

# Polymorphisms of norepinephrine transporter and adrenergic receptor $\alpha_{1D}$ are associated with the response to $\beta$ -blockers in dilated cardiomyopathy

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Recent clinical trials have clearly demonstrated that the administration with  $\beta$ -blockers decreases the mortality in the patients with chronic heart failure (CHF). However, significant heterogeneity exists in the effectiveness of  $\beta$ -blockers among individual cases. We focused on 39 polymorphisms in 16 genes related to adrenergic system and investigated their association with the response to  $\beta$ -blockers among 80 patients with CHF owing to idiopathic dilated cardiomyopathy. The polymorphisms of NET T-182C ( $P=0.019$ ), ADRA1D T1848A ( $P=0.023$ ) and ADRA1D A1905G ( $P=0.029$ ) were associated with the improvement of left ventricular fractional shortening (LVFS) by  $\beta$ -blockers. Furthermore, combined genotype analysis of NET T-182C and ADRA1D T1848A revealed a significant difference in LVFS improvement among genotype groups ( $P=0.011$ ). These results suggest that NET (T-182C) and ADRA1D (T1848A and A1905G) polymorphisms are predictive markers of the response to  $\beta$ -blockers. Genotyping of these polymorphisms may provide clinical insights into an individual difference in the response to the  $\beta$ -blocker therapy in CHF.

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## Introduction

Traditionally,  $\beta$ -blockers used to be contraindicated in chronic heart failure (CHF) because of their negative inotropic effect. However, in the last decade, clinical studies demonstrated that  $\beta$ -blockers substantially improve the prognosis of the patients with CHF.<sup>1,2</sup> Thus the administration with  $\beta$ -blockers is defined to be a standard therapy against CHF. However, one of the difficulties is an inter-individual variation in therapeutic efficacy observed in the clinical use of  $\beta$ -blockers.<sup>3</sup>

To achieve the clinical benefits maximally, great efforts have been made to identify the determinants for the responsiveness to  $\beta$ -blockers in CHF. For example, it has been revealed that the response is influenced by the etiology of CHF.<sup>4</sup> The CHF patients due to dilated cardiomyopathy (DCM) have much improved in cardiac function than those due to ischemic cardiomyopathy. It is

also reported that iodine-123 metaiodo benzyl guanidine (MIBG) image is a predictable marker for the outcome of  $\beta$ -blocker therapy.<sup>5,6</sup> The proportion of the responders is smaller in the patients with impaired MIBG uptake. These findings suggest that the response to  $\beta$ -blockers depends on the pathophysiological status of cardiac remodeling, including the activation of neurohumoral signals. In this context, we hypothesized that adrenergic system-related gene polymorphism would be a candidate predictor for the response.

In the present study, we have selected 39 polymorphisms from 16 genes related to adrenergic system (Table 1). In addition, the association study was performed to identify the gene polymorphisms that would be related with the response to  $\beta$ -blockers in DCM patients. The data presented here might propose a promising strategy for the individualized medicine in the  $\beta$ -blocker therapy for CHF.

## Results

### Polymorphic analysis

Characteristics of 80 of CHF patients in the present study are shown in Table 2. We genotyped 39 polymorphisms and no heterozygosity was observed at three loci, ADRB2 C491T (Thr164Ile), NET intron13 G-1C and DDC G1385A (Gln462Arg), although they were reported to be polymorphic in the databases. The polymorphisms analyzed in the present study were all in Hardy–Weinberg equilibrium with the exception of the polymorphisms in MAOA and MAOB genes. Since MAOA and MAOB genes are located on X chromosome, we did not analyze Hardy–Weinberg equilibrium in the polymorphisms of these genes. We observed that ADRB2 T-1429A, ADRB2 G-1343A, ADRB3 G250C and NET G1287A are in complete linkage disequilibrium with ADRB2 C523A, ADRB2 G-1023A, ADRB3 T190C (Trp64Arg) and NET intron9 G9A, respectively.

