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特集 わが国の降圧薬介入試験：関係者による解説，評価と展望

第2JATE：高齢者高血圧に対する降圧薬治療の効果に関する調査研究Ⅱ*

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Key Words : hypertension, elderly, clinical trial, antihypertensive therapy, Ca antagonists

はじめに

第2JATE(高齢者高血圧に対する降圧薬治療の効果に関する調査研究Ⅱ：Japanese Trial on the Efficacy of Antihypertensive Treatment in the Elderly Ⅱ)は，JATEの後に行われた高齢者高血圧を対象とした臨床試験である．JATEについては他項で詳述されるが，対照群にプラセボを用いることなどの理由で十分な症例数が登録できなかった[1]．第2JATEはカルシウム(Ca)拮抗薬を基礎薬とする治療研究であり，試験の実施を容易にするため対照群は設けられず，降圧目標は参加医師の裁量に委ねられた．しかし，頭部CTやquality of life(QOL)について評価するなどの特徴を有している．本研究はすでに終了し，結果の概要は学会では発表されているが[2)3)]，まだ原著論文としては公表されていない．本稿では，第2JATEに関し，研究の背景や目的，方法，結果の概要について述べていきたい．

研究の背景と目的

第2JATEは，JATEの後を継いで1997年に開始された臨床試験である．当時は，欧米においては高血圧治療の大規模臨床試験が多く報告されており，高齢者高血圧についての知見も集積されつつあった[4]．しかし，日本でもっとも多く用いられているCa拮抗薬についてのエビデンスは少なかった．一方，わが国においては，大規模臨床試験の必要性は認識されていたが実績に乏しい状態であった[5]．本研究の前身であるJATEは，1992年に開始されたプラセボ対照無作為二重盲検試験であり，高齢者高血圧におけるCa拮抗薬の効果を検討するものであったが，登録症例数は目標に遠く及ばなかった[1]．また，わが国では，高血圧による心血管疾患は脳血管障害が多いという特徴がある．しかし，治療前後に頭部CT検査を行い，降圧レベルと脳血管性病変の関係を検討することは，まだ行われていなかった．

第2JATE研究は，高齢高血圧患者にCa拮抗薬による治療を外来で行い，心血管合併症の発症，QOLおよび頭部CT所見に及ぼす影響を，観察期血圧からの降圧度，降圧レベルおよび血圧コントロール状態との関係で検討することを目的とするものである．この研究によって，高血圧治療と無症候性病変を含む脳血管性障害の発症，進展との関係が明らかとなり，わが国に即した高齢者における適正な降圧治療の指針となるべきデータが得られることが期待された．

研究方法

第2JATE研究は，80施設の参加による多施設共同のオープン試験である．循環器病研究振興

* JATE 2 : Japanese Trial on the Efficacy of Antihypertensive Treatment in the Elderly Ⅱ.

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財団の助成によるもので，事務局は同財団内(国立循環器病センター内)に置かれた．研究組織は，研究代表者は国立循環器病センターの瀧下修一で(後に尾前照雄に交代)，21名の中央委員および数名の調査登録管理者，統計解析委員，CT判読者を含むものであった．筆者は事務局に所属した．研究費は，武田薬品工業，バイエル薬品，三菱ウェルファーマの3社により支援された．

対象は，重篤な合併症を有しない65歳以上85歳未満の高血圧外来患者で，未治療あるいはCa拮抗薬以外の降圧薬により治療中で，観察期の血圧が収縮期160mmHg and/or 拡張期95mmHg以上を登録基準とした．拡張期血圧が110mmHg以上の者，重篤な臓器障害や重篤な他疾患を伴う者などは除外された．

観察期の調査の後，ニトレンジピン，マニジピン，ニソルジピン(後にニフェジピンCRが追加)のうち1剤を使用した降圧治療が開始された．各薬剤は原則として1日1回朝食後投与とし，降圧が不十分であれば原則として承認の最大用量まで増量された．治療目標は一律に定めず，主治医の考えに委ねられた．目標血圧に達しない場合は，ほかの降圧薬が追加された．投与期間は3年間とされた．

評価項目は，血圧および心拍数(1か月ごとに坐位で測定)，自覚症状，副作用，合併症，偶発症，臨床検査(胸部X線，心電図，尿，末梢血，血液生化学)，QOL，頭部CTであった．臨床検査とQOL調査は1年ごとに行われ，頭部CT検査は観察期および3年後に行われた．

エンドポイントは，①心血管系合併症の発症，②血圧コントロールが不良で中止が妥当と判断された場合，③その他の理由で中止が妥当と判断された場合，④死亡，とされた．

目標症例数は1,500例であり，症例登録期間は1997年8月から1999年6月までであった．追跡調査は2002年に終了した．

結果の概要

第2JATE研究には，80施設より661症例が登録された．男性280例，女性381例で，平均年齢は72歳であった．441例が完了し，中止は78例，脱落130例(うち健在確認100例)，登録除外12例であった．主な中止理由は偶発症，合併症，副作用で，主な脱落理由は転医，転居，来院せずであった．

血圧値は，観察期は167±17／91±11mmHgで，1年後は141±16／78±9mmHg，2年後は139±15／77±10mmHg，3年後は140±15／77±10mmHgとなった．

心血管系合併症は25例で，脳卒中13例(脳梗塞9例，脳出血2例，くも膜下出血2例)，心筋梗塞3例，心不全3例，腎不全2例，急死4例であった．心血管死亡は6例，全死亡は14例であった．

心血管系合併症を発症した者と発症しなかった者との間には，観察期の血圧には差がなく，脳卒中を発症した者は治療中の血圧が高い傾向にあった(1年後の収縮期血圧148 vs. 140mmHg)．また，頭部CTで3年後に変化がみられた者(n＝16)は，変化がなかった者(n＝416)に比較して，観察期および3年後の収縮期血圧が有意に高値であった(175 vs. 167mmHgおよび145 vs. 140 mmHg)．

1年後のQOLの変化と血圧のコントロール状況についての解析では，収縮期血圧140mmHg未満のコントロール良好群では63％がQOLの改善を示した．収縮期血圧160mmHg以上のコントロール不良群ではその頻度は51％であり，中間群では58％であった．

考察と結論

第2JATE研究は，高齢者高血圧におけるCa拮抗薬による降圧治療の効果を調べる介入研究であるが，オープンで対照群は設けられず降圧目標も担当医に委ねられており，観察的研究に近いといえよう．しかし，Ca拮抗薬を主とした降圧治療により，高齢高血圧患者の収縮期血圧は140mmHg程度にコントロールされた．高齢の日本人におけるCa拮抗薬の優れた降圧効果を支持する成績であろう．

