

**Serum Bilirubin and Ferritin Levels Link Between Heme Oxygenase-1 Gene Promoter Polymorphism and Susceptibility to Coronary Artery Disease in Diabetic Patients**

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**Objective-** Heme oxygenase (HO) leads to the generation of free iron, carbon monoxide, and bilirubin. A length polymorphism of GT repeat in the promoter of human HO-1 gene has been shown to modulate gene transcription. This study aims to assess the association of the length of (GT)<sub>n</sub> repeats in HO-1 gene promoter with serum bilirubin, markers of iron status, and the development of coronary artery disease (CAD).

**Research design and methods-** We screened the allelic frequencies of (GT)<sub>n</sub> repeats in the HO-1 gene promoter in 986 unrelated individuals that underwent coronary angiography. Serum bilirubin and markers of iron status were evaluated.

**Results-** The distribution of numbers of (GT)<sub>n</sub> repeats was divided into 2 subclasses: class S included shorter (<27) repeats, and class L included longer ( $\geq$ 27) repeats. Among those with diabetes, subjects with L/L genotype had significantly lower bilirubin levels than those with S/S and S/L genotypes ( $0.70\pm 0.22$  vs.  $0.81\pm 0.24$  mg/dL,  $P=0.001$ ) and higher serum ferritin values ( $4.76\pm 0.72$  vs.  $4.28\pm 1.05$   $\mu$ g/L for log-ferritin,  $P=0.001$ ). Compared with those carrying S allele, diabetic subjects with L/L genotype had an almost three-fold increase in CAD risk after controlling for conventional risk factors (odds ratio 2.81, 95% confidence interval [CI] 1.22 to 6.47,  $P=0.015$ ). Adjusting for both serum bilirubin and ferritin, the effect of HO-1 promoter polymorphism on susceptibility to CAD disappeared.

**Conclusions-** Length polymorphism in the HO-1 gene promoter is correlated with susceptibility to CAD in diabetic patients and such effect might be conveyed through its influence on serum bilirubin and ferritin.

**H**O is a rate-limiting enzyme in heme degradation, leading to the generation of free iron, biliverdin, and carbon monoxide (CO). Biliverdin is subsequently converted to bilirubin via the action of biliverdin reductase, and free iron is promptly sequestered into ferritin. There are 2 genetically distinct isozymes of HO: the inducible HO-1 and a constitutively expressed HO-2. HO-1 is a cytoprotective enzyme, upregulated in mammals mostly dependent on transcriptional activation of the HO-1 gene to diverse cellular stress.

The relationship of HO to atherosclerotic vascular disease was suggested initially in 1994 by an observational study reporting that low serum concentrations of bilirubin are associated with increased risk of CAD (1). The human HO-1 gene has been mapped to chromosome 22q12, and a (GT)<sub>n</sub> dinucleotide repeat has been identified in the proximal promoter region (2). The (GT)<sub>n</sub> repeat is highly polymorphic and modulates gene transcription by oxidant challenge (3). We and others have demonstrated that longer (GT)<sub>n</sub> repeat exhibits lower transcriptional activity and is associated with susceptibility to CAD in high risk patients (4,5).

Bilirubin, a natural product of heme catabolism by HO, has been recognized to be an antioxidant and can inhibit lipid peroxidation (6). There is accumulating evidence that individuals with high-normal or just above normal plasma bilirubin levels have a lesser incidence of CAD and carotid plaque formation (7,8). HO-1 is also of critical contribution to iron homeostasis. The association between body iron status and the risk of cardiovascular disease was first postulated by Sullivan in the early 1980s (9), and thereafter by a number of epidemiological studies (10). Because HO-1 promoter polymorphism can conceivably affect the development of CAD, in the present study, the associations of the HO-1 promoter

polymorphism with bilirubin levels, markers of iron status, and the development of CAD were examined.

## RESEARCH DESIGN AND METHODS

**Study Subjects:** The study population consisted of 986 unrelated adult patients who consecutively underwent coronary angiography in the Cardiology Division at Taipei Veterans General Hospital from August 1999 to October 2000. CAD was documented by angiographic evidence of  $\geq 75\%$  stenosis of at least one major coronary artery, or a history of prior angioplasty, coronary artery bypass surgery, or myocardial infarction by history validated by electrocardiographic changes. Non-CAD group consisted of subjects who have normal coronary arteries as documented by angiography ( $< 20\%$  intraluminal obstruction) and to have neither a history of atherosclerosis nor clinical or laboratory evidence of atherosclerosis in other vascular beds. This study protocol was approved by the review committee of Taipei Veterans General Hospital and all participants gave their written informed consent.

