Dimethylarginine Dimethylaminohydrolase 2 Gene Polymorphism and Its Association with Asymmetrical Dimethyl Arginine in Hemodialyzed Patients*

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ABSTRACT

Introduction: Patients with CKD have elevated plasma levels of Asymmetrical Dimethyl Arginine (ADMA), impaired EDRF/NO responses in isolated resistance vessels, and a marked increase in the frequency of cardiovascular events that are predicated by plasma levels of ADMA. ADMA is considered as a risk factor for endothelial dysfunction, progression of chronic kidney disease and a marked increase in the frequency of cardiovascular events that are predicated by plasma levels of ADMA. Elevated ADMA in CKD have been related to a combination of a reduced renal ADMA excretion and a reduced catabolism of ADMA by dimethylarginine dimethylaminohydrolase (DDAH). The current study was undertaken to determine whether there is a correlation between ADMA and SNPs at −449 DDAH.

Subjects and Methods: It was a cross sectional analytic study, 56 hemodialysis patients and 30 healthy individuals were enrolled. Based on its etiology, HD patients group was further divided in to hypertension (HT) subgroup and non-HT subgroup. Genotyping of the polymorphisms was performed using PCR-based SNP detection methods based on 5'-exonuclease activity assays for rs805305.

Results: Heterozygotes were observed as the most abundant genotypes in both groups, followed by GG genotype in the HD patients (30%) and CC (27%) healthy individuals. Among the HT subgroup, the mean plasma levels of ADMA were sequentially higher from genotypes CC, G/C and GG (p = 0.037). Further multiple comparisons between groups using post hoc test showed results that genotype GG and CC were different at 0.05 level of significance. These findings were not found among non HT subgroup. Conclusion: Genetic variation in the DDAH genes is significantly associated with serum ADMA levels in hypertensive HD patients. We observed that carriage of a G at position −449 in the promoter region of the DDAH gene is associated with higher ADMA levels.

Keywords: ADMA; DDAH; Gene Polymorphism

1. Introduction

Atherosclerotic cardiovascular diseases (CVD) are major causes of morbidity and mortality in patients receiving ongoing hemodialysis (HD) [1,2]. Despite its increasing incidence and the accelerated worsening of atherosclerosis in patients on chronic hemodialysis, the proportion of individuals with chronic kidney disease (CKD) receiving appropriate cardiovascular (CV) risk modification treatment is lower than that in the general population [3]. In metabolic disorders associated with atherosclerosis (dyslipidemia, hypertension (HT) and diabetes mellitus), a reduced endothelium-mediated nitric oxide (NO)-dependent vasodilation has been observed, which may contribute to the initiation and progression of atherosclerosis associated with these disorders [4].

The mechanisms of endothelial vasodilator dysfunction (EVD) are likely multi-factorial [5]. Evidences from both animal models and clinical studies suggests that accumulation of the endogenous nitric-oxide synthase (NOS) inhibitors, ADMA and NGmethyl-L-arginine (L-NMMA) contributes to reducing nitric oxide (NO) [6,7].
Plasma levels of ADMA are inversely related to the vascular NO bioavailability and ADMA is an independent determinant of the vascular redox-state [8,9]. ADMA is also a risk factor for EVD, CV mortality and progression of CKD [8,10].

The co-localization of DDAH 2 and NOS at several sites supports the hypothesis that DDAH may regulate NOS activity by controlling the metabolism of ADMA [11]. As DDAH 2 regulates ADMA levels, it is believable that functional DDAH 2 gene polymorphisms may account for the variation in ADMA levels [12]. ADMA levels may be genetically determined by a promoter polymorphism in a regulatory gene encoding DDAH 2 [13]. However, the functional significance has not been determined previously in chronic HD patients. Therefore, the current study was undertaken to determine whether or not there is a correlation between single nucleotide polymorphisms (SNPs) at −449 (promoter region) DDAH 2 with ADMA plasma levels in HD patients.

