Association of Genetic Polymorphisms of Renin–Angiotensin–Aldosterone System-Related Genes with Arterio-Venous Fistula Malfunction in Hemodialysis Patients

Yu-Wei Chen 1,2,3,4,†, Yu-Te Wu 3,4,†, Jhin-Shyaun Lin 4, Wu-Chang Yang 3,4, Yung-Ho Hsu 1,2, Kuo-Hao Lee 3,4, Shou-Ming Ou 3,4, Yung-Tai Chen 5, Chia-Jen Shih 6, Pui-Ching Lee 7, Chia-Hao Chan 3,4, Ming-Yi Chung 8,9,* and Chih-Ching Lin 3,4,*‡

1 Division of Nephrology, Department of Internal Medicine, Shuang-Ho Hospital, Taipei Medical University, New Taipei 235, Taiwan; b101091063@tmu.edu.tw (Y.-W.C.); yhhsu@tmu.edu.tw (Y.-H.H.)
2 Department of Internal Medicine, School of Medicine, College of Medicine, Taipei Medical University, Taipei 110, Taiwan
3 Division of Nephrology, Department of Medicine, Taipei Veterans General Hospital, Taipei 112, Taiwan; wyt0406@gmail.com (Y.-T.W.); wcyang@vghtpe.gov.tw (W.-C.Y.); dadabim3520@gmail.com (K.-H.L.); okokyytt@gmail.com (S.-M.O.); box241029@yahoo.com.tw (C.-H.C.)
4 School of Medicine, National Yang-Ming University, Taipei 112, Taiwan; jhshlin0521@gmail.com
5 Division of Nephrology, Department of Medicine, Taipei City Hospital, He-Ping Branch, Taipei 100, Taiwan; ytchen0117@gmail.com
6 Division of Nephrology, Department of Medicine, Yuan-Shan Branch, Taipei Veterans General Hospital, I-Lan 264, Taiwan; b001089010@tmu.edu.tw
7 Department of Medicine, Taipei Veterans General Hospital, Taipei 112, Taiwan; pclee@vghtpe.gov.tw
8 Institute of Genome Sciences, National Yang-Ming University, Taipei 112, Taiwan
9 Department of Medical Research, Taipei Veterans General Hospital, Taipei 112, Taiwan
* Correspondence: lincc2@vghtpe.gov.tw (C.-C.L); mychung@ym.edu.tw (M.-Y.C.); Tel.: +886-2-287-121-21 (ext. 2993) (C.-C.L); +886-2-287-121-21 (ext. 3265) (M.-Y.C.)
† These authors contributed equally to this work as first authors.
‡ These authors contributed equally to this work as corresponding authors.

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Abstract: Hemodialysis (HD) is the most commonly-used renal replacement therapy for patients with end-stage renal disease worldwide. Arterio-venous fistula (AVF) is the vascular access of choice for HD patients with lowest risk of infection and thrombosis. In addition to environmental factors, genetic factors may also contribute to malfunction of AVF. Previous studies have demonstrated the effect of genotype polymorphisms of angiotensin converting enzyme on vascular access malfunction. We conducted a multicenter, cross-sectional study to evaluate the association between genetic polymorphisms of renin-angiotensin-aldosterone system and AVF malfunction. Totally, 577 patients were enrolled. Their mean age was 60 years old and 53% were male. HD patients with AVF malfunction had longer duration of HD (92.5 ± 68.1 vs. 61.2 ± 51.9 months, p < 0.001), lower prevalence of hypertension (44.8% vs. 55.3%, p = 0.025), right-sided (31.8% vs. 18.4%, p = 0.002) and upper arm AVF (26.6% vs. 9.7%, p < 0.001), and higher mean dynamic venous pressure (DVP) (147.8 ± 28.3 vs. 139.8 ± 30.0, p = 0.021). In subgroup analysis of different genders, location of AVF and DVP remained significant clinical risk factors of AVF malfunction in univariate and multivariate binary logistic regression in female HD patients. Among male HD patients, univariate binary logistic regression analysis revealed that right-side AVF and upper arm location are two important clinical risk factors. In addition, two single nucleotide polymorphisms (SNPs), rs275653 (Odds ratio 1.90, p = 0.038) and rs1492099 (Odds ratio 2.29, p = 0.017) of angiotensin II receptor 1 (AGTR1), were associated with increased risk of AVF malfunction. After adjustment for age and other clinical factors, minor allele-containing genotype polymorphisms (AA and CA) of rs1492099 still remained to be a
significant risk factor of AVF malfunction (Odds ratio 3.63, \( p = 0.005 \)). In conclusion, we demonstrated that rs1492099, a SNP of \( AGTR1 \) gene, could be a potential genetic risk factor of AVF malfunction in male HD patients.

**Keywords:** hemodialysis; arteriovenous fistula; thrombosis; angiotensin receptor gene; single nucleotide polymorphism

1. Introduction

Patent and reliable vascular access is of vital importance to end stage renal disease (ESRD) patients who receive hemodialysis [1] as their choice of renal replacement therapy (RRT). Arterio-venous fistula (AVF), in comparison with arterio-venous graft (AVG) and tunneled dialysis catheter (TDC), is the best vascular access in terms of higher blood flow rate, lower infection rate, and longer lifespan [2]. The thrombosis and occlusion of AVFs diminish the dialysis adequacy and increase the morbidity and mortality of HD patients [3]. Neo-intimal hyperplasia (NIH) causes the maturation failure of AVF, while the recurrent thrombosis compromises the patency in the long-term. Excessive strength and duration applied on puncture site, insufficient anticoagulation use and infection of vascular access all contribute to the recurrent thrombosis of AVF. Multiple studies had been conducted to define the genetic risk factors of AVF thrombosis [3–5]. Coagulation-related genes, hyperhomocysteinemia-related methylene tetrahydrofolate reductase (\( MTHFR \)) gene, transforming growth factor-b1 (\( TGFB1 \)), matrix metalloproteinases (MMPs), hypoxia inducible factor-1\( \alpha \) (\( HIF1A \)), heme oxygenase-1 (\( HO1 \)) and vascular endothelial growth factor-A (\( VEGFA \)) and its receptors (\( FLT1 \) for VEGFR-1 and \( KDR \) for VEGFR-2) have all been studies for their association with AVF stenosis or thrombosis.

