Length polymorphism in heme oxygenase-1 is associated with arteriovenous fistula patency in hemodialysis patients

C-C Lin[1,2,3], W-C Yang[2,3], S-J Lin[1,2,4], T-W Chen[2,3], W-S Lee[2,4], C-F Chang[2,3], P-C Lee[5], S-D Lee[2,5], T-S Su[6,7], CS-J Fann[6,8] and M-Y Chung[6,7]

[1]Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan, Republic of China; [2]School of Medicine, National Yang-Ming University, Taipei, Taiwan, Republic of China; [3]Division of Nephrology, Taipei Veterans General Hospital, Taipei, Taiwan, Republic of China; [4]Division of Cardiology, Taipei Veterans General Hospital, Taipei, Taiwan, Republic of China; [5]Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan, Republic of China; [6]Institute of Genome Sciences, National Yang-Ming University, Taipei, Taiwan, Republic of China; [7]Department of Medical Research, Taipei Veterans General Hospital, Taipei, Taiwan, Republic of China and [8]Division of Epidemiology and Genetics, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, Republic of China

Heme oxygenase-1 (HO-1) is a rate-limiting enzyme in heme degradation, producing carbon monoxide (CO), which carries potent antiproliferative and anti-inflammatory effects in the vascular walls. Transcription of the *HO-1* gene is regulated by the length polymorphism of dinucleotide guanosine thymine repeat $(GT)_n$ in the promoter region, which was measured in this study to determine its association with arteriovenous fistula (AVF) failure in Chinese hemodialysis (HD) patients in Taiwan. L allele means $(GT)_n \geqslant 30$ and S allele means $(GT)_n < 30$. Therefore, there are two L alleles for L/L genotype, one L and one S allele for L/S genotype, and two S alleles for S/S genotype. Among the 603 HD patients who were enrolled in this study, 178 patients had history of AVF failure, while 425 patients did not. Significant associations were found between AVF failure and the following factors (hazard ratio): longer HD duration (1.004 month), lower pump flow (0.993 ml/min), higher dynamic venous pressure (1.010 mmHg), location of AVF on the right side (1.587 vs left side) and upper arm (2.242 vs forearm), and L/L and L/S genotypes of *HO-1* (2.040 vs S/S genotype). The proportion of AVF failure increased from 20.3% in S/S genotype and 31.0% in L/S genotype to 35.4% in L/L genotype ($P = 0.011$). Relative incidences were 1/87.6 (1 episode per 87.6 patient-months), 1/129, and 1/224.9 for HD patients with L/L, L/S, and S/S genotypes, respectively ($P < 0.002$). The unassisted patency of AVF at 5 years decreased significantly from 83.8% (124/148) to 75.1% (223/297) and 69% (109/158) in S/S, L/S, and L/L genotypes, respectively ($P < 0.0001$). In comparison with HD patients with S/S genotype, those with L/L genotype had a higher prevalence of coronary artery disease (29.1 vs 14.2%; $P = 0.005$). A longer length polymorphism with $(GT)_n \geqslant 30$ in the *HO-1* gene was associated with a higher frequency of access failure and a poorer patency of AVF in HD patients. The longer GT repeat in the *HO-1* promoter might inhibit gene transcription, and consequently offset the CO-mediated protective effect against vascular injury.

Kidney International (2006) **69,** 165–172. doi:10.1038/sj.ki.5000019

KEYWORDS: arteriovenous fistula; heme oxygenase-1; hemodialysis; polymorphism; stenosis

Correspondence: *M-Y Chung, Department of Medical Research, Veterans General Hospital-Taipei, No. 201, Sec. 2, Shih-Pai Road, Taipei, Taiwan 112, Republic of China. E-mail: l1124@ms12.hinet.net; mychung@vghtpe.gov.tw*

Received 25 May 2005; revised 27 July 2005; accepted 11 August 2005

In Taiwan, more than 85% of patients with end-stage renal disease undergo maintenance hemodialysis (HD). A well-functioning vascular access is necessary for HD, and long-term technical survival is best for native arteriovenous fistula (AVF), which accounts for a prevalence of more than 80% of the vascular access in our patients. About 17–25% of HD patient hospitalizations in the USA result from vascular access complications, at a cost of US $1 billion annually.[1] Failure of dialysis access can result from either inadequate blood flow on account of stenosis of the venous outflow tract or complete occlusion due to thrombosis.[2] About 80–85% of arteriovenous (AV) access failures come from AV access thromboses, more than 80% of which result from AVF stenoses.[3] Decreased access flow (Qa) is associated with an increased risk of access thrombosis. Qa less than 500 ml/min was demonstrated to be predictive of poorer unassisted patency of native AVF by variable pump flow-based Doppler ultrasound method in our previous study.[4] In addition to Qa, some mechanical factors, such as the surgical skill, the puncture technique, and the shear stress on vascular endothelia, influence AVF patency. Several medical factors have also been identified to be associated with AVF stenosis in HD patients, including endothelial cell injury, stasis, hypercoagulability, medications, and red cell mass.[5] Many factors lead to endothelial cell injury or dysfunction, such as

oxidative stress, hyperhomocysteinemia, activated platelets, tumor necrosis factor-α, calcium/phosphate deposition, and pre-existing intimal hyperplasia. The pathological features of stenosis of vascular access consist of intimal hyperplasia, vascular smooth muscle cell (VSMC) proliferation in the media with subsequent migration to intima, and excessive accumulation of extracellular matrix.[6] In spite of the above findings, the causes for development of stenoses still remain unknown in a significant proportion of HD patients. This interindividual variation may relate to genetic differences among patients. In this regard, AV fistula patency has been reported to be associated with specific genotype polymorphisms of transforming growth factor $\beta 1$ (*TGF-β1*)[7] and methylene tetrahydrofolate reductase (*MTHFR*).[8]

In addition to the above factors, the activity of heme oxygenase-1 (*HO-1*) is another factor associated with higher risk of developing some vascular diseases. *HO-1*, an enzyme playing an important role in heme degradation, results in the production of biliverdin, free iron, and carbon monoxide (CO).[9] This enzyme is associated with oxidative stress, platelet activation, and VSMC proliferation, as VSMC proliferation, migration, and extracellular matrix synthesis may be regulated by its reaction product, CO.[10] The human *HO-1* gene was located at chromosome 22q12, and a (guanidinium thiocyanate $(GT)_n$) dinucleotide repeat of different length was identified in the proximal promoter region.[11] However, the actions of *HO-1* are highly variable and may reflect a role for *HO-1* in maintaining tissue homeostasis. Kaneda *et al.*[12] and Chen *et al.*[13] have shown that the $(GT)_n$ repeat is highly polymorphic, and a longer $(GT)_n$ repeat exhibits lower transcriptional activity and is associated with susceptibility to coronary artery disease (CAD). Chen *et al.*[14] also showed that genetic variation influencing *HO-1* expression would interact with traditional risk factors and contribute to the development of restenosis after placement of coronary stents. However, little information is available on the role of genetic background of *HO-1* in the development of AVF stenosis.