本研究における心血管イベントは25例と少なく，治療中の血圧レベルとの関係は明らかでなかった．しかし，脳卒中発症者の血圧は高値傾向を示し，脳卒中の発症はなかったが頭部CT上変化がみられた者の血圧は，そうでない者に比べて高値であった．最近のPROGRESS研究など

においても示されているが[6]，高齢者においても厳格な血圧コントロールが脳血管の障害予防に効果的と考えられる．

本研究では，患者のQOLは降圧治療により悪化することはなく，むしろ改善した．また，血圧がよくコントロールされた群のQOLの改善率は，コントロール不良の群より高い傾向を示した．これらのことは，Ca拮抗薬を基礎薬としての厳格な降圧治療は，高齢高血圧患者のQOLにも好影響を及ぼすことを示唆している．

第2JATE研究における登録症例数は，JATE研究に比べると約2倍であったが，それでも目標の半分にも達しなかった．わが国における大規模臨床試験の実施の困難さと問題点は，JATEやほかの研究においても示されている[1]．今後の臨床試験において，研究組織や費用を含めて研究体制を強化し，研究に参加する医師や患者さんの利得に配慮することが重要であろう．

結論として，第2JATE研究では目標血圧値を一律に定めなかったが，Ca拮抗薬を主とした治療により，高齢高血圧患者の外来収縮期血圧は平均140mmHgにコントロールされた．また，脳血管の障害進展の予防とQOLの改善には，高齢者においても厳格な血圧コントロールが効果的であることが示唆された．

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Plasma Asymmetric Dimethylarginine and Coronary and Peripheral Endothelial Dysfunction in Hypertensive Patients

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Background: The attenuation of coronary flow reserve (CFR) and endothelium-mediated vasodilation of the brachial artery (EMV-BA) have been frequently reported in hypertensive patients. The present study investigated the link between CFR and EMV-BA in hypertensive patients. We hypothesized that changes in serum asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, and concomitant insulin resistance may be underlying factors connecting the two pathologic alterations.

Methods: A total of 75 patients (30 men and 45 women, 61.5 ± 10.1 years of age) with essential hypertension and without coronary artery disease and diabetes mellitus were included in the study. Measurements of CFR were made using adenosine-triphosphate stress transthoracic Doppler echocardiography, and forearm EMV-BA was measured by venous occlusion strain gauge plethysmography. A plasma ADMA assay and a 75-g oral glucose tolerance test were also performed.

Results: Average CFR and EMV-BA values were 2.54 ± 0.63 and 86.0 ± 54.7%, respectively. A significant correlation was found between CFR and EMV-BA (r = 0.493, $P < .001$). Both CFR and EMV-BA were also significantly correlated with age and plasma ADMA concentration, but were not correlated with insulin resistance, plasma insulin, or left ventricular mass. Multiple regression analysis revealed that ADMA was the only statistically independent parameter associated with CFR and EMV-BA.

Conclusions: The similar deterioration in endothelial function in coronary and peripheral vascular territories may be mainly due to increased plasma ADMA concentration. Plasma ADMA appears to play a major role in endothelial dysfunction in hypertensive patients, independent of insulin resistance or left ventricular hypertrophy. Am J Hypertens 2004;17:802–808 © 2004 American Journal of Hypertension, Ltd.

Key Words: Coronary flow reserve, endothelial function, hypertension, asymmetric dimethylarginine, insulin resistance.

Vascular endothelium is known to be altered in the presence of arterial hypertension. The availability of nitric oxide (NO), which is synthesized by vascular endothelium, may be reduced in patients with essential hypertension.[1,2] Because this pathologic alteration becomes manifest in the earliest stage of hypertension, endothelial dysfunction may represent an initial factor predisposing to atherogenesis and microvascular disease. Forearm vasodilator reserve is commonly used to evaluate endothelial function in clinical settings, and a close concordance between systemic endothelial function and the existence of coronary artery disease has been confirmed.[3] Thus, forearm vasodilator reserve has been viewed as a surrogate marker for impaired coronary circulation.

Coronary flow reserve (CFR) is considered to reflect coronary endothelial function; it is usually reduced, along with forearm endothelial function, in patients with essential hypertension.[4–11] Whereas several reports have suggested an association between left ventricular hypertrophy

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(LVH) and attenuated CFR[5,11] or forearm endothelial dysfunction,[12] the mechanisms underlying this association remains controversial.[6–9,13] In addition, in some reports, peripheral and coronary endothelial functions have been suggested to differ in patients with hypertensive heart disease.[14,15]

Nitric oxide is synthesized by endothelial, neuronal, and macrophage isoforms of the enzyme NO synthases (NOS). This enzyme can be selectively inhibited by guanidine-substituted analogs of L-arginine, such as *N*-monomethyl-L-arginine, which act as competitive inhibitor of NOS. Asymmetric dimethylarinine (ADMA) is an endogenous competitive inhibitor of NOS, and serum ADMA levels have been suggested to be markers of endothelial dysfunction.[16] Recently, ADMA was reported to be associated with insulin resistance.[17]

The present study was initiated to examine further the link between CFR and forearm endothelial function in patients with essential hypertension. We hypothesized that changes in ADMA concentration may be underlying mechanisms connecting the two pathologic alterations. In addition, we assessed the involvement of insulin resistance in the pathophysiology of these associations.

Methods

Study Population

The study population consisted of 75 patients with essential hypertension and without coronary artery disease. All hypertensive patients had a well established history of elevated casual blood pressure (BP) >140/90 mm Hg, with at least three sets of readings taken at 1-month intervals. Exclusion criteria for the present study included the presence of valvular heart disease, idiopathic myopathy, and insufficient echocardiographic imaging of the coronary arteries. Patients with arrythmias, including atrioventricular block and atrial fibrillation, as well as those with bronchial asthma were also excluded on the premise that adenosine triphosphate (ATP) might worsen their symptoms. Patients with renal failure or renal insufficiency (serum creatinine level >1.0 mg/dL) were also excluded because ADMA concentrations are influenced by renal function. Coronary artery disease was ruled out based on a lack of symptoms in the clinical records and by negative findings on exercise stress scintigraphy or coronary angiography, which was performed if necessary. In patients taking antihypertensive drugs, medications were withdrawn approximately 1 week before the examination to exclude direct effects on vasodilation. Individuals with a smoking habit reportedly abstained from smoking for 1 week before the examination. The experimental protocols and the process for obtaining informed consent were approved by the appropriate institutional review committee.