The renin-angiotensin-aldosterone system plays an important role in the regulation of blood pressure and homeostasis of body fluid. The impact of genetic polymorphism of ACE on AVF thrombosis has been studied but contradictory results had been found [6–11]. Fewer studies focused on the role of angiotensin II receptor 1/2 (\( AGTR1/2 \)) in the pathogenesis of AVF thrombosis. The aim of this study was to conduct a case-control study to discover whether single nucleotide polymorphism (SNP) of renin-angiotensin-aldosterone system (RAAS) genes (including Angiotensinogen (\( AGT \)), Angiotensin converting enzyme gene (\( ACE \)), angiotensin II receptor 1 (\( AGTR1 \)) and 2 (\( AGTR2 \)) could be genetic risk factors of AVF thrombosis.

2. Results

2.1. Patient Characteristics

Totally, 577 ESRD patients were enrolled into our study. The demographic and clinical characteristics of study subjects are shown in Table 1. Among them, 154 patients had AVF malfunction and the other 423 patients were included as control group. The mean age of the AVF malfunction patients and control group patients was 60.7 ± 16.1 and 59.8 ± 14.0 years old, respectively (\( p = 0.517 \)). There was no difference in the proportion of gender between the two groups: 55.2% of AVF malfunction patients were male and 52.2% of control group were male (\( p = 0.531 \)). Vintage of HD was significantly longer in AVF malfunction group than in control group (92.5 ± 68.1 versus 61.2 ± 51.9 months, \( p < 0.001 \)). No significant difference between patients with and without AVF malfunction in the frequency of smoking was observed (11.7% vs. 9.5%, \( p = 0.432 \)). As to the comorbidity, the prevalence of diabetes mellitus, cerebrovascular accident, peripheral arterial disease and coronary artery disease did not differ between patients with AVF malfunction and control group (Table 1). However, the prevalence of hypertension in AVF malfunction group was significantly lower (44.8% versus 55.3%, \( p = 0.025 \)). As to the hemodialysis-related parameters, ESRD patients with AVF malfunction had significantly higher average dynamic venous pressure (DVP) than control group (147.8 ± 28.3 versus 139.8 ± 30 mmHg,
However, there was no significant difference between patients with and without AVF malfunction in pre-HD mean arterial pressure (104.8 ± 17.6 vs. 109.7 ± 19.1 mmHg, \( p = 0.109 \)) and post-HD mean arterial pressure (92.8 ± 14.4 vs. 96.6 ± 15.1 mmHg, \( p = 0.184 \)). Delivered dialysis dosage was similar between two groups of patients, in terms of Kt/V and urea reduction rate (URR) (Table 1).

### Table 1. Clinical characteristics of Hemodialysis (HD) patients by status of Arterio-venous fistula (AVF) malfunction.

| Characteristic                              | Malfunction \((n = 154)\) | No Malfunction \((n = 423)\) | \( p \) Value |
|--------------------------------------------|-----------------------------|-------------------------------|---------------|
| Age (years)                                | 60.7 ± 16.1                 | 59.8 ± 14.0                   | 0.517         |
| Gender (%)                                 |                              |                               |               |
| Male                                       | 55.2                        | 52.2                          | 0.531         |
| Female                                     | 44.8                        | 47.8                          |               |
| HD duration (months)                        | 92.5 ± 68.1                 | 61.2 ± 51.9                   | <0.001        |
| Smoking (%)                                | 11.7                        | 9.5%                          | 0.432         |
| Hypertension (%)                           | 44.8                        | 55.3                          | 0.025         |
| Diabetes mellitus (%)                      | 26.1                        | 31.9                          | 0.173         |
| Cerebrovascular accidents (%)              | 11.0                        | 7.3                           | 0.192         |
| Peripheral arterial disease (%)            | 5.2                         | 4.0                           | 0.540         |
| Coronary artery disease (%)                | 23.0                        | 20.2                          | 0.462         |
| ACE inhibitor (%)                          | 9.7                         | 11.1                          | 0.643         |
| ARB (angiotensin II receptor blocker) (%)  | 14.9                        | 18.0                          | 0.391         |
| Site of AVF (%)                            |                              |                               |               |
| Right side                                 | 31.8                        | 18.4                          | 0.002         |
| Left side                                  | 66.2                        | 81.6                          |               |
| Location of AVF (%)                        |                              |                               |               |
| Forearm                                    | 73.4                        | 90.3                          | <0.001        |
| Upper arm                                  | 26.6                        | 9.7                           |               |
| Dynamic venous pressure (mmHg) under pump flow at 250 mL/min | 147.8 ± 28.3 | 139.8 ± 30.0 | 0.021 |
| Pre-dialytic mean arterial blood pressure (mmHg) | 104.8 ± 17.6 | 109.7 ± 19.1 | 0.109 |
| Post-dialytic mean arterial blood pressure (mmHg) | 92.8 ± 14.4 | 96.6 ± 15.1 | 0.184 |
| Kt/V                                       | 1.38 ± 0.13                 | 1.49 ± 0.15                   | 0.113         |
| URR (urea reduction rate) (%)              | 73 ± 4.9                    | 76 ± 5.2                      | 0.124         |
| Maximal pump flow (mL/min)                 | 268.6 ± 29.3                | 274.5 ± 36.3                  | 0.106         |

SNPs of RAAS-related genes studied in our study are listed in Table 2. All SNPs tested in our study were within Hardy-Weinburg equilibrium.

