Genetic Variation in Lectin-Like Oxidized Low-Density Lipoprotein Receptor 1 (LOX1) Gene and the Risk of Coronary Artery Disease

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Background—We examined the association of 3 polymorphisms in the lectin-like oxidized LDL receptor-1 (LOX1 or OLR1) gene with coronary artery disease in the Women's Ischemia Syndrome Evaluation (WISE) study population.

Methods and Results—The WISE sample comprised 589 white and 122 black women who underwent angiography for suspected ischemia. The sample was divided into 3 groups: <20% stenosis (38.7%), 20% to 49% stenosis (24.9%), and ≥50% stenosis (35.3%). The three LOX1 polymorphisms (intron 4/G→A, intron 5/T→G, and 3′ UTR/T→C) were in linkage disequilibrium and thus behaved as a single polymorphism. The frequency of the 3′UTR/T allele was significantly higher in whites than blacks (49% versus 19%; $P<0.0001$). Among white women, the frequency of the 3′UTR/T allele carriers (TC+TT genotypes) increased gradually from 67.9% to 75.0% and 79.2% in the <20%, 20% to 49%, and ≥50% stenosis groups, respectively (χ^2 trend=6.23; $P=0.013$). Logistic regression analyses indicated that APOE (odds ratio, 1.90; $P=0.007$) and LOX1 (odds ratio, 1.67; $P=0.025$) genotypes were independently associated with the risk of disease and that there was no interaction between the two genes. The 3′UTR/T allele carriers also had significantly higher IgG anti-oxLDL levels than individuals carrying the CC genotype (0.94±0.20 versus 0.86±0.16; $P=0.032$). Furthermore, our electrophoretic mobility shift assay data show that the 3′UTR polymorphic sequence affects the binding of a putative transcription factor in an allele-specific manner.

Conclusions—Our data suggest that common genetic variation in the LOX1 gene may be associated with the risk of coronary artery disease in white women. **(*Circulation*. 2003;107:3146-3151.)**

Key Words: risk factors ■ coronary disease ■ genetics

The hallmark of the initiation of atherosclerotic lesion is the foam cell formation, and thus those genes that participate in this pathway are candidates to affect the risk of coronary artery disease (CAD). Oxidized LDL (oxLDL) is believed to play a key role in the initiation of the atherosclerotic process. OxLDL is internalized by several receptors, such as SR-AI/II, SR-BI, CD36, macrosialan, and CD68.[1,2] OxLDL is also internalized by endothelial cells, but this uptake depends on receptors other than the classic scavenger receptors. In 1997, a lectin-like oxidized LDL receptor-1 (LOX-1, OLR1) was identified in bovine aortic endothelial cells.[3] LOX1 is expressed and binds with oxLDL in peripheral tissues, including the endothelial cells of large arteries,[4] macrophages,[5] and smooth muscle cells.[6] The binding of oxLDL to LOX1 induces several cellular events in endothelial cells such as activation of transcription factor NF-κB,[7] upregulation of monocyte chemoattractant protein-1,[8] and reduction in intracellular nitric oxide,[9] which may trigger the onset of cardiovascular events or accelerate the development of atherosclerosis.

The LOX1 gene, also known as OLR1, is mapped to chromosome 12p13.1-p12.3. LOX1 is synthesized as a 40-kDa precursor protein and is composed of four domains: an extracellular lectin-like domain at the C-terminal, a connecting neck domain, a transmembrane domain, and a N-terminal cytoplasmic domain.[10] Recent studies suggest that the lectin domain, which is also called carbohydrate recognition domain (CRD), is the functional domain for the oxLDL binding,[11] and the essential residues in CRD for protein cell surface localization and ligand binding have been recognized.[12]

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Recently, we have identified three common LOX1 polymorphisms in intron 4 (G→A), intron 5(T→G), and 3′ UTR (T→C).[13] In this study, we have examined the relation between these three polymorphisms and the risk of CAD in the Women's Ischemia Syndrome Evaluation (WISE) study sample.