Measurement of Coronary Flow Reserve Using Transthoracic Doppler Echocardiography

Measurement of CFR was performed as described elsewhere.[18,19] Briefly, transthoracic Doppler echocardiographic examinations were performed with the Siemens Sequoia digital ultrasound system at a frequency of 7.0 MHz (Siemens USA, Mountain View, CA). The ultrasound beam was transmitted toward the heart to visualize coronary blood flow in the distal portion of the left anterior descending coronary artery (LAD) by color Doppler flow mapping. First, the left ventricle was imaged in the long axis cross-section, and then the ultrasound beam was inclined laterally. Next, coronary blood flow in the distal LAD was searched for under the guidance of color Doppler flow mapping. Positioning a sample volume on the color signal in the distal LAD, Doppler spectral tracings of flow velocity were recorded by fast Fourier transformation analysis. All studies were recorded on 0.5-in S-VHS videotape for off-line analysis.

We first recorded baseline spectral Doppler signals in the distal portion of the LAD. We administered ATP (140 μg/kg/min intravenously) for 3 min to record spectral Doppler signals during hyperemic conditions.[18,19] All patients had continuous heart rate and BP monitoring throughout the study.

Three experienced investigators (ST, HF, AH) who were unaware of the patients' data performed each examination and analyzed the results. Analysis of coronary flow velocity was performed off-line by tracing the contour of the spectral Doppler signal using the computer incorporated in the ultrasound system. Mean diastolic velocity (MDV) was measured at baseline and peak hyperemic conditions. An average of the measurements was obtained for three cardiac cycles. We defined CFR as the ratio of hyperemic to basal MDV (CFR_{MDV}).

Measurement of Forearm Endothelial Function

Endothelium-mediated vasodilation of the brachial artery (EMV-BA), which represents forearm endothelial function, was measured by strain gauge plethysmography (EC-5R; D.E. Hoknson, Inc., Issaquah, WA), as previously described.[20] Briefly, a mercury-in-Silastic strain gauge, that had been electrically calibrated, was placed on the widest part of the forearm. The pressure of the collecting cuff was set at 60 mm Hg, and occlusion pressure of the wrist cuff was set at 200 mm Hg. After obtaining the baseline forearm blood flow value, hyperemic-forearm blood flow was recorded after the upper arm was compressed by inflating a sphygmomanometer at a pressure of 250 mm Hg for 4.5 min. The forearm blood flow was estimated as the slope of the change in forearm volume (mL/min/100 mL forearm tissue). We calculated the ratio of vasodilator response to reactive hyperemia (after 60 sec

of cuff deflation) and the baseline value of forearm blood flow as EMV-BA.

Measurement of Left Ventricular Mass by M-Mode Echocardiography

Two-dimensional guided M-mode echocardiography was performed to measure LV wall mass. Left ventricular diastolic and systolic diameter (LVDd/Ds), in addition to the diastolic thickness of the left ventricular posterior wall (LVPWT) and interventricular septum (IVST), were assessed in M-mode images in the parasternal long axis view. M-mode analysis was performed according to guidelines of the American Society of Echocardiography. Left ventricular mass was indexed for body height powered to 2.7 (Cornell adjustment), and a left ventricular mass index (LVMI) >50 $g/m^{2.7}$ was considered the cut-off point for LVH.[21]

All measurements were performed by three trained investigators (ST, HF, TH) who were unaware of the subjects' clinical data. We recorded echocardiographic images on 0.5-inch S-VHS videotape, and data were analyzed precisely from the retrieved images.

Measurement of Plasma Asymmetric Dimethylarinine Level and Laboratory Determination

Plasma ADMA levels were determined at Fujimoto Biomedical Laboratories (Matsubara, Osaka, Japan) with a novel high performance liquid chromatography (HPLC) method. This method used the Hitachi L-7480 system (Hitachi, Tokyo, Japan) equipped with a fluorescence detector for excitation at 348 nm and emission at 450 nm with an ODS column using orthophthaldialdehyde for fluorescence determination.

An oral glucose tolerance test (OGTT) was performed by measuring the concentrations of plasma glucose and immunoreactive insulin (IRI) immediately before and 30, 60, and 120 min after 75 g of anhydrous dextrose was ingested. Glucose was measured by the glucose dehydrogenase method, and IRI was analyzed by an enzymatic-immunologic assay. In the present study, fasting and sigma IRI (the sum of the insulin levels 0, 30, 60, and 120 minutes after OGTT) were analyzed.[22] Insulin resistance was calculated according to a homeostasis model assessment (HOMA), as follows: fasting IRI (μU/mL) × fasting blood glucose (mg/dL) ÷ 405.

Biochemical factors were measured in blood collected the morning after overnight fasting on the same day of CFR and EMV-BA measurement.

Statistical Analysis

StatView software (SAS Institute, Cary, NC) was used for all statistical analyses. Values are presented as means ± SD. Differences before and after treatment were analyzed by ANOVA, followed by the Fisher protected least significant difference test for continuous variables and the χ^2 test for categorical variables. A value of $P < .05$ was accepted as representing statistical significance.

Table 1. Main characteristics of the study population

Variable	Mean ± SE
Sex (male/female)	30/45
Age (y)	61.5 ± 1.2
BMI (kg/m^2)	24.7 ± 0.4
Systolic/diastolic BP (mm Hg)	159.2 ± 1.9 / 89.7 ± 1.4
Plasma glucose (mg/dL)	96.0 ± 1.6
Plasma insulin (mU/L)	5.9 ± 0.4
Sigma IRI (mU/L)	235.2 ± 12.2
HOMA	1.54 ± 0.1
Total cholesterol (mg/dL)	198.1 ± 3.5
HDL cholesterol (mg/dL)	49.9 ± 1.8
LDL cholesterol (mg/dL)	125.0 ± 3.6
Total protein	6.8 ± 0.1
Albumin	4.1 ± 0.1
Blood urea nitrogen	15.9 ± 0.5
Serum creatinine (mg/dL)	0.74 ± 0.02
Uric acid	5.4 ± 0.1
Potassium	3.86 ± 0.04
Sodium	140.6 ± 0.3
Calcium	9.66 ± 0.04
Homocysteine	11.0 ± 0.1
Hemoglobin	13.6 ± 0.1
CRP (mg/dL)	0.11 ± 0.01
ADMA (nmol/mL)	0.50 ± 0.01
Cardiac ejection fraction	78.0 ± 0.7
LVMI ($g/m^{2.7}$)	57.4 ± 1.5

ADMA = asymmetric dimethylarginine; BMI = body mass index; BP = blood pressure; HOMA = homeostasis model assessment; LVMI = left ventricular mass index; Sigma IRI = sum of immunoreactive insulin levels 0, 36, 60, and 120 min after oral glucose tolerance test.