### 2.2. Univariate Analysis of the Risk Factor of AVF Malfunction

#### 2.2.1. Clinical and Genetic Risk Factor of AVF Malfunction in all Study Subjects

We included each clinical and demographic characteristic and genotype of each SNP for univariate analysis. Patients with two major alleles are grouped as control and patients with one or two minor alleles are grouped as risk group. Logistic regression was performed and the results are expressed as odds ratio with 95% confidence interval and \( p \) value is also listed in Table 3. Right-sided AVF (Odds ratio (OR) 2.064, \( p = 0.001 \)), upper arm location of AVF (OR 3.381, \( p < 0.001 \)) and increasing dynamic venous pressure (OR 1.011 for each increment of 1 mmHg, \( p < 0.001 \)) were statistically significant risk factors for AVF malfunction. Hypertension, in contrast, was a significant protective factor of AVF malfunction (OR 0.656, \( p = 0.026 \)). None of the SNPs of AGT, ACE, AGTR1 and AGTR2 genes were identified as significant risk factor of AVF malfunction. Estrogen has been widely known to possess protective effects on cardiovascular system [12], thus we divided the study subjects according to different genders for further analysis of possible clinical impact of SNPs.
Table 2. Primer sequences and PCR conditions for amplification of polymorphisms within renin-angiotensin-aldosterone system-related genes.

| Gene       | SNP Name Position | Chromosome Position | Genotyping Method | Primer Forward | Sequence (5’ to 3’) | Allele |
|------------|-------------------|---------------------|-------------------|----------------|---------------------|--------|
| AGT        | rs7079 exon5 (c. +1866) | chr1:230838331 | TaqMan Allelic discrimination | Forward | TGAAGATGCAAGCACCCTGAA | C/A * |
| AGT        | rs11568056 Intron (IVS2-551) | chr1:230842497 | RFLP-PCR | Forward Reverse | GACAGCTGGTGGGCTCTG | G/A * |
| AGT        | rs6687360 Intron (IVS2+749) | chr1:230844992 | RFLP-PCR | Forward Reverse | (6Fam)-GTGCCATATTGCTCAGTCAGTTCACACAGTACAGTC | T/C * |
| AGT        | rs4762 Non-synonymous 207 T>M Exon2, c. +620 | chr1:230845977 | RFLP-PCR | Forward Reverse | (6Fam)-CTACAGGCAATCTGGTGTTAGGCTGACTGGCTGCTTACAGTACAGTC | C/T * |
| AGT        | rs11568028 Intron (IVS1-645) | chr1:230847244 | RFLP-PCR | Forward Reverse | (6Fam)-GGACCACAGGGAGAGCAAAATGAGCCATGAGGTGA | G/A * |
| AGT        | rs3789678 Intron (IVS1+350) | chr1:230849482 | RFLP-PCR | Forward Reverse | (6Fam)-GCCACATGCTGGCTGCTTCATACACAGTGCTGCTTACAGTACAGTC | C/T * |
| AGT        | rs5051 5’-UTR (Exon 1, g. +172, c. -41) -6A>G | chr1:230849872 | RFLP-PCR | Forward Reverse | (6Fam)-GCCACACAGGGAGAGCAAAATGAGCCATGAGGTGA | T/C * |
| ACE        | rs4295 Intron (IVS2-70) | chr17:61556298 | RFLP-PCR | Forward Reverse | (6Fam)-CTGACCCCTACTCTCGCCACAGCACCTACACAACTCTGCAC | C/G * |
| ACE        | rs4340 Intron (Intron 16) | chr17:61568893 | Fragment length analysis | Forward Reverse | (6Fam)-GTAAGCCACTGTGAGAGGTGCACCCCTTTGCTGCTTACAGTACAGTC | I/D * |
| ACE        | rs10853044 3’-UTR (Exon17, c. +5150) | chr17:61586549 | RFLP-PCR | Forward Reverse | (6Fam)-CAGGACACAGACAGGCAAGAGAAGAGAGAGAGAAGAGAAG | T/C * |
## Table 2. Cont.

| Gene      | SNP Name Position | Chromosome Position | Genotyping Method | Primer | Sequence (5' to 3') | Allele |
|-----------|-------------------|---------------------|-------------------|--------|---------------------|--------|
| AGTR1     | rs409742 Upstream (g. –3294) | chr3:148412365 | RFLP-PCR | Forward Reverse | TTCCCACCAACAAATATATGAGG (6Fam)-AAAGTAGGAGTAAACCTTGGTACC | T/C * |
|           | rs275653 Promotor (g. –113) –153A>G | chr3:148415545 | TaqMan Allelic discrimination | Forward Reverse | TGAACCGCTGATCTGATAGTGGACA ACGAGGCTCTGGTTGCTATTC | A/G * |
|           | rs10935724 Intron (IVS1-4477) | chr3:148421253 | RFLP-PCR | Forward Reverse | CATTTTAGCAAAATCCCTGAGGT (6Fam)-CAAGCTTTTGGTAAACTACTTATTC | A/C * |
|           | rs1492099 Intron (IVS2+1169) | chr3:148437503 | RFLP-PCR | Forward Reverse | (6Fam)-GCCCTTGCTCTGGCTCAGGGT AACTTTAAATGGTTTACTGGAA ATTCATCATCTCAGGACACAC | G/A * |
|           | rs385338 Intron (IVS2-9620) | chr3:148449156 | RFLP-PCR | Forward Reverse | (6Fam)-TTCTTTTTGGACAGCACGTAATGCAAGGCTGTAAGTAAATGAA | C/G * |
|           | rs5182 Synonymous coding (Exon3, c. +573) | chr3:148459395 | RFLP-PCR | Forward Reverse | (6Fam)-CATCATCATATGGCTGCTG ACAGGCTCAAAATTCAGGAAATTC | T/C * |
|           | rs5186 3'-UTR (Exon3, c. 1166) 1166A>G | chr3:148459988 | RFLP-PCR | Forward Reverse | GAGAACATCTTCTGAGCAACACGACGTCGTCG GAACACATGGCTGCTGCTG | A/C * |
|           | rs1403543 Intron (IVS1-29, g. 218) 1675A>G | chrX:115302192 | TaqMan Allelic discrimination | Forward Reverse | GCAGCCTGAATTTTAAGGGT TCCACCTGAGCTATCTGCTGTTT | A/G * |
|           | rs11091046 3'-UTR (Exon 3, c. +1593) 3123A>C | chrX:115305126 | RFLP-PCR | Forward Reverse | (6Fam)-CATTGCATCTTACAAGACACGACGACTTACGTTGCTG | C/A * |
|           | rs12840631 Downstream (g. +4317) | chrX:115306351 | TaqMan Allelic discrimination | Forward Reverse | TGATATCTCCCATTTTATCTCCACCTGAA AGCTAATGGGAAATATGGGT | C/G * |