### Association analysis

We evaluated the left ventricular fractional shortening (LVFS) in response to  $\beta$ -blocker treatment among genotypes of each polymorphism. Polymorphisms of NET T-182C, ADRA1D T1848A and ADRA1D A1905G were highly associated with the response to  $\beta$ -blockers (Table 3). Patients with NET-182CC genotype showed lower LVFS improvement than those with NET-182T allele (CC:  $3.7 \pm 7.1$ , TT + TC:  $9.9 \pm 7.9\%$ ,  $P = 0.019$ ). In ADRA1D T1848A and A1905G polymorphisms, LVFS change was lower in patients with ADRA1D 1848TT genotype (TT:  $6.7 \pm 7.0\%$ , AA + AT:  $10.9 \pm 8.5\%$ ,  $P = 0.023$ ) and ADRA1D 1905AA genotype (AA:  $6.7 \pm 7.0\%$ , GG + GA:  $10.8 \pm 8.4\%$ ,  $P = 0.029$ ). For the other polymorphisms analyzed in the present study, no significance of the outcome was observed in  $\beta$ -blocker response.

### Baseline characteristics of subjects according to genotypes of NET and ADRA1D polymorphisms

Comparisons of baseline characteristics among genotypes of NET T-182C and ADRA1D T1848A are shown in Tables 4 and 5. We analyzed only ADRA1D T1848A in subsequent analyses because there was strong linkage disequilibrium

**Table 1** The 39 polymorphisms examined in this study

Gene	Polymorphism	rs no.
ADRA1A	Intron2 G549C	rs3739216
	T1039C (Cys347Arg)	rs1048101
	A1395T (Glu465Asp)	rs2229126
ADRA1B	G549A	rs3729604
	T1848A	rs2236554
ADRA1D	A1905G	rs709024
	C-1291G	rs1800544
ADRA2A	G-261A	rs1800545
	G-98C	rs3111873
ADRA2B	Del 301–303	NA
	C-2069T	rs6846820
ADRA2C	Del 322–325	NA
	A145G (Ser49Gly)	rs1801252
ADRB1	C1165G (Arg389Gly)	rs1801253
	T-1429A	rs2895795
ADRB2	G-1343A	rs2400707
	G-1023A	rs17287446
ADRB3	T-47C	rs1042711
	A46G (Arg16Gly)	rs1042713
NET	C79G (Gln27Glu)	rs1042714
	C491T (Thr164Ile)	rs1800888
NET	C523A	rs17858185
	T190C (Trp64Arg)	rs4994
NET	G250C	rs4998
	T-182C	rs2242446
TH	intron7 A-13C	rs5568
	G1287A	rs5569
DDC	intron9 G9A	rs998424
	intron13 G-1C	rs5561
DDC	G241A (Val81Met)	rs6356
	T-11502C	rs921451
MAOA	G1385A (Gln462Arg)	rs11575542
	T941G	rs6323
MAOB	intron13 G-34A	rs1799836
	C-1021T	rs1611115
DBH	G444A	rs1108580
	C186T	rs4633
COMT	A1222G (Met158Val)	rs4680
	A1338G	rs165599

Abbreviations: ADRA1A, adrenergic  $\alpha_{1A}$  receptor; ADRA1B, adrenergic  $\alpha_{1B}$  receptor; ADRA1D, adrenergic  $\alpha_{1D}$  receptor; ADRA2A, adrenergic  $\alpha_{2A}$  receptor; ADRA2B, adrenergic  $\alpha_{2B}$  receptor; ADRA2C, adrenergic  $\alpha_{2C}$  receptor; ADRB1, adrenergic  $\beta_1$  receptor; ADRB2, adrenergic  $\beta_2$  receptor; ADRB3, adrenergic  $\beta_3$  receptor; NET, norepinephrine transporter; TH, tyrosine hydroxylase; DDC, dopa decarboxylase; MAOA; monoamine oxidase A; MAOB, monoamine oxidase B; DBH, dopamine-beta-hydroxylase; COMT, catechol-O-methyltransferase; NA, not available.

For nonsynonymous polymorphisms, the resulting amino-acid change is shown in parentheses.

between ADRA1D T1848A and ADRA1D A1905G ( $D' = 1.00$ ,  $r^2 = 0.94$ ). No significant differences were observed regarding age, sex, NYHA class, LVFS, blood pressure, heart rate or carvedilol/metoprolol dose. We stratified study population by the use of diuretics, since the use of diuretics was different among ADRA1D T1848A genotype groups. Difference in LVFS improvement among ADRA1D T1848A genotypes remained significant in both population

(patients with diuretics:  $P=0.025$ , patients without diuretics:  $P=0.025$ ).

During follow-up, no significant difference was observed in the reduction of blood pressure or heart rate among genotypes of these polymorphisms (data not shown).

