circulating T-cells are capable of generating LTs (24), additional studies will be required to determine whether LT production through lymphocyte-mediated mechanisms, particularly in individuals carrying the shorter alleles, can contribute to plaque formation and increased risk of CVD. It would also be of interest to determine whether the functional differences we observe are specific to T-cells and/or B-cells and whether they also occur in the cellular sub-populations of these lymphocytes.

We recently reported that the 3 and 4 repeat alleles do not affect total 5-LO mRNA levels in buffy coat-derived leukocytes, which we speculated could be due, in part, to the nature of the samples studied (12). For example, isolated buffy coats comprise a mixture of various immune cells, which as we show in the present study, express 5-LO at different levels. As a result, 5-LO expression could vary between individuals regardless of genotype, depending on the cellular composition of buffy coats. Thus, our observations that the promoter repeats affect gene expression in a specific group of leukocytes would be consistent with this hypothesis and suggest that functional experiments to characterize variants of inflammatory genes are important to carry out in homogenous and isolated cell populations, if possible, to avoid such confounding factors. In addition, another potential explanation for these cellular differences, particularly with respect to granulocytes, may be related to the relatively higher expression of 5-LO and FLAP. It is possible that other *cis* and/or *trans* factors, which control transcriptional regulation of these genes in granulocytes, overshadow the effect of the ‘deleted’ alleles on gene expression. For example, previous studies identified vitamin D-responsive elements within the 5-LO gene, including several in the promoter region, that are potent inducers of transcription (26,27), particularly when the 5-LO promoter is not methylated (28). It is possible that such regulatory elements are more active in certain cell types, such as granulocytes, and lead to higher 5-LO expression regardless of the effect of the promoter repeat alleles. Thus, while our experiments only examined gene expression differences under endogenous conditions, it would be important for future studies to address whether there are also cell-specific differences among the promoter alleles with respect to inducers of transcription as well. This may be particularly relevant with respect to plasma levels of vitamin D, which have been epidemiologically associated with CVD risk (29) and, as mentioned earlier, can enhance 5-LO transcription.

The genomic DNA methylation analyses of several 5-LO genomic regions revealed very little DNA methylation of the promoter and slightly greater DNA methylation in the region covering exon 1 and the very proximal portion of intron 1 regardless of cell type or promoter genotype. These results are consistent with our observations that 5-LO is expressed in granulocytes, monocytes and lymphocytes, although to different extents. In contrast, a previous report demonstrated high DNA methylation of the 5-LO promoter in U937 and HL-60TB cells, which do not express the gene (28). Given that our study was carried out in primary human leukocytes from a relatively large number of subjects, our results may indicate a better reflection of the *in vivo* DNA methylation status of the 5-LO gene. It would also be of interest to examine the DNA methylation status of the 5-LO gene in a variety of other tissues and cell types, including those that are not thought to express the enzyme.

Previous *in vitro* studies with engineered promoter constructs, which would not necessarily take into account the genomic DNA methylation status of the endogenous 5-LO promoter, reported a linear relationship between repeat number and gene expression (14,30). In contrast, we showed that the average methylation of the 4 repeat allele is twice that of the 3 allele, which would suggest increased expression from the 3 allele. However, our present study does not provide direct evidence of causality between the degree of DNA methylation and 5-LO expression since we did not detect gene expression differences between 33 and 44 individuals or a correlation between gene expression and percent DNA methylation of the promoter repeats. Interestingly, the latter observation has also been reported in studies of lymphomas (31,32). Nevertheless, our results do provide evidence that the level of DNA methylation throughout the 5-LO promoter occurs at relatively low levels in granulocytes, monocytes and lymphocytes regardless of genotype, with the exception of the 3 and 4 alleles, which exhibit a DNA methylation pattern that is directly related to the number of their repeats.

The relatively high correlation between 5-LO mRNA levels with those of FLAP, and to some extent LTA4H, suggests that these 5-LO pathway genes are coordinately regulated, particularly in granulocytes. Such an observation is not entirely surprising for 5-LO and FLAP since it is known that these proteins physically interact in order for 5-LO to catalyze the first oxidation step of LT biosynthesis (33) and suggest that
the expression of these genes is potentially controlled through a positive feedback mechanism. However, more in-depth studies will be required to gain a better understanding of how 5-LO pathway gene expression is regulated.