This study was designed to determine whether the length polymorphism of the dinucleotide $(GT)_n$ repeats in the *HO-1* gene promoter region would be an independent factor for predicting patency of AVF in HD patients.

RESULTS

Patient characteristics

The demographic and clinical characteristics of the patients are summarized in Table 1. A total of 603 patients were enrolled in the study. Among them, 425 patients did not have any episode of AV fistula failure, but 178 patients did. Compared with HD patients without AV fistula failure, those with AV fistula failure had a longer HD duration (89.2 ± 65.6 vs 62.5 ± 53.0 months, $P < 0.001$), a higher prevalence of AV fistula at the right upper extremity (32 vs 18.6%, $P < 0.001$) and upper arm (27 vs. 9.2%, $P < 0.001$), higher dynamic venous pressure (148.2 ± 29.5 vs 140.9 ± 29.9 mmHg, $P < 0.001$), lower prevalence of hypertension (45.5 vs 56%, $P = 0.019$), and borderline higher prevalence of cardiovascular disease (32.6 vs 24.9%, $P = 0.05$). The allele frequencies of the dinucleotide length polymorphism $(GT)_n$ in the *HO-1* gene promoter found in the studied individuals are shown in Figure 1. The repeat numbers ranged from 16 to 39, with $(GT)_{23}$ and $(GT)_{30}$ being the two most common alleles in our study population (allele frequencies are 20.6% for $(GT)_{23}$ and 30.1% for $(GT)_{30}$). We assigned those with GT repeats $\geqslant 30$

Table 1 | Clinical characteristics of HD patients by the presence of failure of AV fistula

	Overall (*n*=603)	No AVF failure (*n*=425)	AVF failure (*n*=178)	*P*-value
Male (%)	52.6	51.3	55.6	0.33
Age (years)	60.0±14.5	59.8±14.0	60.6±15.8	0.57
HD duration (months)	70.3±58.2	62.5±53.0	89.2±65.6	<0.001
Site of AV fistula				
Right side (%)	22.6	18.6	32	<0.001
Left side (%)	77.4	81.4	68	
Location of AV fistula				
Upper arm (%)	14.4	9.2	27	<0.001
Forearm (%)	85.6	90.8	73	
Survival of AV fistula (months)	56.5±53.3	64.3±52.9	38.1±49.6	<0.001
Time between creation of AV fistula and initial HD (months)	1.64±4.21	1.79±6.95	1.29±3.87	0.368
Dynamic venous pressure under pump flow at 250 ml/min (mmHg)	142.6±29.9	140.9±29.9	148.20±29.5	<0.001
Maximal pump flow (ml/min)	271.9±33.3	273.2±34.4	268.8±30.1	0.14
Hypertension (%)	52.9	56	45.5	0.019
Diabetes mellitus (%)	30.5	30.4	30.9	0.894
Cerebral infarction (%)	8.6	7.8	10.7	0.246
Peripheral arterial obstructive disease (%)	4.6	3.8	6.7	0.113
Coronary artery disease (%)	21.1	19.5	24.7	0.154
Cardiovascular disease (%)	27.2	24.9	32.6	0.054

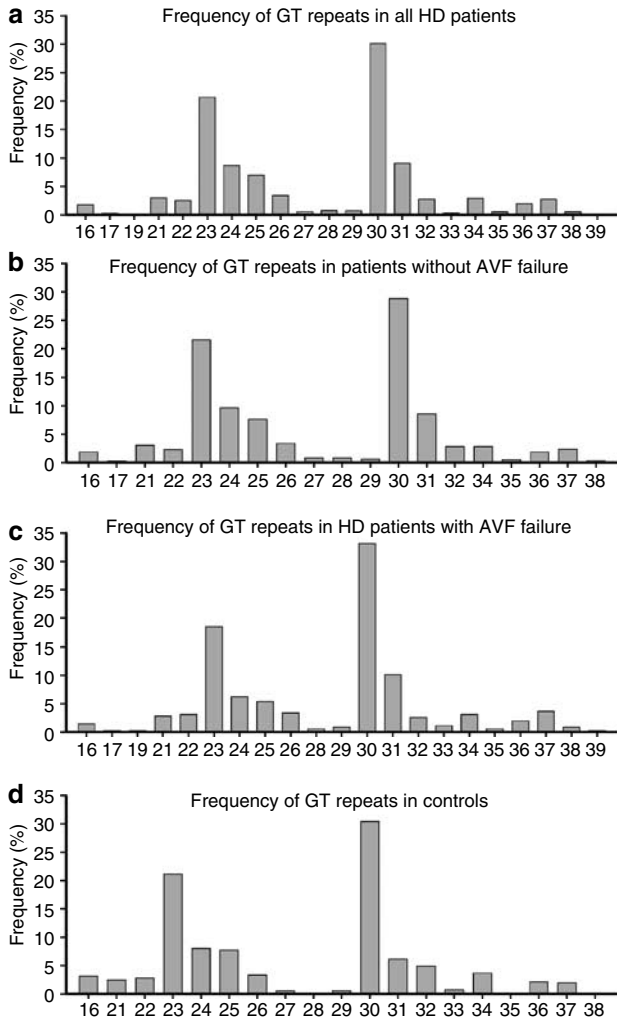

Figure 1 | (a) Distribution of allele frequency of the $(GT)_n$ repeats in all HD patients ($n = 603$), (b) HD patients without AV fistula failure ($n = 425$), (c) those with AV fistula failure ($n = 178$), (d) and controls ($n = 286$). There is no significant difference between (**a**) and (**d**), but there is significant difference between (**b**) and (**c**).

as allele class L (long) and those with GT repeats <30 as allele class S (short) owing to two reasons. First, Yamada *et al.*[15] also defined an allele with the number of GT repeats $\geqslant 30$ as a long allele (class L) in the Japanese population, which, like our Chinese population, is a part of eastern Asian people. Second, the alleles with GT repeats $\geqslant 30$ accounted for about 50% of all the alleles of HD patients and controls in our study. The genotype of the study individual was defined as (1) class L/L if both allele lengths of GT repeats were $\geqslant 30$, (2) class L/S if one GT repeat was $\geqslant 30$ and the other was <30, and (3) class S/S if both allele lengths of GT repeats were <30. Therefore, the case number in any genotype group will not be too small to allow appropriate statistical analysis. The distribution of allele and genotype classes did not differ between whole HD patients and healthy controls (Table 2).