Methods

Subjects

The subjects were derived from the WISE study, which is a National Heart, Lung, and Blood Institute (NHLBI)–sponsored, 4-center study designed to address issues related to ischemic heart disease recognition and diagnosis in women. Women ≥18 years of age were enrolled in the WISE study at one of four clinical centers (University of Alabama at Birmingham, Allegheny University of the Health Sciences at Pittsburgh, University of Florida at Gainesville, and University of Pittsburgh). Major enrollment and exclusion criteria are described elsewhere.[14] A total of 711 DNA samples were available for the present study, including 589 non-Hispanic whites and 122 blacks (not of Hispanic origin) with mean age 57.3±11.6 (SD) years. Samples were obtained with informed consent, and the study was approved by the institutional review board.

Quantitative coronary angiography was performed by using the WISE study protocol, and data were analyzed at the Angiography Core Laboratory at Brown University by investigators blinded to the subjects. All coronary segments identified visually as abnormal were then measured quantitatively. The following definitions were used to categorize the patients into 3 coronary artery groups: (1) normal/minimal disease, <20% stenosis (38.7%); (2) mild disease, 20% to 49% stenosis (24.9%); and (3) significant disease: ≥50% stenosis (35.3%), in any one major epicardial coronary artery.

For allele frequency comparison purposes, a random sample of 339 apparently healthy white women (mean age, 46.3±13.5 years) was obtained from the Central Blood Bank of Pittsburgh after the consent procedures were approved by the University of Pittsburgh Institutional Review Board.

Genetic Screening

Genomic DNA was isolated by using the Puregene Systems DNA purification kit (GENTRA). Genotyping for the LOX1 intron 4 G/A (14 bp from the 5′end of exon 5), intron 5 T/G (27 bp from the start of exon 6), and 3′UTR T/C (188 bp from the stop codon) polymorphisms was done as previously described.[13] The APOE genotypes were determined as previously described.[15]

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) was performed with the LOX1/3′UTR (T→C) polymorphism. Two double-stranded 30-mer oligonucleotides corresponding to the T or C allele were prepared as described elsewhere.[13] The C oligonucleotide was 5′-end–labeled with α-^{32}P ATP and purified by using the QIAquick Purification Kit (Qiagen). Equally concentrated, nonradioactive competitor DNA (C or T oligos) was added 1×, 3×, 10×, 20×, and 50× excess volumes of the labeled probe. The mixture of unlabeled and labeled oligos were incubated with 2 μL of aorta smooth muscle cell nuclear extracts (Geneka) for 20 minutes at room temperature in binding buffer (1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 50 mmol/L NaCl, 10 mmol/L Tris-HCl [pH=7.5], 20% glycerol). The DNA-protein complexes were then separated on 6% nondenaturing polyacrylamide gel at 120 V for 2 hours, and the gel was dried and autoradiographed overnight.

IgG and IgM Anti-oxLDL Measurement

We randomly selected 161 plasma samples from white women in the WISE study for anti-oxLDL assay. IgG and IgM autoantibodies against native and malondialdehyde-modified LDL (m-LDL) were assayed by enzyme-linked immunosorbent assay as described previously,[16] with the modification that sodium azide was omitted from the wash buffer. Data were calculated as the ratio of antibody binding between m-LDL and native LDL (m-LDL/LDL) and then expressed as a percentage of the value for the plasma pool (% M/L).[16] Within-run and between-run coefficients of variation for the IgG anti-oxLDL assay were 10.5% and 5.6%, and for the IgM anti-oxLDL assay were 5.2% and 10.0%, respectively.

Statistical Analyses

Among different patient groups, 1-way ANOVA was used to test for mean differences in lipid traits, anti-oxLDL (IgG, IgM), and continuous variables. Possible differences in proportions of discrete variables were compared among different CAD groups by means of Pearson χ^2 tests. Allele frequency for each polymorphism was calculated by allele counting. The significance of deviations of the observed genotype frequencies from the Hardy-Weinberg equilibrium was tested by a χ^2 goodness-of-fit test. Linkage disequilibrium among the three LOX1 markers was estimated by the |D′| calculation.[17] Comparisons of allele frequencies between racial groups were calculated by using a standard Z test of two binomial proportions.

A χ^2 test was applied to test for a significant linear trend in the association of LOX1 polymorphism with CAD severity.[18] Specifically, in the trend test, we assigned CAD severity levels of 1, 2, and 5 to represent the <20% stenosis, 20% to 49% stenosis, and ≥50% stenosis groups, respectively.