**Analysis of length variability of (GT)<sub>n</sub> repeats in HO-1 gene promoter:** Genomic DNAs were extracted from leukocytes by conventional procedure. The 5'-flanking region containing (GT)<sub>n</sub> repeats of the HO-1 gene was amplified by PCR with a FAM-labeled sense primer, 5'-AGAGCCTGCAGCTTCTCAGA-3', and an antisense primer, 5'-ACAAAGTCTGGCCATAGGAC-3', as previously described (4). The PCR products were mixed together with GenoType™ TAMRA DNA ladder (size range 50-500 bp) (GibcoBRL) and analyzed with automated DNA sequencer (ABI Prism™ 377). Each size of the (GT)<sub>n</sub> repeat was calculated using the GeneScan Analysis software (PE Applied Biosystems).

**Baseline measurements:** Hypertension

was defined as measured systolic blood pressure >140 mmHg, or diastolic blood pressure >90 mmHg. Diabetes mellitus was diagnosed based on the WHO criteria. Patients with hypercholesterolemia were defined as those having a total cholesterol level of >240 mg/dL or who were receiving lipid-lowering therapy. Laboratory measurements were made on 12-hour fasting venous blood samples. The biochemical indicators of iron status in this study included the serum iron concentration, the serum ferritin levels, the serum total iron-binding capacity, and the serum transferrin saturation. Serum iron was measured with a colorimetric assay. Serum ferritin and total iron-binding capacity (TIBC) values were assessed with an immunometric assay (Boehringer Mannheim). Transferrin saturation was calculated as the ratio of serum iron to TIBC.

**Statistical analysis:** All statistical analyses were conducted using the SPSS statistical package, version 10.0. Distributions of continuous variables in groups were expressed as mean  $\pm$  SD and compared by *t* test for two groups or analysis of variance (ANOVA) using least significant different (LSD) method for post hoc multivariate comparison of the means for more than three groups. Values of serum ferritin were log-transformed because of their skewed distributions. Categorical variables were analyzed by chi-square test or Fisher's exact test. The association of CAD status with the allele frequency was assessed with consideration of confounding effects by known coronary risk factors, such as age, sex, diabetes, hypercholesterolemia, hypertension, and smoking habits. After preliminary bivariate analysis using the *t* test and  $\chi^2$  test, multiple logistic regression analysis with forward stepwise selection was performed to evaluate the effect of genotype on CAD after controlling for other established risk factors of CAD. Significance was accepted at  $P < 0.05$ . All the study participants are Chinese from northern Taiwan and have similar ethnic

backgrounds.

## RESULTS

The allele frequencies of (GT)<sub>n</sub> microsatellite polymorphism in HO-1 promoter region were highly polymorphic, ranging from 16 to 38 (4). Since the proportion of allele frequencies of either below or above 27 GT repeats was around 50%, we classified the alleles into two subgroups: the lower component, with repeat number <27, was designated as "class S"; and the upper component, with  $\geq 27$  GT repeats, was designated as "class L". These patients were then classified as having an S/S, S/L, or L/L genotype according to each of their HO-1 alleles.

Supplementary Table 1 shows the distribution of HO-1 promoter genotypes in all subjects and those with hypertension (n=639), diabetes mellitus (n=263), hypercholesterolemia (n=179), or current smoking (n=260) stratified by the status of CAD. No significant difference in genotypic frequencies between the two groups (CAD vs. non-CAD) in the whole study population was observed. But diabetes mellitus was found to have a significant interaction with genotypes: the proportions of S/S, S/L and L/L genotypes were 36.5%, 47.6% and 15.9%, respectively, in diabetic subjects without CAD; and 18.5%, 51.5%, and 30.0%, respectively, in diabetic subjects with CAD.

The characteristics of the whole study population and subjects with diabetes mellitus stratified by HO-1 genotype are presented in Table 1. Across the three genotypes, only serum bilirubin and ferritin concentrations were significantly different in both the whole study population and subjects with diabetes. There were no significant differences in age, gender, percentages of risk factors, levels of serum cholesterol, triglycerides, and fasting blood glucose, or other markers of iron status including serum iron, TIBC and transferrin saturation values.