*Combined genotype analysis of NET T-182C and ADRA1D T1848A polymorphisms*

Finally, we performed combined genotype analysis of NET T-182C and ADRA1D T1848A polymorphisms. Change of LVFS value, based on combined genotypes of these two polymorphisms, is shown in Figure 1. There were no differences in baseline LVFS among the four genotype groups (data not shown); however, the increase of LVFS was significantly different among these genotypes ( $P=0.011$  calculated by ANOVA). The ADRA1D 1848A-carrier/NET-182T-carrier group was compared with the other three combined genotype groups (ADRA1D 1848TT/NET-182T-

carrier, ADRA1D 1848A-carrier/NET-182CC, ADRA1D 1848TT/NET-182CC) using Dunnett  $t$ -test. Apparent LVFS improvement was obtained in only the ADRA1D 1848A-carrier/NET-182T-carrier group, whereas the ADRA1D 1848TT/NET-182CC group exhibited no improvement ( $12.0 \pm 8.1$  vs  $1.9 \pm 3.8$ ,  $P=0.022$ ). Compared to the ADRA1D 1848A-carrier/NET-182T-carrier group, both the ADRA1D 1848TT/NET-182T-carrier group and the ADRA1D 1848A-carrier/NET-182CC group resulted in less LVFS improvement.

**Discussion**

We investigated the significances of 39 adrenergic system-related gene polymorphisms in the interindividual difference of  $\beta$ -blocker response. Polymorphisms of NET (T-182C) and ADRA1D (T1848A and A1905G) were significantly associated with the improvement of LVFS by  $\beta$ -blocker treatment. Furthermore, we demonstrated synergistic effect of NET T-182C and ADRA1D T1848A (A1905G) polymorphisms in the improvement of cardiac function.

We analyzed the relationship between the polymorphism of NET T-182C and ADRA1D T1848A and the other clinical markers of heart failure as well. As a result, the patients with ADRA1D 1848A allele greatly improved in left ventricular diastolic diameter (LVDd), compared to those with ADRA1D 1848TT genotype. Moreover, the patients with NET-182T allele exhibited better improvement in cardiothoracic ratio, brain natriuretic peptide and LVDd than those with NET-182CC genotype (data not shown). These findings suggest that the results of this study were not derived from the bias in echocardiography. However, since clinical studies demonstrated that  $\beta$ -blocker therapy reduces the mortality due to heart failure, large-scale and long-term clinical studies should be needed to address the relation between the gene polymorphisms and survival rate.

Norepinephrine transporter plays an important role in cardiovascular homeostasis. More than 90% of norepinephrine is removed by presynaptic reuptake via NET.<sup>7</sup> In the present study, we demonstrated that the patients with NET-182CC genotype were resistant to  $\beta$ -blocker therapy. Interestingly, it is reported that the response to  $\beta$ -blocker therapy in patients with non-ischemic DCM could be predictable using MIBG myocardial scintigraphy.<sup>5,6</sup> Since MIBG

**Table 2 Characteristics of the CHF patients in this study**

Parameter	CHF (n = 80)
Age	58.5 ± 12.0
Sex (M/F)	60/20
NYHA class	2.6 ± 1.0
LVFS (%)	14.0 ± 6.4
SBP (mm Hg)	116.2 ± 15.9
DBP (mm Hg)	73.3 ± 11.7
HR (beats/min)	79.2 ± 16.8
<b><math>\beta</math>-Blockers</b>	
Carvedilol	81.3%
Metoprolol	12.5%
Others	6.3%
<b>Other medication</b>	
ACE inhibitors/ARBs	80.0%
Diuretics	79.5%
Digoxin	42.5%

Abbreviations: ACE, angiotensin converting enzyme; ARB, angiotensin II receptor blocker; CHF, chronic heart failure; DBP, diastolic blood pressure; HR, heart rate; LVFS, left ventricular fractional shortening; NYHA, New York heart association; SBP, systolic blood pressure.

Values are represented as mean ± s.d. or frequencies.

**Table 3 Improvement of LVFS by  $\beta$ -blocker treatment among genotype groups of each polymorphism**

	Genotype			Genetic model	P-value
NET	TT (n = 27)	TC (n = 41)	CC (n = 12)		
T-182C	10.7 ± 7.3	9.3 ± 8.3	3.7 ± 7.1	Recessive	0.019
ADRA1D	TT (n = 38)	TA (n = 36)	AA (n = 6)		
T1848A	6.7 ± 7.0	10.2 ± 8.4	15.1 ± 8.6	Dominant	0.023
ADRA1D	AA (n = 36)	AG (n = 36)	GG (n = 8)		
A1905G	6.7 ± 7.1	10.2 ± 8.3	13.3 ± 9.1	Dominant	0.029

Abbreviations: LVFS, left ventricular fractional shortening; NET, norepinephrine transporter.