The relations between each genetic polymorphism and CAD severity, which was defined by extent of stenosis (<20% stenosis; 20% to 49% stenosis; ≥50% stenosis), were further determined by logistic regression analysis. In the model, we pooled the moderate (20% to 49%) and significant (≥50%) stenosis groups and compared that with the normal/minimum (<20%) stenosis group to estimate the relation between the LOX1 polymorphism and CAD risk. Odds ratios and 95% CI were estimated by logistic regression while simultaneously adjusting for significant CAD risk factors (age, body mass index [BMI], smoking history, history of diabetes, history of hypertension, lipid-lowering drug [statins/others] intake, and APOE genotype). A forward stepwise logistic regression analysis was used to choose the above significant risk factors (probability value ≤0.05 for a variable to enter the model) from a set of known risk factors including: age, BMI, smoking history, alcohol history, history of diabetes, history of hypertension, lipid-lowering drug (statins/others) intake, family history of CAD, menopausal status, plasma total cholesterol, LDL-cholesterol, HDL cholesterol, TG levels, and APOE genotype.

The relations between levels of plasma anti-oxLDL (IgG and IgM) and LOX1 3′UTR genotypes were examined by multivariate analysis adjusting for significant covariates. Of all the variables examined, age and HDL were significant covariates for IgG anti-oxLDL and TC for IgM anti-oxLDL.

Results

Characteristics of the WISE Samples

Table 1 presents clinical characteristics according to CAD stenosis severity among white women. Age (P<0.001), smoking (P=0.004), statin intake (P<0.001), other lipid-lowering drug intake (P=0.0039), history of hypertension (P=0.001), history of diabetes (P<0.001), menopausal status (P<0.001), and levels of HDL cholesterol (P=0.012) and IgM anti-oxLDL (P=0.003) differed significantly among stenosis groups. Among the black women, age (P=0.002), history of hypertension (P=0.029), and TG level (P=0.021) were significantly associated with severe CAD (data not shown).

Distribution of LOX1 Polymorphisms in Whites and Blacks

Homogeneity tests were performed to determine if the LOX1 genotype distributions at three loci were comparable across

TABLE 1. Patient Characteristics Based on Stenosis of Arteries in White Women

	Stenosis Groups			
	<20% (n=227)	20% to 49% (n=150)	≥50% (n=206)	*P*
Age, y	53±0.7	58±0.9	62±0.8	<0.001
BMI, kg/m^2	29.8±0.5	28.7±0.5	28.7±0.4	0.161
Former smoker, yes, %	29.8	32	37.9	...
Current smoker, yes, %	14.6	16.4	19.9	0.004
Alcohol use within last 6 mo, yes, %	14.2	18.7	10.9	0.118
Lipid-lowering drug intake				
Statin, yes, %	9.3	34.7	38.3	<0.001
Others, yes, %	1.3	8	5.8	0.004
Family history of CAD, yes, %	66.1	69.6	67.3	0.777
History of hypertension, yes, %	43.8	56	62.3	0.001
History of diabetes, yes, %	9.7	15.3	35.9	<0.001
Menopause, yes, %	73.9	86.5	88.7	<0.001
Total cholesterol, mg/dL	195±2.9	201±3.9	194±3.2	0.289
LDL cholesterol, mg/dL	114±2.6	116±3.3	110±2.9	0.350
Triglycerides, mg/dL	148±7.4	170±13.5	170±6.8	0.102
HDL cholesterol, mg/dL	55±0.8	54±1.2	51±0.8	0.012
*IgG anti-oxLDL, %m-LDL/LDL	0.90±0.02	0.92±0.02	0.93±0.04	0.690
*IgM anti-oxLDL, %m-LDL/LDL	1.39±0.05	1.67±0.07	1.60±0.08	0.003

*OxLDL levels were available on selected white individuals, including 58 in the <20% stenosis group, 52 in the 20% to 49% group, and 49 in the ≥50% group.

the three geographic locations within whites and blacks. No significant differences were observed between the locations and thus the samples were pooled within ethnic groups. The distribution of three LOX1 polymorphisms was significantly different between whites and blacks (Table 2). The frequencies of the intron 4/G allele (49.2% versus 18.8%; $P<0.001$), intron 5/T allele (49.1% versus 18.6%; $P<0.001$), and 3′UTR/T allele (49.0% versus 18.8%; $P<0.001$) were significantly higher in whites compared with blacks.