Mean serum bilirubin was higher in carriers of S allele ( $0.85\pm 0.32$  mg/dL) than those with L/L genotype ( $0.79\pm 0.25$  mg/dL) ( $P=0.013$ ) in the whole study population, and the difference was more pronounced ( $0.81\pm 0.24$  vs.  $0.70\pm 0.22$  mg/dL,  $P=0.001$ ) in subjects with diabetes. Serum ferritin levels were highest in subjects with L/L genotype, intermediate in those with S/L genotype, and lowest in those with S/S in the whole study population and in subjects with diabetes. When subjects with L/L genotype and those carrying the S allele were compared, ferritin level was significantly higher in subjects with L/L genotype ( $127\pm 99$  or  $4.54\pm 0.88$   $\mu\text{g/L}$  for log-ferritin) than carriers of S allele ( $114\pm 107$  or  $4.33\pm 0.98$   $\mu\text{g/L}$  for log-ferritin) ( $P=0.008$  for log-ferritin) in the whole study population. Among subjects with diabetes, such difference was again much greater ( $148\pm 104$  vs.  $111\pm 96$   $\mu\text{g/L}$  for ferritin,  $P=0.031$ ; or  $4.76\pm 0.72$  vs.  $4.28\pm 1.05$  for log-ferritin,  $P=0.001$ ).

The baseline characteristics of the whole study population and subject with diabetes stratified by the status of CAD are summarized in Table 2. When all subjects were considered, patients with CAD were older and had a higher percentage of male gender, higher fasting blood sugar and triglyceride levels, and a lower HDL value compared to those without CAD, as expected. Serum bilirubin levels were significantly lower in patients with CAD ( $0.81\pm 0.30$  vs.  $0.87\pm 0.32$  mg/dL,  $P=0.006$ ), and a trend towards a higher serum ferritin level was observed in patients with CAD ( $126\pm 124$  vs.  $110\pm 95$   $\mu\text{g/L}$ ,  $P=0.061$ ). There was no difference in serum iron value, total iron-binding capacity, or transferrin saturation between subjects with and without CAD. On the other hand, the two groups of diabetic patients with and without CAD only differed in percentages of male gender with respect to demographic characteristics. Diabetic patients with CAD had significantly lower serum bilirubin levels ( $0.76\pm 0.23$  vs.  $0.86\pm 0.32$  mg/dL,  $P=0.040$ ) and higher serum ferritin

levels ( $141\pm 139$  vs.  $104\pm 102$   $\mu\text{g/L}$  or  $4.54\pm 1.01$  vs.  $4.16\pm 1.10$   $\mu\text{g/L}$  for log-ferritin,  $P=0.024$  for log-ferritin).

The relations between serum bilirubin and ferritin levels, HO-1 genotypes, and CAD are shown in Figure 1. Among subjects with diabetes, serum bilirubin levels in CAD patients with L/L genotype was significantly lower than those in carriers of S allele, regardless of their CAD status; whereas differences in serum bilirubin levels between carriers of L/L genotype with and without CAD were not statistically significant. On the other hand, log ferritin values in CAD patients with L/L genotype were significantly higher than those in carriers of S allele with or without CAD. Differences in log ferritin values between CAD and non-CAD patients with L/L genotype, though substantial, did not reach statistical significance. Compared to subjects with diabetes, differences in both serum bilirubin and ferritin levels within non-diabetic subjects were much less prominent.

We then performed multivariate analyses to further examine the links between serum bilirubin and ferritin levels, HO-1 genotypes, and CAD in diabetic patients. After controlling for conventional risk factors, carriers of L/L genotype revealed significantly enhanced susceptibility to CAD compared with those carrying S allele, resulting in an odds ratio of 2.81 (95% confidence interval [CI], 1.22 to 6.47,  $P=0.015$ ) (Table 3). As a next step, we investigated the association of serum bilirubin and ferritin levels with CAD separately. When the HO-1 genotype was not included in the model, a 0.1 mg/dL increase in bilirubin levels decreased CAD risk by 16% and 1 log-unit elevation in ferritin values increased CAD risk by 41%. After we included both the HO-1 promoter genotype and bilirubin levels in the logistic regression model, the odds ratio (OR) of HO-1 effect fell to 2.65 and became less significant (95% CI, 1.05-6.69,  $P=0.040$ ). When both the HO-1 promoter genotype and

ferritin values were included, the OR of HO-1 effect decreased to 2.31 and was of borderline significance (95% CI, 0.97-5.49,  $P= 0.058$ ). Adjusting both serum bilirubin and ferritin values, the OR of HO-1 effect reduced further to 1.71 and became non-significant (95% CI, 0.75-3.90,  $P= 0.203$ ) (Table 3).