* Minor allele, also as risk allele in statistical analysis.
Table 3. Univariate logistic regression model of factors associated with AVF malfunction in all HD patients.

| Clinical or Genetic Factors                      | Odds Ratio | 95% CI Lower | 95% CI Upper | Significance |
|------------------------------------------------|------------|--------------|--------------|--------------|
| Age (year)                                      | 1.005      | 0.992        | 1.018        | 0.488        |
| Right side vs. left side                        | 2.064      | 1.358        | 3.138        | 0.001 *      |
| Upper arm vs. forearm                           | 3.381      | 2.090        | 5.469        | <0.001 *     |
| Hypertension                                    | 0.656      | 0.452        | 0.950        | 0.026 *      |
| Diabetes mellitus                               | 0.755      | 0.499        | 1.143        | 0.184        |
| Coronary artery disease                         | 1.183      | 0.757        | 1.848        | 0.462        |
| Peripheral artery disease                       | 1.309      | 0.553        | 3.097        | 0.541        |
| Cerebrovascular accident                        | 1.569      | 0.842        | 2.925        | 0.156        |
| Dynamic venous pressure (mmHg)                  | 1.011      | 1.005        | 1.018        | <0.001 *     |

**AGT**

| rs7079                                           | 1.781      | 0.623        | 5.093        | 0.282        |
| rs11568056                                       | 1.003      | 0.649        | 1.548        | 0.990        |
| rs6687360                                        | 1.205      | 0.824        | 1.761        | 0.336        |
| rs4762                                           | 0.660      | 0.398        | 1.092        | 0.106        |
| rs11568028                                       | 0.854      | 0.570        | 1.278        | 0.442        |
| rs3789678                                        | 1.012      | 0.638        | 1.605        | 0.960        |
| rs5051                                           | 0.922      | 0.609        | 1.398        | 0.703        |

**ACE**

| rs4295                                          | 1.303      | 0.833        | 2.036        | 0.246        |
| rs4340                                          | 1.369      | 0.937        | 2.001        | 0.105        |
| rs10853044                                      | 1.138      | 0.690        | 1.876        | 0.612        |

**AGTR1**

| rs409742                                        | 1.233      | 0.727        | 2.093        | 0.437        |
| rs275653                                        | 1.393      | 0.881        | 2.030        | 0.157        |
| rs10935724                                      | 0.902      | 0.596        | 1.366        | 0.626        |
| rs1492099                                       | 1.437      | 0.846        | 2.441        | 0.180        |
| rs385338                                        | 1.119      | 0.743        | 1.686        | 0.591        |
| rs5182                                          | 1.285      | 0.852        | 1.937        | 0.231        |
| rs5186                                          | 0.729      | 0.374        | 1.421        | 0.353        |

**AGTR2**

| rs1403543                                       | 1.014      | 0.699        | 1.472        | 0.940        |
| rs11091046                                      | 0.970      | 0.652        | 1.444        | 0.882        |
| rs12840631                                      | 1.105      | 0.726        | 1.684        | 0.641        |

*AGT*, Angiotensinogen; *ACE*, Angiotensinogen-converting enzyme; *AGTR1*, Angiotensin II receptor, type 1; *AGTR2*, Angiotensin II receptor, type 2; *p* < 0.05.

2.2.2. Clinical and Genetic Risk Factor of AVF Malfunction in Female Study Subjects

Upper arm location of AVF (Odds ratio (OR) 2.690, *p* = 0.004) and increasing dynamic venous pressure (OR 1.017 for each increment of 1 mmHg, *p* = 0.001) were the only two risk factors for AVF malfunction in female HD patients (Table 4). Laterality of AVF was not a significant factor for AVF malfunction in female HD patients (OR 0.766, *p* = 0.411). Hypertension seemed to have protective effects on AVF, but there is no statistical significance (OR 0.606, *p* = 0.077). None of the SNPs of *AGT, ACE, AGTR1* and *AGTR2* genes is identified as significant risk factor of AVF malfunction in female HD patients.
Table 4. Univariate logistic regression model of factors associated with AVF malfunction in female HD patients.

| Clinical or Genetic Factors          | Odds Ratio | 95% CI Lower | 95% CI Upper | Significance |
|-------------------------------------|------------|--------------|--------------|--------------|
| Age (year)                          | 0.991      | 0.972        | 1.011        | 0.386        |
| Right side vs. left side            | 0.766      | 0.406        | 1.445        | 0.411        |
| Upper arm vs. forearm               | 2.690      | 1.371        | 5.279        | 0.004 *      |
| Hypertension                        | 0.606      | 0.348        | 1.055        | 0.077        |
| Diabetes mellitus                   | 0.664      | 0.342        | 1.287        | 0.225        |
| Coronary artery disease             | 0.933      | 0.455        | 1.912        | 0.850        |
| Peripheral artery disease           | 0.832      | 0.169        | 4.101        | 0.821        |
| Cerebrovascular accident            | 1.500      | 0.494        | 4.552        | 0.474        |
| Dynamic venous pressure (mmHg)      | 1.017      | 1.007        | 1.027        | 0.001 *      |

For **AGT**

| SNP                      | Odds Ratio | 95% CI Lower | 95% CI Upper | Significance |
|--------------------------|------------|--------------|--------------|--------------|
| rs7079                   | 0.981      | 0.523        | 1.840        | 0.952        |
| rs11568056               | 0.835      | 0.429        | 1.625        | 0.595        |
| rs6687360                | 0.867      | 0.499        | 1.509        | 0.615        |
| rs4762                   | 0.510      | 0.234        | 1.110        | 0.090        |
| rs11568028               | 0.766      | 0.413        | 1.423        | 0.399        |
| rs3789678                | 1.310      | 0.662        | 2.590        | 0.438        |
| rs5051                   | 1.011      | 0.560        | 1.825        | 0.972        |