Results

The main characteristics of the study population are listed in Table 1. Only one patient met the criteria for isolated diastolic hypertension (137/97 mm Hg), and 37 patients had isolated systolic hypertension. The prevalence of LV hypertrophy (LV mass index >50 $g/m^{2.7}$ according to the Cornell criteria[21]) was 70.7%. Although none of the patients met the criteria for diabetes mellitus (fasting blood glucose >126 mg/dL or HbA1c >5.8%), the prevalence of impaired glucose tolerance according to the OGTT was 40.0%. The prevalence of insulin resistance, defined as HOMA >1.73, was 28.0%.

Measurements of CFR and EMV-BA were performed successfully in all patients, and no serious side effects

Table 2. Results for EMV-BA and CFR_{MDV} in hypertensive patients

Variable	Mean ± SD (range)
EMV-BA (%)	86.0 ± 54.7 (12.1–262.1)
Baseline MDV (cm/sec)	16.6 ± 4.3 (8.4–29.0)
Hyperemia MDV (cm/sec)	40.8 ± 10.6 (23.6–69.0)
CFR_{MDV}	2.54 ± 0.63 (1.29–4.53)

CFR = coronary flow reserve; EMV-BA = endothelium-mediated vasodilation of the brachial artery; MDV = mean diastolic coronary flow velocity.

(atrioventricular block, marked bradycardia, bronchial asthma, hypotension) occurred as a result of ATP infusion. Administration of ATP induced a significant decrease in BP and increase in heart rate (systolic BP 158.9 ± 21.5 →145.5 ± 22.4 mm Hg; diastolic BP 89.1 ± 13.7 →81.9 ± 14.5 mm Hg; heart rate 65.2 ± 11.3 →75.3 ± 12.2 beats/min). The hemodynamic changes were similar to those previously observed in studies on CFR measurements that used the same protocol.[19,23] The values of mean coronary flow velocity at baseline and during hyperemia, CFR_{MDV} and EMV-BA, are shown in Table 2.

We found CFR_{MDV} to be significantly correlated with EMV-BA ($r = 0.493$, $P < .0001$; Fig. 1a). The correlation coefficients between CFR_{MDV} or EMV-BA and other measured risk factors are shown in Table 3. Age was significantly correlated with both CFR_{MDV} and EMV-BA, but the degree of the correlations was of lesser magnitude than those between CFR_{MDV} or EMV-BA and plasma ADMA concentrations (Table 3, Figs. 1b and 1c). Neither CFR_{MDV} nor EMV-BA showed significant associations with LVH. Moreover, neither CFR_{MDV} nor EMV-BA was significantly correlated with HOMA-R and IRI.

Multiple regression analysis was performed to define independent relationships between CFR_{MDV}, EMV-BA, and other risk factors listed in Table 3. The results of this analysis are shown in Table 4, and indicate that plasma ADMA concentration showed a statistically independent association with both CFR_{MDV} and EMV-BA.

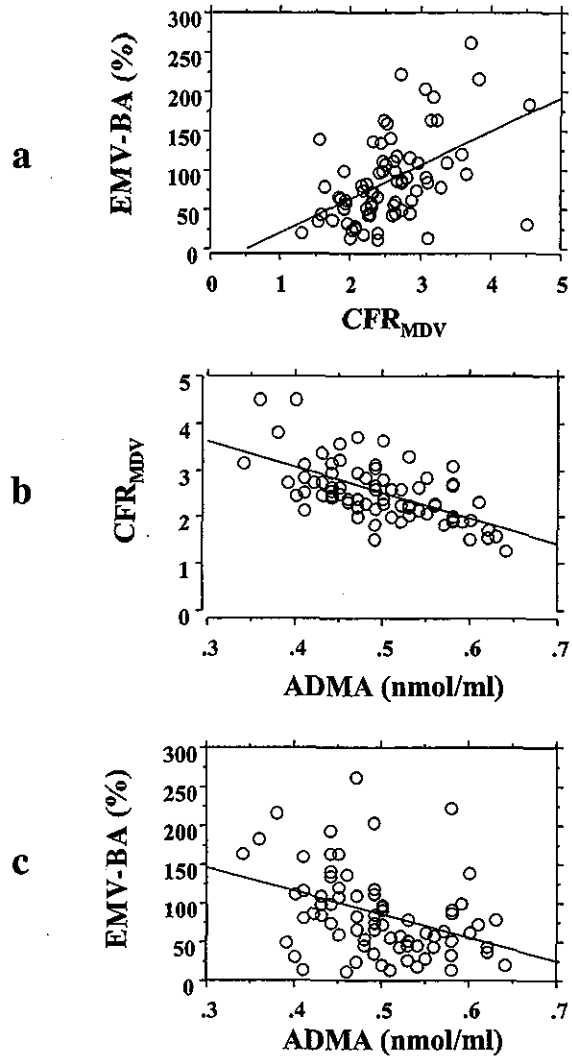

FIG. 1. Correlation of the three parameters: (**a**) coronary flow reserve of mean diastolic coronary flow velocity (CFR_{MDV}) and endothelium-mediated vasodilation of the brachial artery (EMV-BA); (**b**) CFR_{MDV} and plasma asymmetric dimethylarginine (ADMA) concentration; and (**c**) EMV-BA and plasma ADMA concentration.

Discussion

There is accumulating evidence that endothelial cells play a crucial role in vascular tone and structure. One of the major endothelium-derived vasoactive mediators is NO, which is formed in the endothelium by constitutive, endothelial isoforms of NOS. The vascular effects of NO involve vasodilation as well as inhibition of platelet and monocyte adhesion and aggregation, LDL oxidation, superoxide radical elaboration, and smooth muscle cell proliferation. Together, these effects contribute to the antiatherosclerotic properties of the intact vascular wall. The substance ADMA has been recognized as an endogenous, competitive inhibitor of NOS. A number of reports have suggested that an increased concentration of ADMA and its biologically inactive structural isomer, symmetric dimethylarginine (SDMA), might account for endothelial dysfunction in patients with end stage renal disease,[24] hypercholesterolemia,[16,25] and hypertension.[25] In addition, plasma ADMA concentration has been suggested to be associated with carotid intima-media thickness[26] and coronary stenosis.[27] Thus, ADMA was considered as a surrogate marker of subclinical atherosclerosis.