Association of *HO-1* polymorphism with AV fistula failure in HD patients

As shown in Table 2, the proportion of L allele frequency (57.3 vs 48.1%; $P = 0.004$) and the proportion of L/L genotype frequency (31.5 vs 24%; $P = 0.011$) were significantly higher in HD patients with AVF failure than in those without AVF failure. With regard to the frequencies of HD patients with AVF failure in different classes of genotypes, the proportion of AVF failure increases from 20.3% in class S/S to 31.0% in class L/S and 35.4% in class L/L (Table 3; $P = 0.011$).

Association of *HO-1* polymorphism with comorbid vascular diseases

In comparison with HD patients with S/S genotype of *HO-1*, those with L/L genotype had a significantly higher prevalence of CAD (29.1 vs 14.2%; $P = 0.005$) and cardiovascular disease (33.5 vs 20.3%; $P = 0.033$), but similar frequencies of hypertension, DM, cerebral infarction, and PAODs (Table 3).

Cox regression model of multivariate analysis for AVF failure

Table 4 shows the Cox regression model for the association of AVF failure with the selected parameters carrying obvious or borderline statistical significances from Table 1. There was a significant correlation between increasing incidence of AVF failure and longer HD duration, lower pump flow, higher

Table 2 | Comparison of HO-1 promoter genotypes and allele frequencies among controls, total and subgroups of hemodialysis patients (with and without AVF failure)

	Controls	Total HD patients	HD patients without AVF failure	HD patients with AVF failure	*P*-value
Alleles, n (%)	*n*=572	*n*=1206	*n*=850	*n*=356	
S (short)	285 (49.8%)	593 (49.2%)	441 (51.9%)	152 (42.7%)	NS*
L (long)	287 (50.2%)	613 (50.8%)	409 (48.1%)	204 (57.3%)	0.004#
Genotypes, n (%)	*n*=286	*n*=603	*n*=425	*n*=178	
S/S	61 (21.3%)	148 (24.5%)	118 (27.8%)	30 (16.8%)	NS*
L/S	163 (57%)	297 (49.3%)	205 (48.2%)	92 (51.7%)	0.011#
L/L	62 (21.7%)	158 (26.2%)	102 (24%)	56 (31.5%)	

The alleles were classified into two subgroups according to the number of GT repeats: the S group with repeat number <30, and the L group with $\geqslant 30$ GT repeats; the genotypes were classified into three subgroups according to the number of GT repeats at both chromosomes: the S/S group with both repeat numbers <30, L/S group with one repeat number <30 and one repeat number $\geqslant 30$, and L/L group with both repeat numbers $\geqslant 30$. NS=nonsignificant. *Signifies the *P*-value for the comparison between controls and HD patients; #signifies the *P*-value for the comparison between HD patients with and without AVF failure.

Table 3 | HO-1 genotypes and vascular diseases in hemodialysis patients

Genotype, case number (*n*)	S/S *n*=148	L/S *n*=297	L/L *n*=158	*P*-value
Hypertension (%)	51.4	52.9	54.4	NS
Diabetes mellitus (%)	25.7	31.6	32.9	NS
Cerebral infarction (%)	7.4	9.8	7.6	NS
Peripheral arterial obstructive disease (%)	3.4	4.7	5.7	NS
Coronary artery disease (%)	14.2	20.2	29.1	0.005
Cardiovascular disease (%)	20.3	27.3	33.5	0.033
AVF failure (%)	20.3	31.0	35.4	0.011

Table 4 | Cox regression model of factors associated with AVF failure in HD patients

	Significance	Hazard ratio	95% CI lower	95% CI upper
HD duration (months)	0.006	1.004	1.001	1.008
Pump flow (ml/min)	0.006	0.993	0.988	0.998
Maximal venous pressure (mmHg)	<0.001	1.010	1.004	1.015
Right vs left side	0.005	1.587	1.147	2.197
Upper arm vs forearm	<0.001	2.242	1.557	3.228
HO-1 genotype (L/L+L/S vs S/S)	0.002	2.040	1.295	3.215
Cardiovascular disease	0.224	1.424	0.805	2.517
Hypertension	0.858	0.945	0.507	1.760

Table 5 | HO-1 genotypes and relative incidence of AVF failure in HD patients

Genotype	Total observations (patient-months)	Episodes (times)	Episodes/patient-months	
S/S	10794.7	48	1/224.9	$P<0.002$
S/L	20513.6	159	1/129.0	
L/L	11035.2	126	1/87.6	
Total	42343.5	333	1/127.2	

dynamic venous pressure, location of AVF at the right side and upper arm, and L/L and L/S genotypes of *HO-1*. *HO-1* genotype polymorphism was a strongly relevant factor and showed a significant difference, with a hazard ratio of 2.040 and a 95% confidence interval between 1.295 and 3.215 ($P=0.002$).

Relative incidence of AVF failure and *HO-1* genotypes

Some patients experienced multiple episodes of AVF failure during observation. Thus, *HO-1* genotypes and the relative incidence of AVF failure (number of incidences per patient-months of follow-up) were calculated. Table 5 lists the relative incidence of AVF failure in HD patients according to the three different *HO-1* genotypes. Relative incidences were 1 episode per 87.6 patient-months, 1 episode per 129 patient-months, and 1 episode per 224.9 patient-months for HD patients with L/L, L/S, and S/S genotypes, respectively. The relative incidence in the subgroup of L/L genotype (1 episode per 87.6 patient-months) was significantly greater than that in the subgroup of S/S genotype (1 episode per 224.9 patient-months; $P<0.002$; Table 5).