For **ACE**

| SNP                      | Odds Ratio | 95% CI Lower | 95% CI Upper | Significance |
|--------------------------|------------|--------------|--------------|--------------|
| rs4295                   | 1.095      | 0.558        | 2.150        | 0.792        |
| rs4340                   | 1.401      | 0.805        | 2.439        | 0.233        |
| rs10853044               | 1.520      | 0.723        | 3.197        | 0.269        |

For **AGTR1**

| SNP                      | Odds Ratio | 95% CI Lower | 95% CI Upper | Significance |
|--------------------------|------------|--------------|--------------|--------------|
| rs409742                 | 0.856      | 0.366        | 2.003        | 0.720        |
| rs275653                 | 0.933      | 0.455        | 1.912        | 0.850        |
| rs10935724               | 1.104      | 0.584        | 2.087        | 0.761        |
| rs1492099                | 0.680      | 0.267        | 1.735        | 0.420        |
| rs385338                 | 1.204      | 0.669        | 2.167        | 0.535        |
| rs5182                   | 0.952      | 0.510        | 1.777        | 0.878        |
| rs5186                   | 0.483      | 0.179        | 1.304        | 0.151        |

For **AGTR2**

| SNP                      | Odds Ratio | 95% CI Lower | 95% CI Upper | Significance |
|--------------------------|------------|--------------|--------------|--------------|
| rs1403543                | 0.982      | 0.546        | 1.768        | 0.953        |
| rs11091046               | 0.871      | 0.474        | 1.600        | 0.656        |
| rs12840631               | 1.136      | 0.641        | 2.013        | 0.661        |

AGT, Angiotensinogen; ACE, Angiotensinogen-converting enzyme; AGTR1, Angiotensin II receptor, type 1; AGTR2, Angiotensin II receptor, type 2; *p < 0.05.

2.2.3. Clinical and Genetic Risk Factor of AVF Malfunction in Male Study Subjects

Among male HD patients in current study, right-sided AVF (OR 3.051, p < 0.001) and upper arm location of AVF (OR 4.474, p < 0.001) were two risk factors for AVF malfunction. Protective effect of HTN was not observed in male subgroup. Increasing dynamic venous pressure seemed to increase the risk of AVF malfunction, with borderline statistical significance (OR 1.008 for each increment of 1 mmHg, p = 0.061). Two SNPs in AGTR1 gene probably had adverse impacts on AVF patency. The first one is rs275653 (Promotor (g. –113), –153A>G) and male HD patients with minor allele (AG or GG) had higher risk of AVF malfunction than those with major alleles only (GG genotype) (OR 1.900, p = 0.038). Another potential genetic risk factor of AVF malfunction is rs1492099 (Intron (IVS2 + 11689)). Male HD patients having minor allele (CA or AA genotype) were associated with higher prevalence of AVF malfunction than those carrying two major alleles (CC genotype) (Table 5). None of SNPs in AGT gene, ACE gene or AGTR2 gene was significantly associated with AVF malfunction. However, AG or GG genotype of rs6687360 (Intron(IVS2+749)) of AGT gene and TC or CC genotype of rs5182...
(Synonymous coding(Exon3, c. +573)) had potential effects on AVF patency; thus, these two SNPs were also included in the following multivariate analysis (OR 1.615, \( p = 0.076 \) and OR 1.623, \( p = 0.084 \), respectively, Table 5).

**Table 5.** Univariate logistic regression model of factors associated with AVF malfunction in male HD patients.

| Clinical or Genetic Factors                           | Odds Ratio | 95% CI Lower | 95% CI Upper | Significance |
|-------------------------------------------------------|------------|--------------|--------------|--------------|
| Age (year)                                            | 1.014      | 0.997        | 1.031        | 0.115        |
| Right side vs. left side                              | 3.051      | 1.724        | 5.397        | <0.0001 *    |
| Upper arm vs. forearm                                 | 4.474      | 2.215        | 9.038        | <0.0001 *    |
| Hypertension                                          | 1.450      | 0.878        | 2.396        | 0.147        |
| Diabetes mellitus                                     | 0.806      | 0.470        | 1.380        | 0.432        |
| Coronary artery disease                               | 1.374      | 0.771        | 2.447        | 0.281        |
| Peripheral artery disease                             | 1.603      | 0.564        | 4.555        | 0.376        |
| Cerebrovascular accident                              | 1.566      | 0.733        | 3.342        | 0.247        |
| Dynamic venous pressure (mmHg)                        | 1.008      | 1.000        | 1.017        | 0.061        |
| **AGT**                                               |            |              |              |              |
| rs7079                                                | 0.984      | 0.547        | 1.768        | 0.956        |
| rs11568056                                           | 1.185      | 0.668        | 2.107        | 0.563        |
| rs6687360                                             | 1.615      | 0.951        | 2.744        | 0.076        |
| rs4762                                                | 0.817      | 0.418        | 1.594        | 0.552        |
| rs11568028                                           | 0.942      | 0.552        | 1.608        | 0.827        |
| rs3789678                                            | 0.809      | 0.431        | 1.515        | 0.507        |
| rs5051                                                | 0.850      | 0.472        | 1.530        | 0.588        |
| **ACE**                                               |            |              |              |              |
| rs4295                                                | 1.459      | 0.799        | 2.663        | 0.219        |
| rs4340                                                | 1.320      | 0.780        | 2.233        | 0.301        |
| rs10853044                                           | 0.902      | 0.456        | 1.785        | 0.768        |
| **AGTR1**                                             |            |              |              |              |
| rs409742                                              | 1.584      | 0.795        | 3.156        | 0.191        |
| rs275653                                              | 1.900      | 1.035        | 3.489        | 0.038 *      |
| rs10935724                                            | 0.785      | 0.452        | 1.361        | 0.388        |
| rs1492099                                            | 2.287      | 1.160        | 4.509        | 0.017 *      |
| rs385338                                              | 1.053      | 0.592        | 1.872        | 0.860        |
| rs5182                                                | 1.623      | 0.938        | 2.808        | 0.084        |
| rs5186                                                | 1.159      | 0.459        | 2.926        | 0.735        |
| **AGTR2**                                             |            |              |              |              |
| rs1403543                                             | 1.099      | 0.661        | 1.826        | 0.717        |
| rs11091046                                            | 1.133      | 0.654        | 1.964        | 0.636        |
| rs12840631                                            | 1.145      | 0.598        | 2.191        | 0.683        |

*AGT*, Angiotensinogen; *ACE*, Angiotensinogen-converting enzyme; *AGTR1*, Angiotensin II receptor, type 1; *AGTR2*, Angiotensin II receptor, type 2; *p < 0.05*.