2. Patients and Methods

2.1. Study Population and Dialysis Protocol

Fifty-six patients with a creatinine clearance of less than 15 ml/min per 1.73 m² who had been on chronic HD treatment for more than 3 months were recruited for the present study. Thirty-four HD patients (60.7%) were male. Clinical practice was not changed or modified for the purpose of the study. As the control group, 30 healthy individuals paramedics who had no cardiovascular clinical risk factors (HT, diabetes, dyslipidemia, smoking, family history of premature coronary artery disease or CKD), were enrolled. Five healthy individuals (16.7%) as controls were male.

All participants (patients and controls) signed the Free and Informed Consent Form and the study was approved by the Ethics Committee of Al Irsyad Hospital, Surabaya, Indonesia. It was an analytic cross-sectional study. Clinical and demographic details were also collected.

All patients underwent dialysis twice a week for 4 weeks using a commercially available dialyser (type F6-8 HPS polysulfone semi-synthetic, fresenius GMBH, Germany). Each HD session lasted 4 hours.

Blood pressure (BP) was measured using standard mercury sphygmomanometers after participants had been sitting for at least 10 min. Hypertension was defined as blood pressure ≥ 140/90 mmHg at the time of recruitment, history of hypertension before HD, or the use of anti-hypertensive medication to have BP ≤ 130/80 mmHg. Hypertension was considered as an etiology of CKD if a patient had chronic hypertension before HD, and still had hypertension after HD.

The clinical features of HD patients are shown in Table 1. The predominant causes of CKD were hypertension (35 patients, 62.5%) and diabetes (10 patients, 17.9%). Other etiologies such as infection, glomerulonephritis and kidney stone were less than 10% (Figure 1).

2.2. Blood Sampling

Venous blood samples were drawn from the arterial-venous fistula 10 minutes before the first HD session in a week. Peripheral whole blood was obtained from 30 healthy individuals. Blood samples were 3 ml for each participant without additives. Plasma was immediately separated by centrifugation at 800 g for 15 minutes, and then was stored at −20°C prior to use.

2.3. Determination of ADMA

ADMA was measured using an enzyme-linked immunosorbent assay (DLD Diagnostika, Hamburg, Germany) as previously reported [14]. The plasma HCY concentration was measured using The AxSYM HCY assay (Abbott USA), that is a fluorescence polarization immunoassay in the accordance with the procedure previously published [15,16].

2.4. Genomic DNA

Genomic DNA was extracted from whole blood with a commercially available DNA isolation kit (QIAmp DNA blood Mini kit, Qiagen GmbH, Crawley, West Sussex,
Genotyping of the polymorphisms was performed using PCR-based SNP detection methods (Applied Biosystems, Carlsbad, USA) based on 5'-exonuclease activity assays for rs805305 (−449 G/C) [17,18]. Allelic variation was assessed in each participant.

3. Statistics
All variables were examined for normal distribution using the Kolmogorov-Smirnov test. Normally distributed variables were presented as mean ± SD. Pearson correlation was undertaken for associations of clinical covariates with ADMA levels. Distribution differences of gene polymorphism between the groups were analyzed using Mann-Whitney Test. One way Anova and post hoc test were also used to analyze significant difference between the genotypes within subgroups.

4. Results
4.1. Correlation of ADMA and eGFR in HD Patients
Before performing a parametric correlation test (Pearson), a Kolmogorov-Smirnov test was performed, and the results were normal distribution (ADMA: 0.95; eGFR: 1.05). The Pearson correlation test showed a weak (p = 0.134) non-significant correlation between estimated glomerular filtration rate (eGFR) and ADMA in HD patients.

4.2. Gene Polymorphism Distribution HD Patients and Healthy Individuals
Gene polymorphisms were assessed among healthy individuals and HD patients. Heterozygotes were observed as the most abundant genotypes in both groups (70% in healthy individuals and 55% in HD patients), followed by GG genotype in the HD patients (30%), while CC (27%) were the second most common genotype polymorphism in the healthy individuals (Tables 2 and 3). Using the Mann-Whitney U test, a significant polymorphism distribution between both groups was sought, and the result was 0.006. There was a significant difference in polymorphism distribution among both groups.

4.3. Subgroups within HD Patients
The HD patients were divided into two subgroups. The subgroups were categorized based on the etiology of the CKD in HD patients. One subgroup was a hypertension subgroup (35 of 56 HD patients samples). The other was a non-HT subgroup, a subgroup of HD patients in which the etiologies of CKD were DM, infection, glomerulonephritis and kidney stone (21 of 56 HD patients samples). There was no significant difference in the distribution of DDAH gene polymorphism among subgroups, as the results of Mann-Whitney test was 0.364.