Forearm endothelium-mediated vasodilation and CFR are hallmarks of endothelial function, and have been applied extensively in the clinical setting to assess subclinical atherosclerosis of systemic and coronary arteries. Böger et al showed that elevated plasma ADMA levels were associated with impaired endothelium-dependent forearm vasodilation in young hypercholesterolemic individuals.[16] Päivä et al demonstrated a significant increase in

Table 3. Correlation coefficients between CFR_{MDV}, EMV-BA, and other cardiovascular risk factors in hypertensive patients

	CFR_{MDV}		EMV-BA	
	r	*P*	*r*	*P*
Age	0.308	.007	0.336	.003
BMI	0.157	.178	0.106	.364
SBP	0.121	.299	0.071	.546
DBP	0.189	.105	0.129	.270
LDL cholesterol	0.064	.586	0.022	.850
Homocystein	0.220	.058	0.335	.003
HOMA	0.013	.914	0.104	.377
Sigma IRI	0.124	.288	0.027	.818
CRP	0.190	.113	0.230	.053
ADMA	0.609	< .0001	0.382	.0007
LVMI	0.182	.118	0.062	.600
EF	0.020	.862	0.001	.990

ADMA = asymmetric dimethylarginine; BMI = body mass index; CFR_{MDV} = coronary flow reserve of mean diastolic coronary flow velocity; DBP = diastolic blood pressure; EMV-BA = endothelium-mediated vasodilation of the brachial artery; HOMA-R = homeostasis model assessment; IRI = immunoreactive insulin; LVMI = left ventricular mass index; SBP = systolic blood pressure.

plasma ADMA in borderline hypertensive patients with reduced hyperemic myocardial blood flow.[25] In the present study, we found a significant association between CFR and EMV-BA in patients without coronary artery disease. However, the association between CFR and forearm vasodilator reserve is controversial.[14,15] The peripheral and coronary arteries have different properties, such as recruitment of shunt vessels and receptor occurrence and density. The process of readaptation to high BP may also not be distributed homogeneously over the two vascular beds. Therefore, it remains to be determined whether CFR measurement can be used to assess coronary endothelial function.

Table 4. Multiple regression analysis of the relationship between CFR_{MDV}, EMV-BA, and other cardiovascular risk factors in hypertensive patients

	CFR_{MDV}		EMV-BA	
	r	*P*	*r*	*P*
Age	0.204	.171	−0.085	.629
BMI	0.170	.139	−0.112	.408
SBP	−0.088	.509	0.086	.587
DBP	0.292	.037	0.066	.687
LDL cholesterol	−0.093	.424	0.027	.841
Homocystein	−0.029	.809	−0.144	.316
HOMA	0.047	.696	−0.056	.695
Sigma IRI	−0.211	.069	0.057	.677
CRP	−0.090	.404	−0.126	.0329
ADMA	−0.598	< .0001	−0.302	.029
LVMI	0.104	.350	−0.103	.436
EF	−0.097	.809	−0.081	.528

Abbreviations as in Table 3.

Analysis performed with CFR_{MDV} and EMV-BA as the dependent variables. R^2 values for the entire model were 0.378 and 0.244, respectively.

In the present study, ADMA, a marker of endothelial dysfunction, showed a strong association, and EMV-BA showed a significant association, with CFR. These results resolve the debate over whether CFR mainly represents coronary endothelial function. Coronary flow reserve might be regarded as an integrated measure of endothelial dependent and independent vasodilation, both of which become disturbed in early atherosclerosis. However, several patients showed a discrepancy between CFR and EMV-BA. We suspect that endothelial dysfunction is not the only etiology of impaired CFR, and that other mechanisms might contribute selectively to CFR. Thus, further verification is needed to elucidate the other possible mechanisms of reduced CFR.

A number of investigations have found evidence that insulin resistance or compensatory hyperinsulinemia may be an efficient marker of atherosclerosis, although the causal role remains unknown. One of the explanations for the link between insulin resistance and endothelial dysfunction is increased plasma ADMA concentration. Stühlinger et al demonstrated a significant relationship between insulin resistance and plasma ADMA concentrations.[17] Miyazaki et al described that lipid- or hyperglycemia-induced dysregulation of dimethyl-arginine dimethylaminohydrolase (DDAH), an enzyme that metabolizes ADMA, was the main cause for elevated ADMA levels in patients with dyslipidemia or diabetes mellitus.[26] Taken together, elevated serum ADMA by reduced degradation has been suggested to play a significant role in endothelial dysfunction, accounting for the higher prevalence of cardiovascular events in patients with insulin resistance. In the present study, although we demonstrated that patients with reduced CFR and EMV-BA had increased serum ADMA concentrations, we could not find an association between these three parameters and insulin resistance or compensatory hyperinsulinemia. According to an investigation of patients with type 2 diabetes by Yokoyama et al, insulin resistance is not related to reduced CFR.[28] The authors suggested that control of blood glucose concentration rather than insulin resistance is an independent parameter determining impaired CFR. Galderisi et al reported that insulin resistance was independently associated with CFR in the overall population; however, they also demonstrated that only insulin-like growth factor-1 (IGF-1), not insulin resistance, was independently associated with reduced CFR in patients with hypertension.[29] Although more accurate parameters of insulin resistance such as SSPG analysis may be needed, we suggest that insulin resistance is not the major mechanism underlying impaired coronary and systemic endothelial function through increased plasma ADMA concentrations, especially in hypertensive patients.

There are several limitations to the present study. We adopted the cuff occlusion plethysmography method for assessing endothelial function in brachial artery. Although

this conservative method is less popular than ultrasonography, Wu et al demonstrated the high correlation between the two techniques.[30] Second, a relatively large number of patients showed an impaired glucose tolerance pattern in the OGTT. Although we excluded patients with hemoglobin A1c levels greater than 6.0%, we have to consider the effects of impaired glucose tolerance on coronary and systemic hyperemic responses.