2.3. **Multivariate Analysis of Risk Factors of AVF Malfunction in Male HD Patients**

To define the risk factor of AVF malfunction in male HD patients, we included clinical and genetic parameters with stronger association with AVF malfunction, i.e., \( p < 0.200 \) in univariate binary logistic regression, and perform multivariate Cox-regression analysis. Forward likelihood ratio test was applied to construct the model. Age, hypertension, laterality of AVF, upper arm/forearm location of AVF, dynamic venous pressure, rs275653, rs1492099, rs6687360, rs5182 and rs409742 are included into analysis. As shown in Table 6, right-sided AVF (OR 3.611, \( p = 0.001 \)) and upper arm location of AVF (OR 3.384, \( p = 0.003 \)) and CA or AA genotype of rs1492099 (OR 3.355, \( p = 0.008 \)) remain significantly associated with AVF malfunction after adjustment.
Table 6. Multivariate logistic regression model of factors associated with AVF malfunction in male HD patients.

| Clinical or Genetic Factors | Significance | Odds Ratio | 95% CI Lower | 95% CI Upper |
|-----------------------------|--------------|------------|--------------|--------------|
| Right side vs. left side    | 0.001 *      | 3.559      | 1.709        | 7.412        |
| Upper arm vs. forearm       | 0.003 *      | 3.837      | 1.590        | 9.258        |
| \textit{AGTR}1 \textit{rs1492099 CA+AA vs. CC} | 0.005 *      | 3.632      | 1.469        | 8.982        |
| Age                         | 0.543        | 1.007      | 0.985        | 1.029        |
| Hypertension                | 0.313        | 0.702      | 0.353        | 1.395        |
| Dynamic venous pressure     | 0.473        | 1.004      | 0.994        | 1.014        |
| \textit{AGT} rs6687360      | 0.065        | 1.885      | 0.962        | 3.692        |
| \textit{AGTR}1 \textit{rs409742} | 0.522        | 1.500      | 0.433        | 5.195        |
| \textit{AGTR}1 \textit{rs275653} | 0.913        | 0.917      | 0.195        | 4.307        |
| \textit{AGTR}1 \textit{rs5182} | 0.712        | 1.148      | 0.551        | 2.391        |

\textit{AGT}, Angiotensinogen; \textit{ACE}, Angiotensinogen-converting enzyme; \textit{AGTR}1, Angiotensin II receptor, type 1; \textit{AGTR}2, Angiotensin II receptor, type 2 * \( p < 0.05 \).

3. Discussion

3.1. AVF Malfunction, HD Characteristics and Comorbidities

The longer duration of hemodialysis represents much more total needle puncture times. Repeated injury to AVF may induce local inflammation, thrombosis and fibrotic change or stenosis. In our study, hemodialysis patients with AVF malfunction have significantly longer HD duration than control group. The finding is compatible with aforementioned pathophysiology of development of AVF malfunction. In our study population, most of the patients, like other cultural groups in the world, are right-handed. Cardiovascular surgeons tend to establish AVF anastomosis on the forearm of non-dominant upper extremity, \textit{i.e.}, left forearm. HD patients who had their AVF on dominant side and upper arm might have worse vascular condition [13].

In our study, HD patients without AVF malfunction have significantly higher prevalence of HTN (Table 1). Hypertension is also found to be a protective factor for AVF malfunction in univariate analysis of all study subjects (Table 3). Hemodialysis patients with lower blood pressure may be prone to develop intradialytic hypotension, lower blood flow rate and thus may result in clotting within dialyzer or vascular access. Therefore, HD patients who have HTN may probably benefit from a higher blood pressure, whether during or between hemodialysis sessions. However, protective effects of HTN are not significant among male HD patients. One possible explanation is that the male patients tended to exercise their upper limbs more often, whether in their labor work or recreational activities. Larger muscle girdle may potentially interfere with the measurements of blood pressure.

Compared with HD patients without AVF malfunction, HD patients with AVF malfunction have higher average dynamic venous pressure (Table 1). Higher dynamic venous pressure implicates hindrance of venous return, such as thrombosis or stenosis. Thrombosis and stenosis are both the major mechanical causes of AVF malfunction.