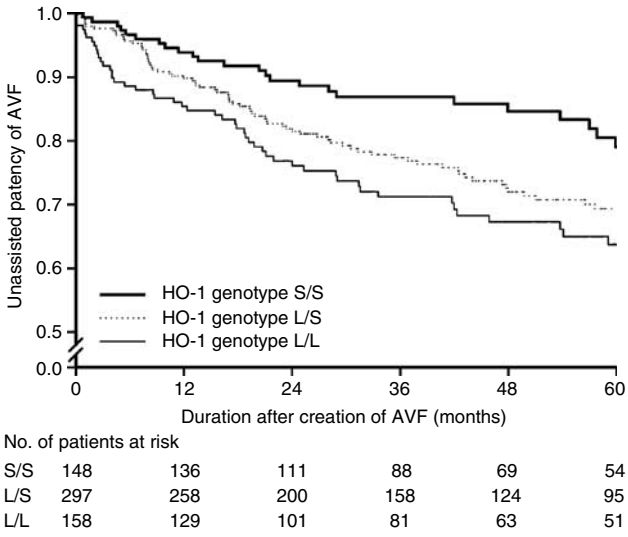

Figure 2 | Five-year unassisted patency of AV fistula comparing HD patients with different HO-1 genotypes (S/S, L/S, L/L); $P<0.0001$ for the comparison between the three survival curves.

Relationship between the *HO-1* genotype and AVF patency

Of the 148 patients with S/S genotypes, fistula failure occurred in 30 patients after 46.4±54.3 months, whereas the remaining 118 patients without fistula failure were followed for 65.1±54.2 months ($P=0.09$). Among the 297 HD patients with L/S genotypes, 92 patients developed AV fistula failure after a mean time of 37.2±45.2 months. The remaining 205 patients without fistula failure were followed for 63.2±53.6 months ($P<0.001$). Among the 158 patients with L/L genotypes, 56 patients had fistula failure that occurred after 35.0±54.1 months. The 102 patients who had no fistula failure had a mean follow-up of 65.7±50.4 months ($P<0.001$). The time of follow-up in patients without fistula failure did not differ significantly among the three genotype subgroups (S/S, L/S, and L/L). We evaluated the effect of different genotypes of *HO-1* on AVF patency for all the HD patients in this study. As shown in Figure 2, AV fistula patency differed significantly when patients were classified according to their *HO-1* genotypes. The unassisted patency of AV fistulae at 5 years decreased significantly from 83.8% (124/148) in patients with S/S genotypes to 75.1% (223/297) in patients with L/S genotypes and further to 69% (109/158) in patients with L/L genotypes ($P<0.0001$ by log-rank test; Figure 2).

DISCUSSION

According to the recommendation of the Disease Outcomes Quality Initiative (DOQI) guidelines, creation of native AV fistulas at the upper extremity is preferable to placement of AV graft account of lower morbidity and higher long-term patency. Nevertheless, stenosis remains the most frequent complication for native AV fistulas and may predispose to

thrombosis and subsequent AVF failure. In this study, both univariate and multivariate analyses showed that the presence of AVF failure could be significantly correlated with several clinical factors, including HD duration, location of AV fistula at the upper arm (cephalic vein-to-brachial artery anastomosis) or right upper extremity, and dynamic venous pressure. As to the preferable location of vascular access, usually the first native AV fistula will be created at the forearm of the nondominant hand. Since more than 95% of our HD patients use the right hand as their dominant hand, the desirable location of AVF placement should be the left forearm (brachiocephalic vein-to-radial artery anastomosis at wrist). Henceforth, the generalized vascular quality was usually poorer in these HD patients, with location of their first AVF at the upper arm (cephalic vein to brachial artery at elbow) or right side because a suitable vein for the creation of AVF was not available at the left forearm. It is reasonable that HD patients with poorer vascular condition may also have higher risk of AVF failure. Patients with AVF stenosis usually have a higher dynamic venous pressure than those without stenosis. From univariate analysis, hypertension was a significant factor in decreasing the frequency of AVF failure. As we know, hypotension may increase the risk of access failure because reducing Qa may predispose to clotting in a dialyzer. HD patients with hypertension should be less prone to hypotension, and therefore may have a lower prevalence of AVF failure than those without hypotension. In comparison with HD patients without hypertension, however, those with hypertension had a lower frequency of location of AVF at the upper arm (11.9 vs 18.2%, $P<0.05$). As location of AVF at the upper arm is a risk factor of AVF failure by our results, its lower frequency might reduce the risk of AVF failure in those with hypertension. Therefore, hypertension was no more significant in reducing the frequency of AVF failure after the confounding effect of AVF location was removed by multivariate analysis. On the contrary, maximal pump flow was not significant in univariate analysis, but was significant in multivariate analysis, which might be caused by higher prevalence of location of AVF at the upper arm in those with AVF failure. The Qa of AVF at the upper arm is usually higher than that of AVF at the forearm. A higher Qa usually permits a higher maximal pump flow. Consequently, the maximal pump flow may increase more in those with AVF failure than in those without AVF failure under the above-mentioned confounding effect between these variables. After adjusting for the confounding effect arising from location of AVF at the upper arm, maximal pump flow was significantly lower in those with AVF failure by multivariate analysis.

In our study, both older age and a history of diabetes mellitus (DM) were not significant risk factors for AVF failure, which is similar to the results of some large-scale single-center[16] and multi-center[8,17] studies on AVF survival. Only a few earlier single-center studies, including smaller case numbers, reported a significantly reduced AVF survival in HD patients with old age or DM.[18] However, these two characteristics were risk factors for failure of synthetic AV grafts in HD patients.[17] Moreover, our study revealed that there was no correlation between cardiovascular disease and AVF failure, which is similar to the studies reporting no association of AVF failure with a history of any vascular disease, including CAD,[8,19] cerebral vascular disease,[8] or peripheral vascular disease.[19] Cardiovascular diseases are more greatly characterized by inflammatory response and endothelial dysfunction than AVF stenosis. In addition, the main pathological feature of AVF stenosis is intimal hyperplasia, which is different from the atherosclerotic lesions specific to cardiovascular diseases.