Polymorphisms except NET T-182C, ADRA1D T1848A and ADRA1D A1905G were not associated with the improvement of LVFS by  $\beta$ -blockade.

**Table 4** Baseline characteristics of patients according to genotypes of NET T-182C

Parameter	TT (n = 27)	TC (n = 41)	CC (n = 12)	P-value*
Age	58.1 ± 10.2	58.1 ± 12.8	60.9 ± 13.8	0.45
Sex (M/F)	23/4	27/14	10/2	0.47
NYHA class	2.8 ± 1.0	2.5 ± 1.0	2.3 ± 1.0	0.41
LVFS (%)	14.0 ± 6.3	14.0 ± 6.6	14.2 ± 5.8	0.91
SBP (mm Hg)	121.8 ± 16.4	113.7 ± 15.0	113.1 ± 16.5	0.48
DBP (mm Hg)	77.0 ± 10.8	71.4 ± 12.0	72.1 ± 11.4	0.72
HR (beats/min)	82.2 ± 18.6	77.3 ± 17.1	79.2 ± 10.7	0.99
<i>Dose of <math>\beta</math>-blockers</i>				
Carvedilol (mg/day)	14.3 ± 7.0 (n = 21)	12.0 ± 5.9 (n = 34)	12.8 ± 6.7 (n = 10)	0.95
Metoprolol (mg/day)	30.0 ± 17.3 (n = 5)	50.0 ± 11.5 (n = 4)	20.0 (n = 1)	—
<i>Other medication</i>				
ACE inhibitors/ARBs (%)	88.9%	68.3%	100.0%	0.062
Diuretics (%)	91.3%	74.4%	72.7%	0.55
Digoxin (%)	47.8%	41.0%	36.4%	0.66

Abbreviations: ACE, angiotensin converting enzyme; ARB, angiotensin II receptor blocker; DBP, diastolic blood pressure; HR, heart rate; LVFS, left ventricular fractional shortening; NET, norepinephrine transporter; NYHA, New York heart association; SBP, systolic blood pressure.

\*P-value was calculated with recessive model (TT+TC vs CC).

**Table 5** Baseline characteristics of patients according to genotypes of ADRA1D T1848A

Parameter	TT (n = 38)	TA (n = 36)	AA (n = 6)	P-value*
Age	57.8 ± 12.4	59.6 ± 11.4	56.3 ± 14.7	0.62
Sex (M/F)	26/12	29/7	5/1	0.20
NYHA class	2.5 ± 1.0	2.5 ± 1.1	3.5 ± 0.8	0.65
LVFS (%)	14.4 ± 6.9	13.6 ± 6.0	14.0 ± 5.0	0.59
SBP (mmHg)	114.6 ± 17.1	117.5 ± 15.5	119.6 ± 9.8	0.39
DBP (mmHg)	72.6 ± 11.9	74.2 ± 11.8	73.0 ± 10.5	0.61
HR (beats/min)	81.5 ± 17.4	75.1 ± 13.1	89.3 ± 26.6	0.24
<i>Dose of <math>\beta</math>-blockers</i>				
Carvedilol (mg/day)	12.0 ± 6.7 (n = 33)	13.8 ± 6.0 (n = 27)	13.0 ± 6.7 (n = 5)	0.28
Metoprolol (mg/day)	30.0 ± 26.5 (n = 3)	44.0 ± 8.9 (n = 7)	— (n = 0)	0.39
<i>Other medication</i>				
ACE inhibitors/ARBs (%)	81.6	77.8	83.3	0.74
Diuretics (%)	91.7	68.8	60.0	0.011
Digoxin (%)	44.4	46.9	0	0.74

Abbreviations: ACE, angiotensin converting enzyme; ARB, angiotensin II receptor blocker; DBP, diastolic blood pressure; HR, heart rate; LVFS, left ventricular fractional shortening; NET, norepinephrine transporter; NYHA, New York heart association; SBP, systolic blood pressure.