4.3.1. Subgroup Hypertension Analysis
As the most frequent cause of CKD in this study, DDAH gene polymorphisms and the levels of plasma ADMA were analyzed, including as to whether or not there were differences between genotypes. The distribution of polymorphism is shown in Table 4.

The table also reveals that the mean plasma levels of ADMA were sequentially higher from genotypes CC, G/C and GG. These different findings between genotypes were confirmed statistically using a one-way anova test with the results of 0.037. Further multiple comparisons between groups using post hoc test showed results that genotype GG and CC were different at 0.05 level of significance (Table 5).

4.3.2. Subgroup Non-Hypertension Analysis
The gene polymorphism distributions and their correlation with plasma levels of ADMA were also analyzed in non-hypertension HD patients. Compared to the HT subgroup, there was a similar composition of gene polymorphism frequency among Non-HT (Table 6). But, when statistical tests were used to define association between mean plasma levels of ADMA and DDAH gene polymorphism, there were no significant differences found between groups of genotypes; either by using the Anova test (p = 0.322), or using the multiple comparison post hoc test (p > 0.05).

5. Discussion
There are considerable data on the role of ADMA in hemodialysis patients and likewise on the association of DDAH gene polymorphism and ADMA in certain popu-
Table 4. ADMA levels among HT subgroups.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>N</th>
<th>Mean</th>
<th>Std deviation</th>
<th>Std Error</th>
<th>95% confidence interval for mean</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>11</td>
<td>0.9491</td>
<td>0.18157</td>
<td>0.05475</td>
<td>0.8271 1.0711</td>
<td>0.80</td>
<td>1.45</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>21</td>
<td>0.8248</td>
<td>0.16540</td>
<td>0.03609</td>
<td>0.7495 0.9000</td>
<td>0.60</td>
<td>1.30</td>
</tr>
<tr>
<td>CC</td>
<td>3</td>
<td>0.6800</td>
<td>0.12124</td>
<td>0.07000</td>
<td>0.3788 0.9812</td>
<td>0.61</td>
<td>0.82</td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>0.8514</td>
<td>0.18103</td>
<td>0.03060</td>
<td>0.7892 0.9136</td>
<td>0.60</td>
<td>1.45</td>
</tr>
</tbody>
</table>

ADMA.

Table 5. Multiple comparison of ADMA levels among DDAH gene polymorphisms.

<table>
<thead>
<tr>
<th>(I) DDAH</th>
<th>(J) DDAH</th>
<th>Mean difference (I-J)</th>
<th>Standard error</th>
<th>Sig.</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>G/C</td>
<td>0.12433</td>
<td>0.06263</td>
<td>0.056</td>
<td>−0.0033 0.2519</td>
</tr>
<tr>
<td>CC</td>
<td>GG</td>
<td>0.26909*</td>
<td>0.10961</td>
<td>0.020</td>
<td>0.0458 0.4924</td>
</tr>
<tr>
<td>G/C</td>
<td>GG</td>
<td>−0.12433</td>
<td>0.06263</td>
<td>0.056</td>
<td>−0.2519 0.0033</td>
</tr>
<tr>
<td>CC</td>
<td>G/C</td>
<td>0.14476</td>
<td>0.10387</td>
<td>0.173</td>
<td>−0.0668 0.3563</td>
</tr>
<tr>
<td>GG</td>
<td>CC</td>
<td>−0.26909</td>
<td>0.10961</td>
<td>0.020</td>
<td>−0.4924 −0.0458</td>
</tr>
</tbody>
</table>

Dependent Variable: ADMA; LSD. *The mean difference is significant at the 0.05 level.