Although the above-mentioned risk factors of AVF failure have been identified in this study, stenosis still develops with wide individual variation. As the stenotic lesions are characterized by intimal hyperplasia originating from VSMC migration, proliferation, and exuberant synthesis of extracellular matrix,[20] some factors (such as TGF-β and homocysteine) were associated with these pathologic characteristics. Further, according to the genetic reports by Heine *et al.*[7] and Fukasawa *et al.*,[8] genotype polymorphisms of *TGF-β* and *MTHFR* are independent factors for predicting AVF stenosis and patency.

We investigated *HO*, another genetic factor other than *TGF-β* and homocysteine, possibly contributing to the pathophysiologic features of AVF stenosis.

HO catalyzes the rate-limiting step in the degradation of heme by cleaving the α-meso carbon bridge of heme, leading to the generation of equimolar quantities of CO, free iron, and biliverdin.[21] There are three isoforms of HO: (i) *HO-1*, an inducible 32-kDa protein, is ubiquitously distributed, and its encoding gene is located at chromosome 22q12;[11] (ii) *HO-2*, a constitutively expressed 36-kDa protein, is present in high levels in the brain and testes, and its encoding gene is located at chromosome 16p13.3; (iii) *HO-3* is a 33-kDa protein resembling HO-2, but with lower catalytic activity. *HO-1* is a stress-responsive protein that can be induced by various oxidative agents, including heavy metals, inflammatory mediators, ultraviolet radiation, endotoxin, heme, and hemoglobin.[22] Moreover, *HO-1* plays an important role in growth regulation, cell proliferation, cell death (apoptosis), and cell hypertrophy.

HO-1-derived CO may act in three ways to regulate the function and numbers of cells in vascular walls.[10] First, it can work as an autocrine factor to inhibit VSMC proliferation by blocking cells in the G_0/G_1 phase of the cell cycle.[10] Second, the release of CO from luminal VSMCs can function in a paracrine manner to inhibit the release of vascular mitogens from circulating platelets and adjacent endothelial cells. Third, *HO-1*-mediated release of CO, in an endocrine-like fashion, may participate in the process of 're-endothelialization of the vessel wall' at sites of vascular injury by stimulating growth and preventing apoptosis of endothelial cells. Through the combination of the above-mentioned effects, VSMC proliferation is inhibited further, as several antiproliferative factors, including nitric oxide and heparan sulfate proteoglycan, can be released from endothelial cells.[23] Finally, the *HO-1*-catalyzed production of biliverdin and bilirubin may aggravate the loss of VSMCs in injured vascular walls by prompting VSMC apoptosis.[24]

Augmented synthesis of CO after upregulation of *HO-1* gene expression will inhibit the proliferation of VSMC through the following mechanisms:[10] (i) enhancing expression of cyclic 3′,5′ guanosine monophosphate by increasing the activity of guanylate cyclase; (ii) arresting VSMCs in G1/S phase of the cell cycle; (iii) decreasing the expression of cyclin A mRNA, leading to downregulation of cyclin A-associated kinase and cdk-2 activity, which are key regulators of both G1- and S-phase cell progression; (iv) enhancing the activity of p21, which is a cyclin-dependent kinase inhibitor; (v) lowering the release of endothelin-1 and platelet-derived growth factor from endothelial cells; (vi) inhibiting platelet aggregation; and (vii) incremental synthesis of bilirubin inhibiting monocyte transmigration and leukocyte adhesion induced by oxidized LDL or other oxidants.[25]

Dinucleotide repeat $(GT)_n$, the most frequent simple repeat in the human genome, is usually characterized by length polymorphism and may modify gene transcription.[26] A $(GT)_n$ dinucleotide repeat was located in the proximal promoter region *HO-1* gene, which was mapped at chromosome 22q12.[11,27] It is suggested that the highly polymorphic $(GT)_n$ repeat in the *HO-1* gene could alter transcriptional activity. Chen *et al.*[13] showed that the more the $(GT)_n$ repeats in the promoter region, the less the transcription of the *HO-1* gene by using *HO-1* promoter/luciferase reporter genes carrying different lengths of $(GT)_n$ repeats in rat aortic smooth muscle cells. This result is in accord with that shown earlier in Hep3B cells.

In this study of 603 HD patients, we analyzed the correlation between AVF failure and the dinucleotide length polymorphism $(GT)_n$ in the *HO-1* gene promoter. There was no difference in primary diseases between the *HO-1* genotype groups. The frequencies of DM and hypertension, suspected to have a correlation with vascular disease, were similar among the three patient groups with different *HO-1* genotypes. However, the proportion of AVF failure increased from 20.3% in group S/S to 31.0% in group L/S and 35.4% in group L/L ($P = 0.011$). A similar trend of frequency increase from the *HO-1* genotype group S/S to L/S to L/L could also be found for CAD and cardiovascular disease, but not for peripheral artery obstructive disease (PAOD) or cerebral infarction.

Recently, the role of the dinucleotide repeat length polymorphism of the *HO-1* gene promoter has been reported in several human vascular diseases. Emerging evidence shows that a longer $(GT)_n$ repeat in the promoter region of the *HO-1* gene is associated with (i) restenosis and increased vascular inflammation after percutaneous transluminal angioplasty[28] (odds ratio = 5 for $(GT)_n \geqslant 25$ vs $(GT)_n < 25$), (ii) susceptibility to CAD in Japanese patients with coronary risk factors[12] (odds ratio = 2.5–4.8 for $(GT)_n > 27$ vs $(GT)_n \leqslant 27$), and (iii) the development of abdominal aortic aneurysms[29] (odds ratio = 1.9 for $(GT)_n \geqslant 25$ vs $(GT)_n < 25$). It was shown that a longer $(GT)_n$ repeat was associated with emphysema in smokers (odds ratio = 4.2 for $(GT)_n \geqslant 30$ vs $(GT)_n < 30$).[15] Chen *et al.*[13] further reported that type II diabetics carrying longer $(GT)_n$ repeats may have higher oxidative stress and increased susceptibility to the development of CAD (odds ratio = 4.7 for $(GT)_n \geqslant 32$ vs $(GT)_n < 32$). As excessive growth of VSMCs is an important contributing factor to a number of vascular disease states such as restenosis following angioplasty,[30] atherosclerosis,[31] and hypertension,[32] increasing *HO-1* expression in blood vessels may offer a promising approach in treating these vascular disorders.