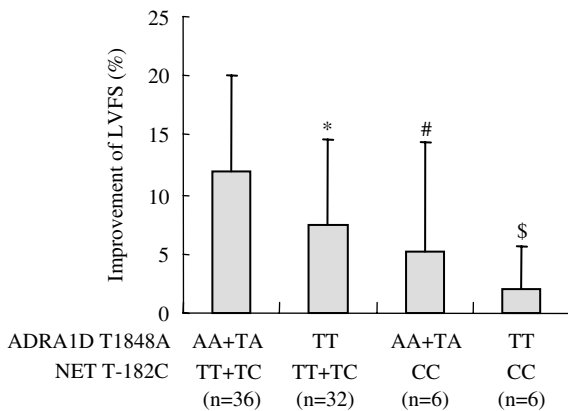
\*P-value was calculated with dominant model (TT vs TA+AA).

scintigraphy is influenced by norepinephrine uptake via NET, functional analysis of this polymorphism, especially by using MIBG, would be informative.

Our results suggest that ADRA1D T1848A and A1905G polymorphisms correlate with the response to  $\beta$ -blockers. Several studies have investigated the possible associations between adrenergic receptor  $\alpha_{1D}$  and hypertension.<sup>8,9</sup> In our subjects, no difference in blood pressure was observed among genotypes of ADRA1D T1848A and A1905G polymorphisms either before or after treatment. Therefore, these polymorphisms are likely to be associated with the drug

response independently of blood pressure. It should be noted that more than 80% of the patients in the present study were treated with carvedilol, a blocker of  $\alpha_1$  and  $\beta$  adrenergic receptors.

Recently, Carvedilol or Metoprolol European Trial (COMET) has proposed that carvedilol extends survival compared with metoprolol in heart failure patients;<sup>10</sup> however, some investigators raised the concerns against the conclusion of COMET investigators.<sup>11</sup> Among them, one of the most serious concerns is that the reduction in heart rate is more remarkable in carvedilol group than in metoprolol,



**Figure 1** Improvement of LVFS by  $\beta$ -blocker treatment among combined genotypes of NET T-182C and ADRA1D T1848A. Data are presented as means  $\pm$  s.d. \* $P=0.065$ , # $P=0.136$ , \$ $P=0.022$  vs the ADRA1D 1848A-carrier/NET-182T-carrier group.  $P$ -values were calculated by Dunnett  $t$ -test.

suspecting that the dosage of metoprolol was insufficient, compared with carvedilol dosage. In our study,  $\beta$ -blockers were administered at their maximal tolerated dosage, and consequently no significant difference was observed in the improvement of LVFS between carvedilol- and metoprolol-treated groups (carvedilol vs metoprolol,  $8.5 \pm 7.8$  vs  $10.5 \pm 8.5\%$ ,  $P=0.48$ ).

In the previous studies, ADRB1 Ser49Gly ADRB1 Arg389-Gly and ADRB2 Gln27Glu were associated with the response to  $\beta$ -blockers in CHF patients.<sup>12-14</sup> However, we failed to detect the significant association between the response to  $\beta$ -blockers and these polymorphisms. The inconsistency might be derived from the difference in the cause of heart failure. Subjects in the previous studies included the patients with ischemic cardiomyopathy as well as DCM, while we focused exclusively on DCM.

Average doses of  $\beta$ -blockers in our study were lower than those in Europe and the United States. Actually, the lower doses of  $\beta$ -blockers are used for CHF treatment in Japan than in Western countries. Less than 20 mg/day of carvedilol remarkably improves the prognosis in Japanese CHF patients, probably due to a difference in  $\beta$ -adrenergic receptor sensitivity.<sup>15</sup>

Metoprolol and carvedilol are mainly metabolized by the polymorphic CYP2D6 enzyme and their plasma concentrations are expected to be affected by CYP2D6 genotypes.<sup>16-18</sup> Therefore, we assessed the direct contribution of CYP2D6 genotypes to the efficacy of  $\beta$ -blockers with dose adjustment. As a result, no significant association was detected in our population (data not shown). Pharmacodynamic response should be more important than pharmacokinetic variation in predicting the response to  $\beta$ -blockade.

Finally, we performed combined genotype analysis of NET T-182C and ADRA1D T1848A polymorphisms. It is important that combined genotype was more informative than NET T-182C or ADRA1D T1848A alone. For example, the response in NET-182T-carrier was modified by the ADRA1D

T1848A genotype, as supported by the present results that the ADRA1D 1848A-carrier/NET-182T-carrier group showed the better response than the ADRA1D 1848TT/NET-182T-carrier group.

The present study is designed as an exploratory study to identify potential polymorphisms associated with  $\beta$ -blocker response. Multiple testing in our study of 39 polymorphisms might possibly lead to a false positive result due to type II error. However, traditional correction for multiple testing such as Bonferroni correction controls very tightly for false positives: the consequence of this is the maximization of the number of false negatives. Therefore, we did not perform adjustment for multiple testing.