In conclusion, we demonstrate that 5-LO promoter alleles with less than five Sp1-binding motifs lead to increased expression of three LT pathway genes in lymphocytes. Taken together, our results provide a functional basis for recent genetic studies that have associated these alleles with increased carotid atherosclerosis and MI risk (11,12) and support the notion that increased 5-LO pathway activity can play an important role in atherogenic processes.

MATERIALS AND METHODS

Study subjects

Samples for the experiments described in this study came from the baseline visit of a randomized, double blind, placebo-controlled intervention trial to examine the effect of omega-3 fatty acid supplementation in subjects with different 5-LO promoter variants. The registry number for this study is NCT00536185, and details of the study design can be found at the ClinicalTrials.gov website. Healthy adults between 20 and 59 years of age, who self-identified as African American, black or a person of African descent, were recruited into the study from three study sites: Davis, Sacramento and Oakland, California. Recruitment of study participants occurred at health fairs, community meetings, churches and other public venues from May 2005 to March 2007. Potential study participants received a brief interview, ~15 min in length, by trained recruitment personnel to characterize general health, previous diagnosis of major diseases, smoking and alcohol usage, medication, nutritional supplements and other factors. After the initial screening by questionnaire, a buccal swab (Isohelix, DNA Swab Pack, SK-1, Kent, UK) was taken for DNA isolation and genotyping. Eligible subjects who had one of the six 5-LO promoter repeat genotypes of interest (33, 34, 44, 35, 45, 55) were subsequently invited to participate in the study. A fasting blood sample was used for a complete blood count, lipid and chemistry panel analyses to identify any undiagnosed medical problems. Study participants who had a physician-diagnosed chronic inflammatory disease (arthritis, autoimmune disease or asthma), CVD, hypertension, diabetes or a lipid disorder that required regular use of anti-inflammatory or lipid-lowering medication were excluded. Subjects with abnormal results on standard chemistry and lipid panels, including hypertriglyceridemia (fasting plasma triglyceride >500 mg/dl), hypercholesterolemia (fasting plasma LDL cholesterol >160 mg/dl), hemoglobin <12 g/dl for females and <14 g/dl for males or a complete blood count that suggested underlying undiagnosed disease were also excluded and referred to their physician for further evaluation. However, we cannot exclude the possibility that some subjects may have subclinical disease that would not be detectable through this screening process. The institutional review boards of The University of California, Davis, Alta Bates Summit Medical Center, the USC Keck School of Medicine reviewed and approved ethical permission for all procedures involving human volunteers and the protocols. Written informed consent was obtained from all study participants.

Genotyping

Genotyping of the 5-LO promoter repeat polymorphism was performed using previously described methods (11) from genomic DNA isolated from buccal swabs. The genotypes for each individual were confirmed using genomic DNA obtained from lymphocytes.

Leukocyte isolations

At the baseline visit subsequent to enrollment, a fasting blood sample (80 ml) was collected into heparinized tubes and processed within 4 h. Plasma was separated after low-speed centrifugation, and the buffy coat containing mononuclear cells (lymphocytes and monocytes) and granulocytes was then removed and diluted with approximately one volume of Hank’s balanced salt solution (HBSS) into a total volume of ~32 ml. Two step-gradients per subject were then prepared in 50 ml Blue Max (BD Biosciences, San Jose, CA, USA) centrifuge tubes using room-temperature Histopaque 1077 and 1119 (Sigma Aldrich, St Louis, MO, USA) (12 ml of each density in each tube). The diluted buffy coat was gently layered onto the step gradients (16–24 ml per gradient) and centrifuged at 700 g for 30 min at 25°C with the brake off. Mononuclear cells were removed from above the low-density step, and granulocytes from above the high-density step. Mononuclear cells were washed once with HBSS, and residual erythrocytes were lysed with hypotonic buffer. The same procedure was used for granulocytes. Monocytes were then purified from lymphocytes by positive selection using CD14-labeled magnetic microbeads with an LS magnetic column (Miltenyi Biotec, Auburn, CA, USA). Unselected mononuclear cells were saved as the lymphocyte preparation. Purity of monocytes was assessed by FACS analysis (BD FACSCalibur) using PE-labeled anti-CD14 antibody (Miltenyi Biotec) and an isotype control reagent (IgG2a; BD Biosciences). Purity of granulocytes and lymphocytes was assessed by differential count using a hematology analyzer (CellDyne 3200). Granulocytes (0.5–2 million cells), monocyte (0.5–2 million cells) and lymphocytes (20 million cells) were suspended in RLT buffer (Qiagen, Valencia, CA, USA) and phosphate-buffered saline for RNA and DNA isolation, respectively, and frozen at ~80°C prior to analysis. The median percent purity (25th/75th percentiles) of granulocytes was 95.1 (89.9/98.0%; n = 72), of lymphocytes was 92.8 (89.6/94.6%; n = 107) and of monocytes was 92.6 (89.1/96.1%; n = 103). Two granulocyte samples had low purities (<30%) and were excluded from all subsequent analyses.