To our knowledge, we have provided the first published evidence regarding the link between systemic and coronary endothelial function and increased plasma ADMA concentration. Plasma ADMA appears to play a similarly major role in endothelial dysfunction in the two different vascular territories, and its effects seemed to be independent from insulin resistance, left ventricular hypertrophy, and BP. We speculate that analysis of ADMA together with the two vascular physiologic assessments might provide more practical information for evaluating endothelial dysfunction.

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Original Article

The Thiazide-Sensitive Na^+-Cl^- Cotransporter Gene, *C1784T*, and Adrenergic Receptor-β3 Gene, *T727C*, May Be Gene Polymorphisms Susceptible to the Antihypertensive Effect of Thiazide Diuretics

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The response of blood pressure to thiazide diuretics (TZDs) differs among individuals. The prediction of the antihypertensive effect of TZDs is important for realizing individualized therapy in the management of hypertension. The aim of this study was to identify the single nucleotide polymorphisms (SNPs) susceptible to the antihypertensive effect of TZDs, particularly focusing on genes related to water-electrolyte absorption in the kidney. Seventy-six outpatients (mean age, 65.4±9.0 years) with essential hypertension (EHT) taking TZDs were retrospectively assessed. We defined as responders (R) those whose mean blood pressure was lowered by more than 5 mmHg after the use of TZDs. Forty-eight SNPs in 17 genes (*ADD1*, *GNB3*, *TSC* [*SLC12A3*], *MLR* [*NR3C2*], *NCX1* [*SLC8A1*], *WNK1*, *WNK4*, *AGT*, *ACE*, *AT1* [*AGTR1*], *CYP11B2*, *ADRB1*, *ADRB2*, *ADRB3*, *ADRA1A*, *ADRA1B*, *ADRA2A*) were genotyped in the 76 patients. The SNPs in *TSC*, *MLR*, *NCX1*, *WNK1*, and *WNK4* were identified by direct sequencing and those with minor frequencies of greater than 5% were genotyped in this study. The comparison of polymorphism prevalence between R and non-responders (NR) showed significant differences in *TSC C1784T* (*C* allele *vs.* *T* allele, odds ratio (OR)=3.81, p=0.016, confidence interval (CI): 1.25–11.63) and *ADRB3 T727C* (*Trp64Arg*) (*T* allele *vs.* *C* allele, OR=4.59, p=0.005, CI: 1.54–13.68). The blood pressure (BP) in patients homozygous for the major alleles of both *TSC C1784T* and *ADRB3 T727C* were significantly reduced by TZD treatment; however, the BP in those homozygous for the minor allele and heterozygous (*TSC C1784T*: TT+CT; *ADRB3 T727C*: CC+CT) for both SNPs were not significantly changed after TZD treatment. Both newly detected *TSC C1784T* and *ADRB3 T727C* are gene polymorphisms susceptible to the antihypertensive effect of TZDs in patients with EHT. Thus, the prediction of BP reduction by TZDs may be possible by evaluating these two SNPs.
(*Hypertens Res* 2004; 27: 821–833)

***Key Words*: thiazide diuretics, gene polymorphism, essential hypertension**

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Introduction

Thiazide diuretics (TZDs) have been most widely used as a first line antihypertensive drug (*1*, *2*). Recently, the ALLHAT study confirmed the usefulness of TZDs for the reduction of blood pressure (BP) and cardiovascular diseases in comparison with newer antihypertensive drugs, including Ca channel blockers (CCBs) such as amlodipine and angiotensin converting enzyme inhibitors (ACEIs) such as lisinopril in about 40,000 hypertensive patients with high risk factors (*3*). TZDs are not only effective as a monotherapy for hypertension, but are also very useful for combination therapy with other antihypertensive drugs (*4*). Moreover, the use of a TZD as a drug therapy for hypertension, which is a chronic and lifelong disease, would be very good from the viewpoint of the cost of drugs, because TZDs are the cheapest of all antihypertensive drugs. However, the response of BP to TZDs differs among individuals, and TZDs often induce side effects, such as hypokalemia and lipid, glucose and uric acid metabolism abnormalities (*4*). Therefore, it would be useful to determine the individual sensitivity to a TZD before prescribing it.

Regarding previous findings about gene polymorphisms that influence TZD-sensitivity, Turner *et al.* (*5*) reported that the β3-subunit of the G protein (*GNB3*) *C825T* polymorphism was related to the antihypertensive effect of a TZD in Caucasian and African-American subjects with essential hypertension (EHT). Glorioso *et al.* (*6*) also demonstrated that the α-adducin (*ADD1*) *Gly460Trp* polymorphism is the gene conferring susceptibility to the antihypertensive effect of TZDs in Italian hypertensives. This *ADD1 Gly460Trp* polymorphism was also suggested to confer susceptibility to salt-sensitivity in Caucasians and Asians with EHT (*7*).

Mutations of causative genes have recently been detected in several monogenic electrolyte disorders, such as mutations in the thiazide-sensitive Na-Cl cotransporter (*TSC*) gene for Gitelman syndrome (*8*, *9*), the WNK1 and 4 genes for Gordon syndrome (pseudohypoaldosteronism type II) (*10*) and the mineral corticoid receptor (*MLR*) for pseudohypoaldosteronism type I (PHA I) (*11*). TZDs are commonly effective for treating Gitelman syndrome and Gordon syndrome. We also focused on the Na^+/Ca^{2+} exchanger gene (*NCX1*), because its impairment was recently reported in mesangial cells from salt-sensitive hypertensive rats (*12*). TZDs are known to be effective for salt-sensitive hypertension. It is also known that the renin-angiotensin-aldosterone system (RAAS) and sympathetic nervous system (SNS) are activated in response to changes in circulating blood volume after TZD administration. Therefore, it is expected that gene polymorphisms related to the RAAS and SNS might be involved in the antihypertensive effect of TZDs. The present study investigated the gene polymorphism influencing the TZD-sensitivity by analyzing mainly single nucleotide polymorphisms (SNPs) of several water-electrolyte-related genes, including *GN3B*, *ADD1*, *TSC*, *MLR*, *NCX1*, *WNK1*, *WNK4* and RAAS- and SNS-related genes, to anticipate the effect of TZDs on BP in patients with hypertension.