Linkage Disequilibrium

The three LOX1 polymorphisms were in significant linkage disequilibrium in whites and blacks. The |D′| value of 1 indicates a complete linkage disequilibrium between two markers. In the white women, all three markers were essentially in complete linkage disequilibrium with each other (|D′|=0.996; $P<0.001$), indicating that the three variants behaved as one polymorphism. Therefore, we report the analyses of the 3′UTR polymorphism only. Although in black women, the three polymorphisms were in disequilibrium ($P<0.0001$), it was not complete (|D′| ranged from 0.32 to 0.37). However, because the results of analyses of the polymorphisms were similar in blacks, we present only the 3′UTR polymorphism results.

LOX1 Polymorphisms and CAD Severity

Before analyzing the effects of the LOX1 polymorphisms on CAD severity, we examined the association of the LOX1 polymorphisms with total cholesterol, LDL cholesterol, HDL cholesterol, and TG levels. However, no significant association was found (data not shown).

The distribution of the 3′UTR genotypes among the three stenosis groups is shown in Table 3. Among white women, the frequency of the 3′UTR/T allele significantly increased as the stenosis severity increased (χ^2 trend=6.23, $P=0.013$). The age, BMI, lipid-lowering drug intake, smoking history, history of diabetes, history of hypertension, and APOE genotype (*APOE*4* versus non-*APOE*4*), adjusted OR between T allele carriers (TC and TT genotypes) compared with the CC genotype for having at least moderate CAD (≥20% stenosis) was 1.67 (95% CI, 1.07, 2.63; $P=0.025$). However, there was no significant association with CAD within the black women, probably because of small sample size in each category (<20%: 45 subjects; 20% to 49%: 25 subjects; ≥50%: 45 subjects). Because the *APOE*4* allele of the APOE polymorphism is a well-known risk factor for CAD, and in our white sample the adjusted OR for *APOE*4* was 1.90 (95% CI, 1.19, 3.02; $P=0.007$), we performed regression analyses to test for an interaction between the LOX1 and APOE polymorphisms. However, no significant interaction was observed between the two polymorphisms ($P=0.23$).

LOX1 3′UTR Polymorphism and Anti-oxLDL (IgG and IgM) in Whites

Table 4 shows the distribution of mean IgG anti-oxLDL and IgM anti-oxLDL levels among the LOX1 3′UTR genotypes in white women. The mean IgG values were significantly higher in the T carriers (TC+TT genotypes) compared with the CC genotype (0.94±0.20 versus 0.86±0.16; $P=0.032$). No significant difference was observed for the IgM values among genotypes. It is interesting that whereas IgM anti-oxLDL levels related to the severity of disease (Table 1), IgG

TABLE 2. Distribution of OLR1 Intron 4, Intron 5, and 3′UTR Polymorphisms in Whites and Blacks

Genotype	Whites, n (%)	Blacks, n (%)	*P*
Intron 4			
GG	145 (24.7%)	5 (4.2%)	
GA	287 (49.0%)	35 (29.2%)	
AA	154 (26.3%)	80 (66.7%)	
Total	586	120	
	χ^2=77.44; *P*<0.0001		
Allele frequency: G	0.492	0.188	<0.0001
Intron 5			
TT	144 (24.6%)	5 (4.1%)	
TG	287 (49.0%)	35 (28.9%)	
GG	155 (26.5%)	81 (66.9%)	
Total	586	121	
	χ^2=77.80; *P*<0.0001		
Allele frequency: T	0.491	0.186	<0.0001
3′UTR			
TT	143 (24.5%)	5 (4.3%)	
TC	286 (49.0%)	34 (29.1%)	
CC	155 (26.5%)	78 (66.7%)	
Total	584	117	
	χ^2=74.54; *P*<0.0001		
Allele frequency: T	0.490	0.188	<0.0001

anti-oxLDL levels were affected by genotype. This may not be surprising, considering that serum total IgM levels normally reflect an acute inflammatory response, whereas total serum IgG is a long-term immune response indicator.

Electrophorectic Mobility Shift Assay

The 3′UTR T or C oligonucleotides formed a DNA-protein complex with smooth muscle cell nuclear extracts (Figure 1). However, by using increasing amounts of unlabeled competitor oligos (T or C allele) to compete with the labeled C oligo, we found that the C allele had an average ≈3-fold higher binding affinity compared with the T allele (see Figure). Identical allele-dependent differences in binding were observed on repeating EMSA at least 5 times. The EMSA data indicate that the 3′UTR sequence binds regulatory protein(s) and that the C allele has a higher affinity for binding compared with the T allele.