Real-time RNA quantitation

Total RNA was isolated using RNeasy kits from Qiagen, Inc., of which 1 μg was reverse-transcribed using cDNA Archive kits from ABI (Foster City, CA, USA). Real-time mRNA levels for 5-LO, FLAP, LT44H and beta glucuronidase (as an endogenous control) were determined in triplicate using pre-developed assays from Applied Biosystems (Foster City, CA, USA).
DNA methylation analyses
Quantitative DNA methylation analysis using the MassARRAY system (Sequenom, San Diego, CA, USA) was carried out as described previously (34,35). Briefly, 1 μg of genomic DNA was treated with sodium bisulfite using an EZ-96 DNA Methylation Kit (Zymo Research, Orange, CA, USA). Amplicons for the four identified CpG islands (regions 1, 2, 3 and 4) in the 5-LO promoter region and exon/intron 1 were PCR-amplified, in vitro-transcribed and subjected to RNase A cleavage. The fragments for each amplicon/region that were generated from this process were then quantitatively assessed for DNA methylation status using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. Primer sequences used to amplify the four regions were as follows: region 1F: 5'-AGGAAGAGGTTTTTGGGGATTTTATATTTT-3'; region 1R: 5'-CAGTATAATACGACTCACTATAGGGAGAAGGCTCCTCCCAAGGTGAAGAGTGGGAGAGAAGTAT-3'; region 2F: 5'-AGAGAGGGTTTATAGGAGTTTGGTTTTG-3'; region 2R: 5'-CAGTATAATACGACTCACTATAGGGAGAAGGCTCCTCCCA-3'; region 3F: 5'-AGGAAGGGTTTATAGGAGTTTGGTTTTG-3'; region 3R: 5'-CAGTAATACGACTCACTATAGGGAGAAGGCTCCTACCACAATCCTATC-3'; region 4F: 5'-AGGAGGTATTGATTTGTATTATTATTTT-3'; region 4R: 5'-CAGTAATACGACTCACTATAGGGAGAAGGCTCCTACAACACCCACCAATCCTATC-3'. Bisulfite sequencing of the same amplicon encompassing the GGGCGG repeats (region 3) that was subjected to mass spectrometry was carried out on individually cloned PCR products according to previously described methods (36) using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA).

Statistical analyses
Gene expression differences between genotype groups were initially assessed with a two-way ANOVA. A significant ANOVA P-value of <0.05 was followed by unpaired t-tests to assess expression differences between the individual 33/44 and 35/45 genotype groups relative to the 55 group. These specific comparisons were pre-planned on the basis of a priori hypotheses that the shorter alleles lead to higher gene expression given our previous studies that these variants predispose to increased atherosclerosis and risk of MI (11,12). Unpaired t-tests were also used to determine methylation differences between promoter repeat alleles. P-values <0.05 from the t-tests were considered statistically significant. All data were analyzed with Statview software (Version 5.0; SAS Institute Inc., Cary, NC, USA).

SUPPLEMENTARY MATERIAL
Supplementary material is available at HMG online.

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