## CONCLUSIONS

A decreased HO-1 expression has been shown in humans and experimental animals with diabetes (11,12), and an inverse relationship between the HO-1 activity and vascular complications associated with diabetes was demonstrated (13). In line with these findings, our previous study (4) has revealed that the length polymorphism in the HO-1 gene promoter is correlated with susceptibility to CAD in diabetic patients. In the present study, we further demonstrated that such effect might be conveyed through its influence on bilirubin and ferritin.

The concept that HO-1 may be causally related to cardiovascular diseases in humans has been suggested by studies assessing (GT)<sub>n</sub> dinucleotide-length polymorphism in the 5'-flanking sequence of the human HO-1 gene. By using HO-1 promoter/luciferase reporter genes carrying different lengths of (GT)<sub>n</sub> repeats, we previously demonstrated that the more (GT)<sub>n</sub> repeats in promoter region, the less transcriptional activity of HO-1 gene in rat aortic smooth muscle cells (4), similar result was also shown earlier in Hep3B cells (3).

Bilirubin is a natural product of heme catabolism by HO. Here we demonstrated that there is an association between HO-1 promoter polymorphism and serum bilirubin levels, which are correlated with the development of CAD. Mean serum bilirubin was significantly higher in carriers of S allele than those with L/L genotype. In a previous case-control study of individuals with early familial CAD, higher serum bilirubin concentrations within the normal range were associated with a significant and marked reduction in CAD risk

(7). In the prospective Framingham Offspring Study, higher serum bilirubin concentrations were associated with associated with a decreased incidence of ischemic heart disease (8). Considering the antioxidant and antiatherogenic properties of bilirubin, the beneficial influence on serum bilirubin in carriers of S allele might exert protective effect against the development of CAD.

HO releases free ferrous ( $Fe^{2+}$ ) iron from heme. The toxic effect of free iron has been linked to oxidative stress through the Fenton reaction, where  $Fe^{2+}$  oxidizes  $H_2O_2$  leading to the generation of hydroxyl radicals (13) which in turn initiate lipid peroxidation. The amount of free ferrous iron is normally maintained at a very low level in humans. Of all the iron in the body (4 g), about 2/3 is found in association with hemoglobin in the ferrous form and the majority of the remainder is stored as ferritin. In 1981, Sullivan suggested that a state of iron depletion was potentially protective against CHD (9). Although the majority of animal research and the in vitro human studies support a role of iron in the pathogenesis of atherosclerosis, prospective human studies have provided inconsistent results in terms of clinical cardiovascular outcomes (10). Some investigators have hypothesized that iron may be primarily involved in the early stage of atherosclerosis, and focusing on cardiovascular morbidity and mortality (reflecting later stages of the disease) may not give insight into the potential mechanistic role of iron (15). Likewise, one recent study demonstrated that reduction of body iron stores by phlebotomy in patients with peripheral arterial disease achieved a significant improvement in cardiovascular outcomes in patients aged <60 years but not in those with an older age (thus more advanced atherosclerosis) (16).

In the present study, for the first time, we demonstrated that there is an association between HO-1 promoter polymorphism and serum ferritin concentrations, a measure of the

body's iron stores, and an association between ferritin concentrations and the development of CAD in diabetic subjects. The mechanisms by which HO-1 polymorphism confers the variance in ferritin values remain to be elucidated. Nevertheless, a few animal studies and clinical data provided some indirect clues. A mouse model deficient in mammalian HO-1 (*Hmox1*) developed pathological accumulation of tissue iron stores associated with an increase in serum ferritin levels (17). HO-1 deficiency is very rare in humans; however, an autopsy report from a 6-year-old boy with HO-1 deficiency presented with growth retardation, anemia, elevated serum levels of ferritin and heme, low serum bilirubin concentrations, and hyperlipidemia associated with fatty streaks and fibrous plaques in the aorta (18). Moreover, treatment with HO inhibitors in healthy volunteers, patients with primary biliary cirrhosis and idiopathic hemochromatosis substantially increased serum ferritin concentrations (19). We hence postulated that the lower expression level of HO-1 imposed by L allele under higher oxidative stress, like in the setting of diabetes, increases iron load in vascular system, which might contribute to the development of atherosclerosis in such a virulent status.