TABLE 3. Distribution of OLR1 3′UTR Polymorphism by Stenosis

	Stenosis Groups			
	<20%	20% to 49%	≥50%	Trend Test **P*
Whites				
TT	48 (21.4%)	37 (25.0%)	56 (27.7%)	
TC	104 (46.4%)	74 (50.0%)	104 (51.5%)	
CC	72 (32.1%)	37 (25.0%)	42 (20.8%)	0.013
T allele frequency	0.446	0.500	0.535	
Blacks				
TT	0 (0%)	2 (8.0%)	3 (6.7%)	
TC	14 (31.1%)	7 (28.0%)	13 (28.9%)	
CC	31 (68.9%)	16 (64.0%)	29 (64.4%)	0.707
T allele frequency	0.156	0.22	0.211	

*Trend tests were performed among three stenosis groups (TT+TC vs CC).

Discussion

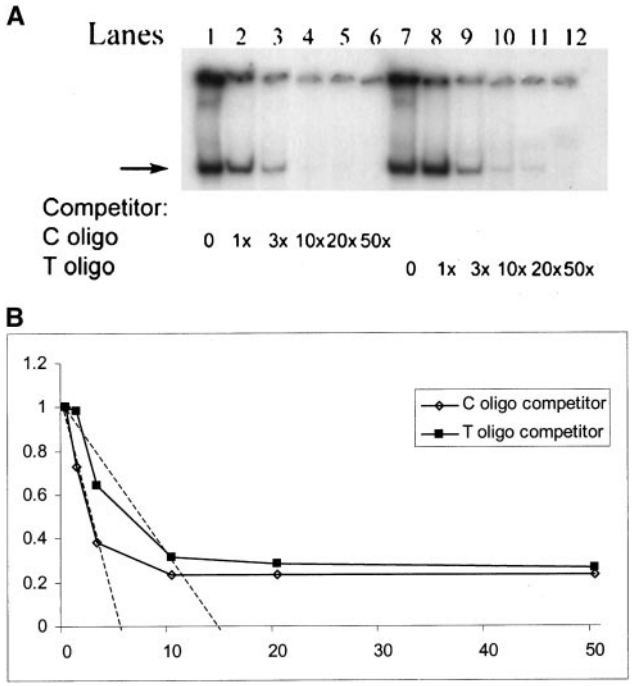
We report the association of common genetic variation in the LOX1 gene in relation to angiographic CAD. All three polymorphisms were in significant linkage disequilibrium and therefore exhibited similar genotype/allele frequencies. Thus, they could be considered a single marker for discussion purpose. Since the 3′ untranslated region of mRNA has been shown to affect the regulation of gene expression in conjunction with the promoter sequence,[19] we focused on the 3′UTR polymorphism because of its potential functional relevance. The frequency of the 3′UTR/T allele increased significantly from 44.6% in the <20% stenosis group to 50% in the 20% to 49% stenosis group and 53.5% in the ≥50% stenosis group. The odds of presenting with at least moderate CAD (≥20% stenosis) for 3′UTR/T carriers versus noncarriers was 1.67 after adjusting for the effects of other significant covariates associated with CAD. This estimated risk is equivalent to the well-known reported risk of the *APOE*4* allele (OR=1.26 for clinical CAD and OR=1.11 for angiographic CAD from a meta-analysis).[20] Since all women in this study were evaluated because of chest pain, there is concern that the control group used in this study (<20% stenosis) for calculating odds ratios may not represent the true control group. To address this question, we screened 339 apparently healthy, white, blood donor women for the LOX1/3′UTR polymorphism. The frequencies of the TT (22.7%), TC (49.3%), and CC (28.0%) genotypes and T allele (47.3%) in blood donor women were very similar to the <20% stenosis group (*P*=0.373; see Table 3) but different from the ≥50% stenosis group (*P*=0.048; see Table 3). This observation provides confidence that the results of our association studies are real rather than representing a chance observation resulting from the unique composition of the WISE sample.