The absence of significant difference in HO-1 genotype frequency between HD patients and healthy controls in our study may result from the following reasons. First, the development of end-stage renal disease (ESRD) is associated with various causes and risk factors, including susceptibility factors (such as older age and family history of ESRD), initiation factors (such as DM, hypertension, nephrotoxic medications, etc.), and progression factors (such as proteinuria, poorly controlled blood pressure and glucose, smoking, etc.). All these factors may play more important roles than *HO-1* in the development of ESRD. Second, the protective effect of *HO-1* on renal injury has been reported mainly in hematuric renal disease[33] and acute renal failure induced by various factors (such as cisplatin, mercury, myoglobulin, etc.).[34–36] However, most cases of ESRD result from nonhematuric chronic kidney diseases (such as hypertension and diabetes). Third, *HO-1* overexpression may worsen renal injury. Suttner *et al.*[37] found that low levels (two- to five-fold that of control) of *HO-1* expression are protective, whereas high (>15-fold) levels of *HO-1* expression actually worsen the damage. This damage may derive from greater elevations of intracellular free iron produced by overexpressed *HO-1* gene.

In this investigation, we studied the association of AVF failure with *HO-1* genotype polymorphisms. However, many factors may predispose HD patients to AVF failure: (i) technique of the vascular surgeon; (ii) endothelial dysfunction and shear stress under excessive extracorporeal blood flow; (iii) vascular injury on account of frequent puncture; (iv) prolonged external compression after HD; (v) reduced Qa (frequent hypotension in association with HD or insufficient dilatation due to poor vascular anatomy); and (vi) hypercoagulable status due to the presence of factors for thrombophilia, such as factor V Leiden, prothrombin gene mutation (*G20210A*), factor XIII genotype (*Val34Leu*), *MTHFR* (*C677T*) genotype T/T, and higher concentrations of lupus anticoagulant, anticardiolipid antibody, factor VIII, homocysteine, and lipoprotein (a).[38] A sudden onset of AVF failure may develop in some patients, without previous appearance of elevated venous pressure or insufficient extracorporeal blood flow. The possibility of coagulation abnormality or another cause of AVF failure must be taken into account.

In the present investigation, we focused on the dinucleotide length polymorphisms of *HO-1*. There was a significant difference between S/S, L/S, and L/L genotypes by patient number and incident episodes (per patient-month). Here, *HO-1* genotype polymorphisms (class L/S and L/L) were proved to be associated with a higher failure frequency as well as a shorter patency of AVF in HD patients.

We acknowledge that some limitations should be considered in the interpretation of this study result. First, all our study subjects are Chinese living in Taiwan, so the

characteristic of distinct ethnicity may prevent the results of this study from being extrapolated to other ethnic groups. Second, the association may not be present for other types of vascular access, such as AV graft, because our result comes from patients with AV fistulae. Third, a selection bias might exist because this study is based on a retrospective cohort study. Only those who survived at the time of study enrollment had the opportunity of being selected and of receiving venous sampling; all those who had dropped out from maintenance HD treatment would be excluded from this study. The selection bias may be avoided by conducting a randomized prospective cohort study.

In conclusion, stenosis-related AVF failure remains a major clinical problem although the programs for regular monitoring and surveillance of AVF are encouraged. Recently, there is increasing evidence that genetic background may explain at least part of the excessive risk for stenosis observed in certain HD patients. In this study, for the first time, we demonstrated that genotype polymorphisms in the promoter of *HO-1* gene may be associated with stenosis-related AVF failure in HD patients. The long dinucleotide GT repeat in the promoter region may diminish *HO-1* gene transcription, and consequently offset the protective effect (through CO) against vascular injury. More studies are needed to investigate whether selective pharmacological regulation of *HO-1* activity in the subgroup carrying higher risk will improve AVF function or reduce the frequency of AVF failure for HD patients in the future.

MATERIALS AND METHODS

Study population

In this study, we screened all patients undergoing maintenance HD therapy at four institutions – Taipei Veterans General Hospital, Shin-Jen HD Center, Hwa-Jong HD Center, and Wen-Lin HD Center – between January and March 2005, for whom medical records and blood samples for genotyping were available. We included patients in this study if they met the following criteria: (1) creation of a native AV fistula as the first vascular access for HD between January 1, 1980 and December 31, 2004; (2) creation of AV fistula by similar surgical skills with end-to-side anastomosis at the upper extremity and (3) receiving HD therapy for 4 h three times weekly for more than 3 months. Patients were excluded if they fulfilled one of the following criteria: (1) receiving an AV graft as the first vascular access and (2) the first episode of fistula failure or interventional procedure did not result from stenosis-related events, such as infectious complication, progressive aneurysmal formation, or steal syndrome. We also included 286 healthy men ($n = 179$) and women ($n = 107$) without renal disease as controls. This retrospective study was based on the Declaration of Helsinki (edition 6, revised 2000).[39] Informed consent was obtained from all study patients and controls, and the study was approved by the Institutional Research Board of Taipei Veterans General Hospital. For HD patients, we recorded the following clinical factors: age (at HD therapy initiation and at the beginning of the study), gender (HD therapy duration), side (right or left) and location (elbow or wrist) of the first created native AV fistula, underlying cause of end-stage renal disease (presence or absence of diabetic nephropathy), time of AV fistula failure, peak dynamic venous pressure, and maximally delivered extracorporeal blood flow during the 30 days prior to AVF failure or the date of termination of observation. We also recorded the history of the following vascular diseases: presence of hypertension, presence of PAOD, presence of cerebrovascular disease (CVD), and presence of CAD prior to AVF failure for those with fistula failure or until March 31, 2005 for those without AVF failure. The primary end point was unassisted patency, which was defined as the time from fistula surgery to the first episode of fistula failure for patients meeting the inclusion criteria and without the presence of any exclusion criteria. We defined fistula failure as the need for any interventional procedure (surgery or angioplasty) to correct a malfunctioning or occlusive fistula, which appeared at least 3 months after fistula surgery. Fistula failure within the first 3 months after fistula construction was regarded as inadequate dilatation rather than intimal hyperplasia; so the appropriate timing for cannulation was 3–4 months after placement of AVF, as suggested by DOQI guidelines.[40] These patients were excluded from our analysis. Patients were assessed at the time of renal transplantation ($n = 1$), death with a functioning access ($n = 3$), shifting to peritoneal dialysis ($n = 2$), or loss to follow-up ($n = 6$). This observation study was terminated on March 31, 2005.