Table 6. ADMA levels among Non-HT & HD patients.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>N</th>
<th>Mean</th>
<th>Std deviation</th>
<th>Std error</th>
<th>95% confidence interval for mean</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>6</td>
<td>0.6950</td>
<td>0.38935</td>
<td>0.15895</td>
<td>0.2864 1.1036</td>
<td>0.00</td>
<td>1.05</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>10</td>
<td>0.9010</td>
<td>0.21153</td>
<td>0.06897</td>
<td>0.7497 1.0523</td>
<td>0.56</td>
<td>1.17</td>
</tr>
<tr>
<td>CC</td>
<td>5</td>
<td>0.7840</td>
<td>0.13278</td>
<td>0.05293</td>
<td>0.6191 0.9489</td>
<td>0.57</td>
<td>0.92</td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>0.8143</td>
<td>0.26423</td>
<td>0.05766</td>
<td>0.6940 0.9346</td>
<td>0.00</td>
<td>1.17</td>
</tr>
</tbody>
</table>

ADMA.

lations. Nonetheless, later studies have not only drawn different conclusions (DDAH isoforms, invitro/invivo, human/animal), but also have found opposite results. In this study, we described the correlation between a novel genetic polymorphism DDAH 2 and ADMA in hypertensive HD patients. The study also revealed a significant distribution difference in DDAH polymorphism between healthy individuals and hemodialysis patients. We believe that this is the first study that has reported such correlations among HD patients.

In the first evaluation, we examined the correlation of estimated glomerular filtration rate (eGFR) and ADMA. There was no significant correlation between eGFR and ADMA. An inverse relationship between plasma ADMA and glomerular filtration rate (GFR) was apparent in patients with coronary artery disease, which has been implicated in their increased cardiovascular risk [19]. However, it has been questioned whether elevated circulating levels of ADMA are merely a marker of kidney disease, coinciding with serum creatinine, or part of the pathophysiological process [20]. Indeed, plasma levels of SDMA correlated more closely with parameters of renal function than did plasma levels of ADMA [21].

ADMA is a naturally-occurring non-selective inhibitor of NOS, and Free ADMA ≥ 80% is actively metabolized by the intracellular enzyme DDAH [11,22]. DDAH is most likely a key determinant of plasma ADMA concentration [14]. Thus, DDAH plays a crucial role in the regulation of NO synthesis via modulating endogenous ADMA levels [23]. Alterations in DDAH activity may
effect vascular structure, as well as vascular reactivity [24]. As research has progressed, DDAH has emerged as an important regulator of NO bioavailability and the integrity of renal and vascular function [10].

In higher organisms, including humans, two isoforms of DDAH exist which have distinct tissue distributions but similar enzymatic activity [25]. Based on tissue messenger ribonucleic acid (mRNA) levels, it has been suggested that the expression pattern of DDAH 1 overlaps more closely with the expression pattern of neuronal NOS, whereas DDAH 2 expression has greater similarities with endothelial NOS expression [26,27]. However, it is still unclear which DDAH isoform represents the principal methylarginine-metabolizing enzyme.

Both groups, the HD patients and the healthy individuals, showed a significant difference in the distribution DDAH 2 gene polymorphism. They shared the same most abundant genotype, which was G/C, followed by GG in the HD group and CC in the healthy individuals group. The polymorphisms were in the non-coding part of the gene, putatively affecting promoter activity, designated DDAH 2 −449. Although our study may not have been adequately powered to detect outcome variations, these findings implied possible functionality polymorphism in the DDAH 2 promoter. As DDAH 2 regulates ADMA levels, it is plausible to think that functional DDAH 2 gene polymorphisms may account for variation in ADMA levels [12]. Data suggest that expression of DDAH 2 may be affected by polymorphisms of the promoter region [17].

For the primary analysis, the HD patients group was divided into a HT subgroup and a Non-HT subgroup. In the subgroup HT, we demonstrated correlations of ADMA with the carriage of the DDAH 2 −449 G allele, where plasma level of ADMA in GG > G/C > CC. Average blood pressure of containing −449 G allele (GG and G/C) was also higher than that of CC allele. O’Dwyer et al. recently reported that the −449G allele of the DDAH 2 gene was associated with increased ADMA concentrations [13]. Higher prevalence of hypertension with an odds ratio of 1.80 (95%CI: 1.29 - 2.49, p < 0.001) for individuals homozygous for the −449G allele was also reported [18]. In the secondary analysis, we could not find any correlation of ADMA and DDAH 2 gene polymorphism among non-hypertensive HD patients.