The present study has strengths and limitations. Strengths include large number of patients and all subjects had coronary arteriography and measures of bilirubin and ferritin. Furthermore, the homogeneous ethnic background possibly reduces variability in measurements. Among the study limitations, it is primarily a sample of Chinese, and therefore, generalization to the other ethnic groups is uncertain. Furthermore, the present study design was cross-sectional, and we cannot infer causality.

In conclusion, we have demonstrated that the microsatellite polymorphism in the promoter of HO-1 gene imposes modulation on serum bilirubin and ferritin levels, which

might be associated with the development of CAD among diabetic subjects.

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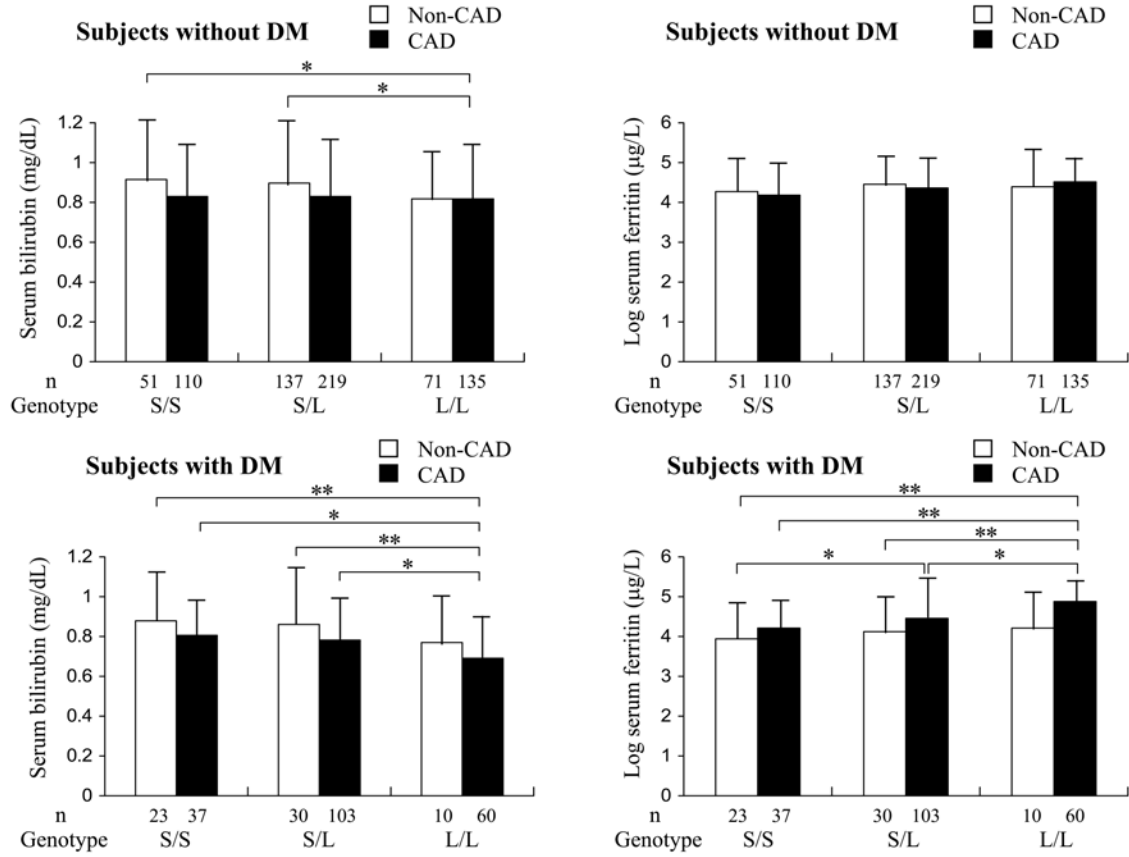
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**Figure Legends**

**Figure 1**—Serum bilirubin and log ferritin levels according to three genotypes and CAD status. Data are expressed as mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ .



**Table 1—General characteristics of the study population stratified by HO-1 promoter genotypes**

Genotype	S/S		S/L		L/L		<i>P</i> in all subject	<i>P</i> in DM
	All subject (n = 221)	With DM (n = 60)	All subject (n = 489)	With DM (n = 133)	All subject (n = 276)	With DM (n = 70)		
Age (yr)	70±8	69±8	68±10	68±9	69±9	68±9	NS	NS
Male, %	90	87	89	85	88	80	NS	NS
Hypertension, %	68	78	65	66	64	73	NS	NS
Current smoker, %	34	28	28	26	29	29	NS	NS
Fasting BS, mg/dl	109±36	148±45	112±46	155±65	110±44	151±62	NS	NS
Total cholesterol, mg/dl	192±42	187±43	187±35	180±35	189±36	188±40	NS	NS
LDL cholesterol, mg/dl	124±35	120±31	119±30	111±33	122±28	116±32	NS	NS
HDL cholesterol, mg/dl	40±11	37±11	39±11	36±9	40±11	40±15	NS	NS
Serum triglycerides, mg/dl	144±91	171±109	146±97	168±109	159±150	176±104	NS	NS
Bilirubin, mg/dl	0.85±0.27	0.83±0.21	0.84±0.30	0.81±0.25	0.79±0.25	0.70±0.22	0.021	0.006
Serum iron, µg/dl	80±42	82±90	78±40	73±84	73±39	112±17	NS	NS
Serum ferritin, µg/l	99±105	82±63	121±107	123±105	127±99	148±104	0.031	0.009
Log ferritin	4.19±0.95	4.10±0.87	4.40±0.98	4.36±1.12	4.54±0.88	4.76±0.72	0.003	0.008
TIBC, µg/dl	272±102	279±92	297±160	296±114	278±124	287±127	NS	NS
Transferrin saturation, %	27±17	27±17	27±13	23±16	25±15	24±17	NS	NS

Data are expressed as mean±SD. DM indicates diabetes mellitus; BS, blood glucose; LDL, low-density lipoprotein; HDL, high-density lipoprotein; and TIBC, total iron-binding capacity.

**Table 2—Characteristics of the study population (n = 986)**

	Non-CAD subjects		CAD patients		P	P
	All subject	With DM	All subjects	With DM		
	(n = 322)	(n = 63)	(n = 664)	(n = 200)		
Age (yr)	67±10	68±9	69±9	68±9	0.002	NS
Male, %	82	71	92	88	<0.001	0.009
Hypertension, %	63	78	66	68	NS	NS
Current smoker, %	26	25	31	29	NS	NS
Fasting BS, mg/dl	104±34	147±47	114±47	154±63	<0.001	NS
Total cholesterol, mg/dl	187±35	184±33	190±38	184±40	NS	NS
LDL cholesterol, mg/dl	118±26	111±26	122±33	115±34	NS	NS
HDL cholesterol, mg/dl	42±11	38±12	39±11	37±11	0.001	NS
Serum triglycerides, mg/dl	134±75	164±94	157±128	173±111	0.001	NS
Bilirubin, mg/dl	0.87±0.32	0.86±0.32	0.81±0.30	0.76±0.23	0.006	0.04
Serum iron, µg/dl	80±43	70±32	75±37	77±42	NS	NS
Serum ferritin, µg/l	110±95	104±102	126±124	141±139	NS	NS
Log ferritin	4.31±0.98	4.16±1.10	4.42±0.99	4.54±1.01	NS	0.024
TIBC, µg/dl	285±129	312±197	286±144	299±158	NS	NS
Transferrin saturation, %	30±14	26±12	28±12	28±12	NS	NS

Data are expressed as mean±SD. DM indicates diabetes mellitus; BS, blood glucose; LDL, low-density lipoprotein; HDL, high-density lipoprotein; and TIBC, total iron-binding capacity.

**Table 3—Association of HO-1 promoter genotypes with the risk of CAD among diabetic patients**

	OR (95% CI)		
	L/L vs. L/S + S/S	Bilirubin (per 0.1 mg/dl)	Log ferritin (per 1 unit)
Adjusted for traditional risk factors*	2.81 (1.22-6.47), <i>P</i> = 0.015	0.84 (0.73-0.97), <i>P</i> = 0.016	1.41 (1.04-1.89), <i>P</i> = 0.025
Additionally adjusted for bilirubin	2.65 (1.05-6.69), <i>P</i> = 0.040		
Additionally adjusted for ferritin	2.31 (0.97-5.49), <i>P</i> = 0.058		
Additionally adjusted for both bilirubin and ferritin	1.71 (0.75-3.90), <i>P</i> = 0.203		

OR indicates odds ratio; and CI, confidence intervals.

\*Adjusted for age, gender, history of hypertension, hypercholesterolemia and status of smoking..