Assessment of comorbid vascular diseases

PAOD was defined as having undergone peripheral bypass surgery, nontraumatic amputation of extremity, and angioplasty for peripheral arteries. CVD was established by a history of carotid endarterectomy or carotid angioplasty, a history of cerebral infarction, or a history of carotid artery stenosis with imaging diagnosis (Doppler scan of carotid arteries, computed tomography, or magnetic resonance imaging of brain). This study excluded patients who had lacunar infarctions without apparent symptoms. CAD was diagnosed from medical records (history of coronary artery angioplasty or coronary artery bypass graft surgery, history of myocardial infarction, or a confirmatory coronary angiography by the medical doctor of each institute). Cardiovascular disease was defined as having a history of PAOD, CVD, or CAD.

Determination of length polymorphism of $(GT)_n$ repeats in *HO-1* gene promoter

Peripheral blood without dietary control during voluntary HD was collected in a tube containing ethylenediamine tetraacetate (EDTA)-2K and immediately subjected to genomic DNA extraction procedures. Genomic DNA was isolated using the Puregene DNA purification kit (Gentra, Minneapolis, MN). The $(GT)_n$ repeat at the promoter region of the *HO-1* gene was amplified by polymerase chain reaction (PCR) with an FAM-labeled sense primer 5′-AGA GCCTGCAGCTTCTCAGA-3′, and an antisense primer 5′-ACAAA GTCTGGCCATAGGAC-3′, according to Kimpara *et al.*,[41] in a 7.5 μl PCR reaction containing 7.5 ng of genomic DNA, 0.333 μM of each primer, 1.5 mM $MgCl_2$, and 0.1875 U of *Taq* DNA polymerase (Amersham Biosciences, GE Healthcare, Sunnyvale, CA) in 200 μM of dNTPs and 1 × PCR buffer supplied with the enzyme. The PCR conditions consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 60 min on MJ Research PTC-100 thermal cyclers (MJ Research Inc., Watertown, MA) or ABI PRISM 9700/2700 thermal cyclers (Applied Biosystems, Foster City, CA). The diluted PCR products with internal size standard, MegaBACE™ ET400-R (Amersham Biosciences, GE Healthcare, Sunnyvale, CA), were analyzed using an ABI PRISM 377 automated DNA sequencer (Applied Biosystems) and GeneScan software (version 2.1) (Applied

Biosystems). The GT-repeat length was determined and statistically analyzed using the Genotyper (version 2.0) program (Applied Biosystems). All fragment analyses contained appropriate negative and positive controls.

Statistical analysis

Data management and statistical analysis were carried out using the SPSS statistical software (version 11.0; USA). Distributions of continuous variables in groups were expressed as mean $\pm$ s.d. and compared by Student's *t*-test. All data were tested for normal distribution before using *t*-tests. Categorical variables, such as the distribution of genotype frequencies in patients and controls, were analyzed by the χ^2 test. The Cox regression model was used to adjust for other established risk factors of AV fistula stenosis. Adjusted odds ratio and 95% confidence interval for every explanatory variable were also calculated. Survival curves of unassisted patency of AV fistula were calculated by the Kaplan–Meier method and compared by the log-rank test. $P < 0.05$ was considered to be statistically significant.

ACKNOWLEDGMENTS

We thank the following clinicians who referred the patients and collected blood samples: Dr Yee-Yung Ng, Dr Tsai-Hun Wu, Dr Der-Cherng Tarng, Dr Jinn-Yang Chen, Dr Yao-Ping Lin, and Dr Chiao-Lin Chuang at Taipei Veterans General Hospital; Dr Hong Hsiang Liou at Shin-Jen Hemodialysis Center; Hai-Ming Sheng at Wen-Lin Hemodialysis Center, Jeng Hu at Hwa-Jong Hemodialysis Center. We also thank Mei-Shiang Wang for technical support. This work was supported by a grant (2004-A042) from Taipei Veterans General Hospital and the Integrated Genome Project (2005-E02) of Taipei Veterans General Hospital in Taiwan.