3.2. Genetic Risk Factors of AVF Malfunction

Various studies have been conducted to delineate the association between genetic polymorphisms and arteriovenous fistula patency. In our previous study, length polymorphism of dinucleotide guanosine thymine repeat (GT)\(n\) in the promoter region of heme oxygenase-1 (\textit{HO1}) was found to be associated with AVF patency. Longer GT repeat in promoter region of \textit{HO1} may hinder the transcription of the gene, and eventually attenuate the protective effect of heme oxygenase-1 on AVF [5]. Recent publication from our group also discovered that SNP of protein arginine methyltransferase 1 (\textit{PRMT1}) gene has statistically significant association with primary patency, assisted primary patency and secondary patency of AVF in male HD patients [14].
Clinical implication of genetic polymorphism of ACE gene, especially ACE insertion/deletion (ACE I/D), has been extensively studied [6–11]. On microvascular scale, Hadjadj et al. [9] conducted a large, family-based, case-control study in 3 different European populations and they discovered that five ACE polymorphisms (rs1800764-C, rs4311-T, Insertion/deletion (I/D or rs1799752)-D, rs4366-G, and rs12449782-G alleles) had statistically significant association with increased risk of diabetic nephropathy. However, contradictory results were shown by different studies and ethnic group and gender may contribute to the disparities of results. In a Taiwanese study completed by Tien et al. [15], female diabetic patients with ACE ID or DD genotype were associated with higher risk of diabetic nephropathy than those with ACE II genotype. On macrovascular scale, ACE polymorphisms have been found to be associated with cerebrovascular accident [16]. However, a recent Spanish case-control study by Domingues-Montanari et al. demonstrated that there was no significant association between ACE polymorphism and ischemic stroke, despite elevated serum level of ACE in ACE DD genotype group [11]. In our study, none of the selected SNPs of ACE gene was associated with AVF malfunction, which was compatible with previous studies [3,6]. In a longitudinal study conducted by Heine et al., 137 HD patients with AVF were enrolled and AVF patency 12 months after creation of AVF were not significantly different among patients with different genotypes (ACE II, ID and DD). Effects of angiotensin converting enzyme inhibitor (ACE-I) and angiotensin receptor blocker (ARB) were evaluated and the results showed ACE-I/ARB had no positive effects on AVF patency [6]. However, a Korean study with similar scale but longer follow-up period had the opposite discoveries [17]. Recently, another smaller study revealed possible association between ACE DD genotype and higher risk of early thrombosis of AVF in HD patients, in comparison with HD patients with ACE II or ID genotype [7].

Genetic polymorphisms of AGTR1 have been found to be closely related to cardiovascular diseases, metabolic disorders and even longevity in previous studies [18–22]. The most widely studied SNP of AGTR1 gene, rs5182 (A1166C) is located on the 3’-UTR of AGTR1 gene exon 3. Recently, a meta-analysis by Liu et al. included 56 studies and 28,952 study subjects and the study found that AGTR1 A1166C was associated with higher risk of hypertension, whether in Asian or Caucasian populations [23]. Significant association between AGTR1 rs5182 and hypertension was also found in Mexican population [19]. Interestingly, previous epidemiology-genomic studies have found the patients with AGTR1 CC genotype were at increased risk of in stent restenosis (ISR) after percutaneous coronary intervention, whether in Caucasian or Asian ethnic groups [24,25]. However, in our study, there was no statistically significant association between rs5182 and AVF malfunction (Tables 3–5). Even it is included into multivariate analysis of male HD patients, it did not appear to be a significant risk factor of AVF malfunction (Table 6). In our study, another 2 SNPs of AGTR1 gene were found to be associated with AVF malfunction: rs275653 and rs1492099. AGTR1 rs275653 was included by the gene association study in Mexican population, it turned out that rs275653 was not associated with hypertension [19]. In a gene association study involving Italian centenarian cohort and Japan validation centenarian cohort, rs275653 C-G-C genotype (minor allele) has been found to be associated with longevity, less AGTR1-expressing peripheral blood neutrophils and lower blood pressure [20]. In our study, patients with minor allele of rs275653 were at increased risk of AVF malfunction, probably due to lower blood pressure. AGTR1 rs1492099 was the other significant risk factor for AVF malfunction. Unfortunately, limited literature revealed the clinical implication of this SNP. Sotoodehnia et al. have conducted a case-control study on adverse cardiovascular effects of SNPs of angiotensin-converting enzyme-related genes [18]. In this study, minor allele of rs1492099 had significant association with decreased risk of sudden cardiac death (OR 0.62, 95% CI 0.4–0.9). In our study, male HD patient with rs1492099 minor allele were at increased risk of AVF malfunction (Tables 5 and 6). The proportion of AVF malfunction in all male HD patients was 26.7%; however, the proportion of AVF malfunction in male HD patients with minor allele of AGTR1 rs1492099 was 43.9%. Although AGTR1 rs1492099 locates on intron of AGTR1 gene, it could possess regulatory effects on transcription of AGTR1, which may cause increased/decreased expression. Altered expression of AGTR1 may cause decreased
systemic expression, which may result in lower prevalence of hypertension. In contrast, minor allele of rs1492099 may result in increased expression of AGTR1, which renders HD patients become more susceptible to angiotensin II, augmented vascular smooth muscle cell proliferation, neointimal hyperplasia and eventually stenosis and malfunction of AVF [26–28]. ACE-I or ARB may be the “double-blade sword.” One possible therapeutic approach is that ACE-I/ARB should be considered as the “first” antihypertensive in HD patients with hypertension, to gain their beneficial effects on inhibition of vascular smooth muscle cell proliferation, meanwhile, to avoid hypotension episodes that may increase the risk of AVF malfunction or thrombosis.

Adverse effects of AGTR1 SNPs were observed only in male HD patients but not in female HD patients. An animal study of estrogen effects on adrenal gland secretion showed that estrogen repressed the expression of AGTR1 and obtunded the aldosterone release stimulated by angiotensin II [29]. Generally speaking, estrogen may lower the AT1 receptor density and increased AT2 receptor density [30]. Therefore, it is possible that the up-regulation effect of SNPs on AGTR1 are attenuated or “overwritten” by hormone effects in female patients.

3.3. Limitations

There are several limitations in our study: First, lack of time dependent variable, such as the prospective follow-up period, makes analysis of causal relationship between clinical/genetic factors and AVF malfunction inapplicable. HD vintage in patients without AVF malfunction is significantly shorter. The actual prevalence of AVF malfunction among control patients when their HD vintage reaches to similar duration as AVF malfunction group is unknown. Second, robust statistical analysis cannot be performed due to relatively small numbers of study subjects. Third, all the study subjects are Taiwanese and the results in our study may not be extrapolated to other ethnic groups. A similar cohort with detailed SNP data in Taiwan may be used to validate our findings, unfortunately, there was no such independent cohort in previously published studies. Fourth, although we advocated the estrogen effect as an interpretation of gender differences on effects of SNPs, further subgroup analysis of premenopausal and menopausal female patients was not available because of limited patient number in our study. Fifth, selection bias may exist in the cross-sectional study design. Sixth, detailed information on medication use of ACE-I or ARB is not available, thus it is difficult to delineate the true clinical effects of angiotensin or angiotensin receptor. Last, but not least important, no blood chemistry data were available to calculate Kt/V or urea reduction rate and no CRP (C-reactive protein) or albumin level data were available to determine the status of inflammation or malnutrition, respectively.