Methods

Study Subjects

Peripheral blood samples for genetic analysis were collected with written informed consent from Japanese patients with EHT at an outpatient clinic of the Division of Hypertension and Nephrology, National Cardiovascular Center, Suita, Japan. This study protocol was approved by the Ethical Committee of the National Cardiovascular Center. Seventy-six patients, who had been newly prescribed TZDs as monotherapy or in addition to other antihypertensive agents, and whose blood-pressure data could be obtained from patients' records in 3 consecutive outpatient visits before and after starting TZDs, were retrospectively enrolled. BP was measured in the subjects after at least 10 min of rest in a sitting position. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) values were the means of three physician-obtained measurements. All subjects visited the outpatient clinic every month. The TZDs were a combination of indapamide (64.5%), trichlormethiazide (26.3%), mefruside (7.9%), and hydrochlorothiazide (1.3%). We defined patients who could achieve a BP reduction greater than 5 mmHg of mean blood pressure (MBP) after taking the TZDs as responders (R), and patients who could not achieve a BP reduction greater than 5 mmHg of MBP or showed increased BP after taking TZDs as non-responders (NR), according to the common evaluation criteria of antihypertensive drug effectiveness in Japan.

DNA Studies

Direct Sequencing for Detection of Polymorphisms in TSC, MLR, WNK1, WNK4, *and* NCX1

Genomic DNA was extracted using an NA-3000 nucleic acid isolation system (KURABO, Osaka, Japan) and stored at −80 °C until use. We sequenced the 32–48 Japanese samples with written informed consent for genetic analysis. The methods used for the direct sequencing have been described previously (*13*). Briefly, all exons, part of the intron and an approximately 1,000-bp upstream region of exon 1, which would include the promoter regions of the *TSC*, *WNK1*, *WNK4* and *NCX* genes, were individually amplified by polymerase chain reaction and sequenced on an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, USA). In the *MLR* gene, exon 2 was sequenced. The polymorphisms were identified using the Sequencer software package (Gene Codes Corp., Ann Arbor, USA), followed by visual inspection.

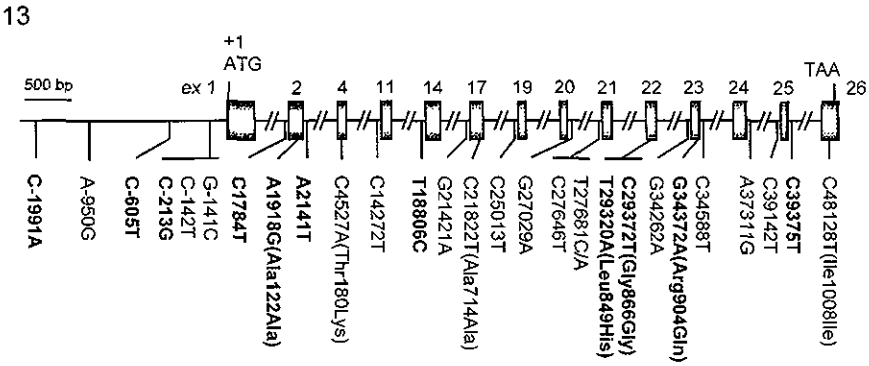

Fig. 1. *Location of genetic variants identified in* TSC. *Nucleotide numbers were counted from the initiation codon (ATG). Sequencing regions are indicated by the bars above the schematic gene structure. The initiation codon, ATG, and stop codon, TAA, are also shown. The single nucleotide polymorphisms presented in bold were genotyped in this study.*

Genotyping of Polymorphisms

The polymorphisms were genotyped using the TaqMan-polymelase chain reaction (PCR) system. Regarding genotyped SNPs, *C825T* of *GNB3* and *G29071T-Gly460Trp* of *ADD1* were selected according to previous studies (*5*, *6*, *14*). As the RAAS-related genes, *angiotensinogen* (*AGT*: *A−20C*, *G−6A*) (*15*, *16*), *ACE* (*G12568C* for *I/D*) (*17*), *angiotensin II type I receptor* (*AT1*: *A1166C*, *A−153G*) (*18*, *19*), and *aldosterone synthase* (*CYP11B2*: *C−344T*) (*20*) were tested. Furthermore, as the SNS-related genes, *adrenergic receptor β-1* (*ADRB1*: *A393G-Ser49Gly*, *G1413C-Arg389Gly*) (*21*), *β-2* (*ADRB2*: *C−47T*, *G2118A-Gly16Arg*, *G2151C-Glu27Gln*) (*22*, *23*), *β-3* (*ADRB3:T727C-Trp64Arg*) (*24*), *α-1a* (*ADRA1A*: *T44653C-Arg492Cys*) (*25*), *α-1b* (*ADRA1B*: *G834A*, *G1167A*) (*26*) and *α-2a* (*ADRA2A*: *A3023G*) (*27*) were tested for TZD sensitivity. Regarding *ACE*, we genotyped *G12568C* instead for the *I/D* polymorphism of 287 bp in intron 16 because *G12568C* showed almost complete linkage disequilibrium (LD) with the *I/D* polymorphism, as reported previously (*17*). For the directly sequenced genes, SNPs having a minor allele frequency of greater than 5% were selected for genotyping. As a result, 11 SNPs of *TSC* (Fig. 1), 2 SNPs of *MLR*, 7 SNPs of *WNK1*, 2 SNPs of *WNK4* and 7 SNPs of *NCX1* were genotyped. The sequences of the allele-specific probes and PCR primers used for the genotyping are shown in Table A1 in Appendix.

Statistical Analysis

Values are expressed as the means±SD. Hardy-Weinberg equilibrium was assessed by χ^2 analysis. The overall distribution of alleles was analyzed by χ^2 analysis. The distribution of genotypes between R and NR was analyzed by 2×2 contingency tables with a 2-sided Fisher exact probability test. The statistical significance was established at $p<0.05$. Comparison of BP reduction between allelic variants was performed by ANOVA followed by the Fisher protected least significant difference test using Stat-View version 5.0 (SAS Institute Inc., Cary, USA). LD and haplotype analyses were performed using the SNPAlyze statistical package version 2.1 (DYNACOM Co., Ltd., Mobara, Japan). The LD between SNPs was calculated by r^2. Tight LD was regarded as $r^2 \geq 0.5$. Haplotype estimation was performed by the expectation-maximization algorithm.