American College of Cardiology/American Heart Association and European Society of Cardiology guidelines recommend that all CHF patients without contraindications should be treated with  $\beta$ -blockers. According to these guidelines,  $\beta$ -blockers are considered to be a first choice in CHF therapy, as well as angiotensin converting enzyme (ACE) inhibitors/angiotensin II receptor blockers (ARBs). Pharmacogenomics-based information, described in this study, may provide a clue to select the optimal pharmacological treatment against CHF.

In conclusion, we demonstrated that NET (T-182C) and ADRA1D (T1848A and A1905G) polymorphisms were the potential predictor for the response to  $\beta$ -blockers. Genotyping of these polymorphisms should probably be helpful in the individualized medicine of  $\beta$ -blockers for CHF patients.

## Materials and methods

### Study population

The study subjects consisted of 80 unrelated consecutive patients with CHF due to DCM. They attended or were admitted to Hokkaido University Hospital, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases and Osaka City University Medical School Hospital. This study was approved by the Ethics Committees of Osaka University and each hospital described above. All subjects gave their informed consent to participate in this study. The dose of  $\beta$ -blockers was progressively increased up to the maximal tolerated dose. Patients in this study were treated with carvedilol ( $n=65$ ), metoprolol ( $n=10$ ) and other  $\beta$ -blockers (bisoprolol, betaxolol, acebutolol,  $n=5$ ). They were followed more than 6 months after initiation of  $\beta$ -blocker therapy. We evaluated LVFS as the parameter of cardiac function, because LVFS can be measured reliably even in the cases with poor echocardiographic images as well as in the multicenter trials. In our study population, the improvement of LVFS was well correlated with that of LVEF ( $R^2=0.825$ ,  $P<0.001$ ).

### Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Maxi Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. We selected 16 genes involved in adrenergic system as candidate genes. Thirty-nine polymorphisms in these genes were

identified based on the literature, the dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) and JSNP ([http://snp.ims.u-tokyo.ac.jp/index\\_ja.html](http://snp.ims.u-tokyo.ac.jp/index_ja.html)) databases.

We genotyped ADRA2C Del 322–325, ADRA2B Del 301–303 and ADRB2 C523A polymorphisms as described previously<sup>19–21</sup> with minor modification. For genotyping ADRB2 promoter polymorphisms (T-1429A, G-1343A and G-1023A), forward and reverse primers were 5'-TGCTTTCTA-TAGCTTCAAAATGTTATTA-3' and 5'-ATCACTCACTCATTACTGTG-3' (T-1429A and G-1343A), 5'-CTAAGGAGGGCACTAAAGTA-3' and 5'-TAAACACACGCTGGCTTGAG-3' (G-1023A), respectively. The underlined nucleotide was artificially exchanged from C to generate a polymorphism specific restriction site. The PCR products were digested with 2U of *Psh*BI (T-1429A), *Hha*I (G-1343A), *Alw*NI (G-1023A) and subjected to 3% agarose gel electrophoresis.

Genotyping of other gene polymorphisms was performed using a chip-based matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry analysis of PCR-generated primer extension products.

#### Statistical analysis

Data are represented as mean  $\pm$  s.d. for continuous variables. Comparisons of baseline parameters among genotypes of each polymorphism were performed using unpaired *t*-test, ANOVA or Mann–Whitney's *U*-test as appropriate.  $\chi^2$ -test was used to analyze the significant differences in male to female ratio and the use of other anti-failure medications, including ACE inhibitors/ARBs, diuretics and digoxin, among genotypes. Hardy–Weinberg equilibrium was assessed by  $\chi^2$ -test with one degree of freedom. Each polymorphism was assessed with the use of dominant (comparison of major allele homozygous with heterozygous plus minor allele homozygous), recessive (comparison of major allele homozygous plus heterozygous with minor allele homozygous) and additive (comparison of each three genotypes) genetic models. *P*-value < 0.05 was considered to be significant. Using a two-sided 0.05 unpaired *t*-test, statistical analysis achieves at least 80% power to detect a LVFS difference of 4.5% between genotype groups, based on statistical power analysis. We did not perform adjustment for multiple testing so as to avoid false negative findings.<sup>22</sup> All statistical analyses were performed with SPSS for Windows version 11.0 software (SPSS Inc., Chicago, IL, USA).

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#### Duality of Interest

The authors declared no duality of interest.

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