REFERENCES

1. Feldman HI, Kobrin S, Wasserstein A. Hemodialysis vascular access morbidity. *J Am Soc Nephrol* 1996; **7**: 523–535.
2. Paun M, Beach K, Ahmad S *et al.* New ultrasound approaches to dialysis access monitoring. *Am J Kidney Dis* 2000; **35**: 477–481.
3. Windus DW. Permanent vascular access: a nephrologist's view. *Am J Kidney Dis* 1993; **21**: 457–471.
4. Lin CC, Chang CF, Chiou HJ *et al.* Variable pump flow-based Doppler ultrasound method: a novel approach to the measurement of access flow in hemodialysis patients. *J Am Soc Nephrol* 2005; **16**: 229–236.
5. Abularrage CJ, Sidawy AN, Weiswasser JM *et al.* Medical factors affecting patency of arteriovenous access. *Semin Vasc Surg* 2004; **17**: 25–31.
6. Weiss MF, Scivittaro V, Anderson JM. Oxidative stress and increased expression of growth factors in lesions of failed hemodialysis access. *Am J Kidney Dis* 2001; **37**: 970–980.
7. Heine GH, Ulrich C, Sester U *et al.* Transforming growth factor beta1 genotype polymorphisms determine AV fistula patency in hemodialysis patients. *Kidney Int* 2003; **64**: 1101–1107.
8. Fukasawa M, Matsushita K, Kamiyama M *et al.* The methylenetetrahydrofolate reductase C677T point mutation is a risk factor for vascular access thrombosis in hemodialysis patients. *Am J Kidney Dis* 2003; **41**: 637–642.
9. Maines MD. The heme oxygenase system: a regulator of second messenger gases. *Annu Rev Pharmacol Toxicol* 1997; **37**: 517–554.
10. Durante W. Heme oxygenase-1 in growth control and its clinical application to vascular disease. *J Cell Physiol* 2003; **195**: 373–382.
11. Lavrovsky Y, Schwartzman MC, Levere RD. Identification of binding sites for transcription factors NF-κB and AP-2 in the promoter region of the human heme oxygenase-1 gene. *Proc Natl Acad Sci USA* 1994; **91**: 5987–5991.
12. Kaneda H, Ohno M, Taguchi J. Heme oxygenase-1 gene promoter polymorphism is associated with coronary artery disease in Japanese patients with coronary risk factors. *Arterioscler Thromb Vasc Biol* 2002; **22**: 1680–1685.
13. Chen YH, Lin SJ, Lin MW *et al.* Microsatellite polymorphism in promoter of heme oxygenase-1 gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients. *Hum Genet* 2002; **111**: 1–8.
14. Chen YH, Chau LY, Lin MW *et al.* Heme oxygenase-1 gene promoter microsatellite polymorphism is associated with angiographic restenosis after coronary stenting. *Eur Heart J* 2004; **25**: 39–47.
15. Yamada N, Yamaya M, Okinaga S. Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema. *Am J Hum Genet* 2000; **66**: 187–195.
16. Konner K, Hulbert-Shearon TE, Roys EC, Port FK. Tailoring the initial vascular access for dialysis patients. *Kidney Int* 2002; **62**: 329–338.
17. Saran R, Dykstra DM, Wolfe RA. Association between vascular access failure and the use of specific drugs: The Dialysis Outcomes and Practice Patterns Study (DOPPS). *Am J Kidney Dis* 2002; **40**: 1255–1263.
18. Golledge J, Smith CJ, Emery J. Outcome of primary radiocephalic fistula for haemodialysis. *Br J Surg* 1999; **86**: 211–216.
19. Dixon BS, Novak L, Fangman J. Hemodialysis vascular access survival: upper-arm native arteriovenous fistula. *Am J Kidney Dis* 2002; **39**: 92–101.
20. Lemson MS, Tordoir JH, Daemen MJ, Kitslaar PJ. Intimal hyperplasia in vascular grafts. *Eur J Vasc Endovasc Surg* 2000; **19**: 336–350.
21. Tenhunen R, Marver HS, Schmidt R. The enzymatic conversion of heme to bilirubin by microsomal heme oxgyenase. *Proc Natl Acad Sci USA* 1968; **244**: 6388–6394.
22. Maines MD. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J* 1989; **2**: 2557–2568.
23. Van Belle E, Bauters C, Asahara T, Isner JM. Endothelial regrowth after arterial injury: from vascular repair to therapeutics. *Cardiovasc Res* 1998; **38**: 54–68.
24. Liu X, Chapman GB, Wang H, Durante W. Adenovirus-mediated heme oxygenase-1 gene expression stimulates apoptosis in vascular smooth muscle cells. *Circulation* 2002; **105**: 79–84.
25. Ishikawa K, Sugawara D, Wang X *et al.* Heme oxygenase-1 inhibits atherosclerotic lesion formation in LDL-receptor knockout mice. *Circ Res* 2001; **88**: 506–512.
26. Naylor LH, Clark EM. d(TG)*n*. d(CA)*n* sequences upstream of the rat prolactin gene from Z-DNA and inhibit gene transcription. *Nucleic Acids Res* 1998; **18**: 1595–1601.
27. Kutty RK, Kutty G, Rodorigues IR. Chromosomal localization of the human heme oxygenase genes: heme oxygenase-1 (HMOX1) maps to chromosome 22 q12 and heme oxygenase2 (HMOX2) maps to chromosome 16p13.3. *Genomics* 1994; **20**: 513–516.
28. Exner M, Schillinger M, Minar E *et al.* Heme oxygenase-1 gene promoter microsatellite polymorphism is associated with restenosis after percutaneous transluminal angioplasty. *J Endovasc Ther* 2001; **8**: 433–440.
29. Schillinger M, Exner M, Mlekusch W *et al.* Heme oxygenase-1 gene promoter polymorphism is associated with abdominal aortic aneurysm. *Thromb Res* 2002; **106**: 131.
30. Zierler RE, Bandyk DF, Thiele BL, Strandness DE. Carotid artery restenosis following endarectomy. *Arch Surg* 1982; **117**: 1408–1415.
31. Ross R. The pathogenesis of atherosclerosis: an update. *N Engl J Med* 1986; **314**: 488–500.
32. Schwartz SM, Campbell GR, Campbell JH. Replication of smooth muscle cells in vascular disease. *Circ Res* 1986; **58**: 427–444.
33. Nath KA, Vercellotti GM, Grande JP *et al.* Heme protein-induced chronic renal inflammation: suppressive effect of induced heme oxygenase-1. *Kidney Int* 2001; **59**: 106–117.
34. Shiraishi F, Curtis LM, Truong L *et al.* Heme oxygenase-1 gene ablation or expression modulates cisplatin-induced renal tubular apoptosis. *Am J Physiol* 2000; **278**: F726–F736.
35. Yoneya R, Ozasa H, Nagashima Y *et al.* Hemin pretreatment ameliorates aspects of the nephropathy induced by mercuric chloride in the rat. *Toxicol Lett* 2000; **116**: 223–229.
36. Ishizuka S, Nagashima Y, Numata M *et al.* Regulation and immunohistochemical analysis of stress protein heme oxygenase-1 in rat kidney with myoglobinuric acute renal failure. *Biochem Biophys Res Commun* 1997; **240**: 93–98.
37. Suttner DM, Dennery PA. Reversal of HO-1 related cytoprotection with increased expression is due to reactive iron. *FASEB J* 1999; **13**: 1800–1809.
38. Knoll GA, Wells PS, Young D *et al.* Thrombophilia and the risk for hemodialysis vascular access thrombosis. *J Am Soc Nephrol* 2005; **16**: 1108–1114.
39. The World Medical Association Inc.: World Medical Association Declaration of Helsinki. Available at: http://www.wma.net/e/policy/b3.htm.
40. NKF-K/DOQI Clinical Practice Guidelines for Vascular Access: update 2000. *Am J Kidney Dis* 2001; **37**: S148.
41. Kimpara T, Takeda A, Watanabe K *et al.* Microsatellite polymorphism in the human heme oxygenase-1 gene promoter and its application in association studies with Alzheimer and Parkinson disease. *Hum Genet* 1997; **100**: 145–147.