4. Materials and Methods

4.1. Study Subjects

All ESRD patients receiving regular HD therapy at eight different medical institutions during January to March 2008 were screened for eligibility of current study. The eight medical institutions included five local hemodialysis centers, two regional hospitals and one medical center. The inclusion criteria were listed as follows: adult age elder than 18 years old, prevalent ESRD patients who received stable HD (HD therapy for more than 3 months), using native AVF at upper extremities. Definition of AVF malfunction in current study is the loss of functional patency (patent AVF which can tolerate extracorporeal blood flow more than 200 mL/min during dialysis), any angioplasty or surgery to re-establish AVF patency and total occlusion of the AVF. To investigate specifically on AVF thrombosis, patients with AVF malfunction which were attributed to complications other than thrombosis are excluded, i.e., patients with vascular access infection, aneurysm formation with or without surgical ligation, and dialysis access-associated steal syndrome. Patients with AVF maturation failure (AVF malfunction within 3 months after surgery) were also excluded. Informed consent was obtained from each participant of the study; clinical information and blood sample were then collected. We recorded demographic and clinical information of all the patients recruited, including age, gender, HD vintage,
location (upper arm or forearm) and laterality (right or left) of the AVF. Underlying comorbidities such as hypertension (HTN), diabetes mellitus [31], peripheral arterial occlusive disease (PAOD), cerebrovascular disease (CVA), coronary artery disease (CAD) were all recorded. Hemodialysis-related parameters including maximal blood flow rate during HD and dynamic venous return pressure (DVP) were also documented. The conduction of the study was under the surveillance of the Institutional Research Board of Taipei Veterans General Hospital.

4.2. DNA Isolation and Genotyping of Renin-Angiotensin-Aldosterone System-Related Genes

Venous blood samples with anticoagulant were collected from all study subjects and isolation of genomic DNA was done by using Puregene DNA purification kit (Gentra, Minneapolis, MN, USA). Fifty to 100 ng of DNA was amplified into a final volume of 10 µL, which contained 0.5 U HotStar Taq polymerase (Qiagen, Hilden, Germany), 1× solution Q (Qiagen), 10× reaction buffer (Qiagen), MgCl$_2$ (Qiagen), 200 µmol each deoxynucleoside triphosphate (Roche Applied Science, Mannheim, Germany) and 10 µmol of each primer. The protocol of thermal cycler for DNA amplification was the following: 95°C for 12 min, 30 cycles of 95°C for 1 min, 60°C for 30 s, 72°C for 1 min, 72°C for 8 min. Electrophoresis with 2% agarose gel was used for fractionation of amplified DNA products. The DNA bands in each lane were visualized by ultraviolet light and ethidium bromide staining (0.5 mg/mL). Table 1 listed the primer sequences of selected SNP in current study. Direct sequencing (allelic discrimination with TaqMan MGB probe by ABI 7700) and restriction fragment length polymorphism (RFLP) were applied to determine the SNP genotyping. Positive and negative controls were included in all typing analysis.

4.3. Statistical Analysis

We checked Hardy–Weinberg equilibrium for the genotype polymorphisms [32]. Continuous variables were expressed as mean ± SD and Student’s t-test was used to compare differences between groups. Categorical variables were depicted as median with interquartile range and we applied χ-square test to determine differences between groups. Logistic regression was used to define whether each clinical factor or SNP could be the risk factor for AVF malfunction. Multivariate logistic regression model was used for adjustment of confounders and determination of independent risk factors of AVF thrombosis. We performed two-sided tests in statistical analysis and p value less than 0.05 was considered to imply statistical significance. Statistical Package for the Social Sciences (SPSS) version 18.0 (SPSS, Chicago, IL, USA) was applied in statistical analysis.

5. Conclusions

In the current study, rs1492099, a SNP of AGTR1 gene, is an independent risk factor for AVF malfunction, even after adjustment of other candidate SNPs and clinical factors. Further well-designed study with larger scale or related animal study is needed to determine the exact effects of this SNP.

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Author Contributions: Chih-Ching Lin and Ming-Yi Chung conceived and designed the experiments; Jhin-Shyaun Lin and Yu-Wei Chen performed the experiments; Yu-Wei Chen and Yu-Te Wu collected the clinical data; Yu-Wei Chen, Yu-Te Wu, Chih-Ching Lin, Ming-Yi Chung, Kuo-Hua Lee, Yung-Tai Chen, Shou-Ming Ou, Chia-Jen Shih, and Chia-Hao Chan analyzed the data; Chih-Ching Lin, Wu-Chang Yang, Yung-Ho Hsu, Pui-Ching Lee and Ming-Yi Chung contributed reagents, materials, and analysis tools; and Yu-Wei Chen and Chih-Ching Lin wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.
Abbreviations
The following abbreviations are used in this manuscript:

ACE  Angiotensin converting enzyme
ACE-I  Angiotensin converting enzyme inhibitor
AGT  Angiotensinogen
AGTR-1/2  Angiotensin II receptor 1/2
ARB  Angiotensin receptor blocker
AVF  Arterio-venous fistula
CI  Confidence interval
ESRD  End stage renal disease
HTN  Hypertension
HD  Hemodialysis
HIF-1α  Hypoxia inducible factor-1α
HO-1  Heme oxygenase-1
MMPs  Matrix metalloproteinases
MTHFR  Methylene tetrahydrofolate reductase
NIH  Neo-intimal hyperplasia
OR  Odds ratio
PRMT1  Protein arginine methyltransferase 1
RAAS  Renin-angiotensin-aldosterone system
RRT  Renal replacement therapy
SNP  Single nucleotide polymorphism
TGF-β1  Transforming growth factor-β1
VEGF-A  Vascular endothelial growth factor-A
VEGFR-1/2  Vascular endothelial growth factor receptor-1/2

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