The exact mechanism by which LOX1 can affect the risk of CAD is not clear. No significant associations were observed between the LOX1 polymorphisms and plasma lipid levels in these women. However, since LOX1 is a receptor for oxLDL, it would affect CAD risk through its direct effect on the

TABLE 4. IgG and IgM Anti-OxLDL Among LOX1 3′UTR Genotypes in White Women (Absorbance Ratio of m-LDL/LDL Expressed as Percentage of Plasma Pool)

	LOX1 3′UTR			
	CC (n=49)	TC (n=69)	TT (n=43)	*P*
Mean age	57.35±1.50	55.41±1.45	56.42±1.62	0.822
IgG anti-oxLDL	0.86±0.02	0.95±0.02	0.92±0.04	0.032[a]
IgM anti-oxLDL	1.55±0.07	1.55±0.05	1.51±0.08	0.742[a]

[a]Probability values obtained by comparing TC+TT with CC genotype.



A, EMSA result for LOX1/3′UTR polymorphism: Each sample contains a mixture of 5 μg of nuclear extract derived from aorta smooth muscle cells and 30-mer ^{32}P-labeled 3′UTR oligonucleotide containing the C allele. Arrowhead indicates specific DNA-protein complex associated with the 3′UTR T→C polymorphic site. Competition assay performed by adding excess cold oligonucleotides containing either the 3′UTR C allele (lanes 2 to 6) or the T allele (lanes 8 to 12). Lanes 1 and 7 have no competitor; lanes 2 to 6 have increasing amounts of C oligo competitor (1×, 3×, 10×, 20×, and 50×, respectively); lanes 8 to 12 have increasing amounts of T oligo competitor (1×, 3×, 10×, 20×, and 50×, respectively). B, Densitometric analysis graph showing relative binding of T allele compared with C allele from EMSA data shown in A. Slopes of dashed lines that represent initial linear phases of competition are described by equations $y=-0.2045x+1.00$ for the C oligo competitor and $y=-0.0682x+1.00$ for the T oligo competitor.

metabolism of oxLDL. To examine if the association of the 3′UTR polymorphisms with CAD can be explained through its direct affect on oxLDL, we examined the impact of 3′UTR genotypes on anti-oxLDL levels. Consistent with the association of the T allele carriers (TT/TC) with CAD, we found that the T allele carrier women also had significantly higher IgG anti-oxLDL than women with the CC genotype. These data suggest that the 3′UTR polymorphism may affect the risk of CAD by affecting the oxLDL metabolism.

To provide support for our association studies, we performed EMSA on the 3′UTR sequence variation to assess if it affects the binding of a regulatory element and, if it does, whether there is an allele-specific binding. The results from EMSA indicate that the 3′UTR sequence surrounding the T→C polymorphic site is involved in specific-binding to a nuclear protein (see Figure). Furthermore, there was an allele-dependent binding to a transcriptional factor such that the 3′UTR/T allele binds with a weaker affinity compared with the C allele. Whether the 3′UTR region encompassing the polymorphic sequence harbors a negative or positive *cis*-acting element and how it interacts with regulatory elements in the LOX1 promoter region is not known. It is also possible that the 3′UTR site may directly affect the mRNA stability or translation regulation of LOX1. Additional functional studies of the 3′UTR polymorphism along with the characterization of the LOX1 promoter may shed more light about the functional nature of this mutation. The intron 4 and intron 5 polymorphisms, which reside 14 bp and 27 bp from exon 5 and exon 6, respectively, may also be functional by possibly affecting the exon splicings.

CAD is a multifactorial disease in which the complex pathogenesis may only be explained by potential gene-environment or gene-gene interactions. For this reason, we performed a series of exploratory multiple regression analysis to test for possible interactions between the LOX1 polymorphisms and environmental or genetic factors such as *APOE*4*, which is a significant risk factor for CAD. No significant interactions were revealed from the logistic regression analyses. Our results suggest that the association between the LOX1 polymorphism and CAD risk is independent of the known CAD environmental or genetic factors.

In summary, our data suggest that common genetic variation in the LOX1 gene is associated with angiographic CAD in women. To our knowledge, this is the first study reporting significant association and thus this should be considered provisional until validated in independent case-control samples. Other limitations of this study were that only women were used and that the black sample size was small. Future studies including both men and women and larger cohorts of black cases and control subjects should further elucidate the genetic and biological significance of the LOX1 polymorphisms in the underlying cause of CAD.

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