Oxidative stress is defined as tissue damage caused by the disequilibrium between pro-oxidants and anti-oxidants [28]. Vascular oxidative stress is a major factor in the pathogenesis of atherosclerosis [29]. One of the main effects of oxidative stress is the decrease in the biological activity of NO [28]. Among the mechanisms for impaired NO synthesis is the accumulation of the endogenous NOS inhibitor asymmetric ADMA [7]. ADMA inhibits all three isoforms of nitric oxide synthase and therefore has the potential to produce diverse biological effects, particularly in the cardiovascular system [26,30]. Some studies of human subjects with essential HT report elevated plasma levels of ADMA [31,32]. Investigations of ADMA concentration by independent methods in patients with renal failure, have identified plasma ADMA concentration as a strong independent predictor of disease progression. Of particular interest was the finding that a 0.1 µmol/L increase in plasma ADMA was associated with a 20% increase in cardiovascular event rate [7,33].

The enzyme DDAH specifically hydrolyzes these asymmetrically methylated arginine residues to citrulline and methylamines [34,35]. Several groups have demonstrated that modulating DDAH activity can have a profound effect on endothelial NO production [7,36,37]. Two isoforms of DDAH have been identified. Since DDAH 2 is the predominant isofrom expressed in the cardiovascular system and since hypercholesterolemia impaired endothelial DDAH activity while DDAH 1 protein expression remained unchanged [23,38,39], DDAH 2 gene polymorphism was studied in the present study. The finding that the gene for DDAH is evolutionarily well conserved suggests that the enzyme confers an important survival advantage [10]. And it can be postulated that genetic variations in DDAH that affect the ability of the enzyme to metabolize ADMA might increase cardiovascular risk in patients with renal failure and might also determine, in part, the rate of disease progression [26].

Growing and irresistible epidemiological evidence links ED with cardiorenal syndrome. Recently, a human study, using artery and vein graft suggests that ADMA may be directly implicated in the regulation of the vascular redox state of atherosclerosis by affecting superoxide generation and NO bioavailability [8]. Therefore, any therapeutic modulation of ADMA levels, via genetic modification of DDAH expression or activity, may represent a new strategy for the treatment of cardiovascular disorders, including in HD patients.

6. Conclusions

Genetic variation in the DDAH 2 genes is significantly associated with serum ADMA levels in hypertensive HD patients. We observed that carriage of a G at position −449 in the promoter region of the DDAH 2 gene is associated with higher ADMA levels.

Further studies are required to determine the pathophysiological significance of elevated serum ADMA in HD patients and to better understand how DDAH gene variation influences ADMA levels. And we propose that a polymorphism at position −449 in the DDAH 2 may be functional and that it has the potential to be used as a marker for susceptibility to cardiovascular events among...
hypertensive hemodialyzed patients.

7. Limitations

This study has some limitations. The number of patients was relatively small, we used only one marker of oxidative stress and we did not evaluate other factors potentially involved in endothelial dysfunction. Furthermore, our study was limited by its cross-sectional design, allowing no causal interferences.

REFERENCES


The document provides a detailed list of references, each numbered from [23] to [39]. The references cover a range of topics related to hypertension, cardiovascular disease, and the role of dimethylarginine dimethylaminohydrolase (DDAH) in regulating nitric oxide (NO) production. The references include studies on the effects of DDAH on blood pressure, endothelial function, and the mechanisms of DDAH action in various diseases such as atherosclerosis, thrombosis, and vascular biology. The text also includes a discussion on the therapeutic potential of targeting endogenous inhibitors of NO synthesis and the roles of oxidative stress and retinoic acid in modulating NO production. The references are from reputable journals such as Arteriosclerosis, Thrombosis, and Vascular Biology, Circulation, and Nature Reviews Drug Discovery. The authors of the references include M. Yusuf, H. Dayoub, J. Leiper, A. Tojo, M. Feng, H. Dhall, J. Achan, F. Perticone, J. Santa Maria, J. Leiper, N. Dhalla, W. Welch, M. Omata, K. Sudaoka, M. E. Widlansky, T. Ogawa, C. Smith, A. Ito, R. H. Böger, and others.