Results

Group Characteristics

Overall BP was significantly reduced after TZD administration (Fig. 2). Table 1 shows the group characteristics of R and NR. Forty-five patients who showed an MBP reduction of greater than 5 mmHg were defined as R, and 31 patients were defined as NR. Neither averaged age nor body mass index (BMI) showed a significant difference between R and NR. The BP before TZD administration was significantly higher in R than in NR. After TZD treatment, the averaged BP in R was remarkably decreased; however, the averaged BP in NR was slightly higher than that at pretreatment (Table 1). Control for deviation from Hardy-Weinberg equilibrium gave non-significant results in most SNPs examined in the present study, except *ADRA1B G1167A*. In the genotyping of *ADRA1B G1167A*, all of the study subjects were homozygous for the major allele, GG. This suggests that polymorphism of *ADRA1B G1167A* might not exist in the Japanese population.

Detection of Genetic Variants

We detected 52 SNPs of *TSC*, 7 SNPs of *MLR*, 35 SNPs of *WNK1*, 22 SNPs of *WNK4* and 15 SNPs of *NCX1* (Table A2 in Appendix). We confirmed some of the identified SNPs in the public database, dbSNPs (*http://www.ncbi.nlm.nih.gov/SNP/*). As shown in Table A2 in Appendix, some SNPs were very rare. Therefore, we chose SNPs that had a minor allele frequency of greater than 5% for genotyping by the TaqMan

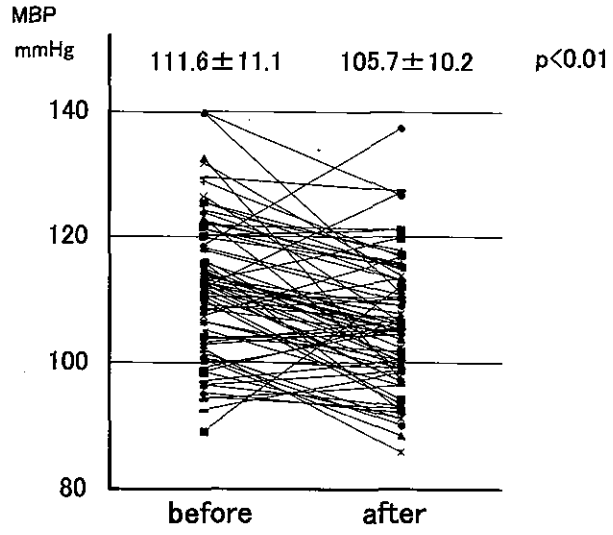

Fig. 2. *Mean blood pressure (MBP) before and after treatment with thiazide diuretics (TZDs) in all subjects. The average MBP in all subjects was significantly reduced by treatment with TZDs.*

method. Furthermore, some of these polymorphisms showed LD with other polymorphisms. Thus, we chose one SNP from among the polymorphisms with tight LD (*28*) ($r^2 \geq 0.5$) for genotyping the subjects. Therefore, genotyping by the TaqMan method was finally performed for 11 SNPs in *TSC*, 2 SNPs in *MLR*, 7 SNPs in *WNK1*, 2 SNPs in *WNK4* and 7 SNPs in *NCX1* (Table A1 in Appendix).

Association Study for the Effect of TZDs

No polymorphisms of the *MLR*, *WNK1*, *WNK4*, *NCX1*, and RAAS genes, and no polymorphisms of most of the SNS genes examined in the present study, including *C825T GNB3* and *Gly460Trp ADD1*, were significantly related to the effect of the TZDs, based on the comparison of each allele frequency between R and NR (Table 2). Two SNPs, *TSC C1784T* and *ADRB3 T727C*, showed a significant correlation with the effect of the TZDs (Table 3). The BP in patients with the homozygotes of the major allele of both *TSC C1784T* and *ADRB3 T727C* were significantly reduced by TZD treatment; however, the BP in those with the homozygotes of the minor allele and heterozygote (*TSC C1784T*: TT+CT; *ADRB3 T727C*: CC+CT) of both SNPs were not significantly changed after TZD treatment (Fig. 3). Furthermore, there was a much more significant difference in prevalence between the patients with the homozygotes of the minor alleles and heterozygote of these two SNPs, *TSC C1784T* and *ADRB3 T727C*, combined and those with the homozygotes of the major allele in R and NR than in *TSC C1784T* or *ADRB3 T727C* alone (odds ratio [OR]=5.09, $p=0.003$, confidence interval [CI]: 1.82–14.23).

Table 1. Comparison of Patients Characteristics between R and NR of TZD

	R (±SD)	NR (±SD)	*p* value
Number	45	31	
Age (years)	64.5±9.3	66.7±8.6	N.S.
Sex (male/female)	18/27	20/11	<0.05
BMI (kg/m^2)	24.7±3.2	24.7±3.2	N.S.
Pre SBP (mmHg)	157.9±14.3	142.9±15.0	<0.01
Pre DBP (mmHg)	95.0±10.7	86.5±10.1	<0.01
Pre MBP (mmHg)	115.9±9.7	105.3±9.9	<0.01
Pre HR (/min)	71.0±7.9	72.4±7.9	N.S.
Post SBP (mmHg)	138.8±15.0	147.2±17.4	<0.05
Post DBP (mmHg)	87.0±9.9	88.2±11.0	N.S.
Post MBP (mmHg)	104.3±9.3	107.9±11.2	N.S.
Post HR (/min)	72.4±9.3	72.8±9.0	N.S.
Monotherapy (%)	28.9	25.8	N.S.
Kind of TZD (%)			
Indapamide	66.7	61.3	N.S.
Trichlormethiazide	26.7	25.8	N.S.
Mefruside	6.7	9.7	N.S.
Hydrochlorothiazide	0.0	3.2	N.S.

R, responder; NR, non-responder; TZD, thiazide diuretics; BMI, body mass index; Pre SBP, systolic blood pressure at pretreatment; Pre DBP, diastolic blood pressure at pretreatment; Pre MBP, mean blood pressure at pretreatment; Pre HR, heart rate at pretreatment; Post SBP, systolic blood pressure at posttreatment; Post DBP, diastolic blood pressure at posttreatment; Post MBP, mean blood pressure at posttreatment; Post HR, heart rate at posttreatment; Monotherapy, prevalence of monotherapy by thiazide diuretics; Kind of TZD, prescribed kinds of TZD; N.S., not significant.

Haplotype Analysis

We measured the LD to understand the haplotype distribution of *TSC C1784T* in Japanese. There was a strong LD between the multiple SNPs within *TSC*. *TSC* was composed of three LD blocks, and the LD block containing *C1784T* consisted of two SNPs, *C−213G* and *C1784T*. The haplotype frequency was calculated for these two SNPs and the differences in haplotype distribution were compared between R and NR (Table 4). The results showed that the haplotype H2 with the 1784T allele tended to be different between the two groups, although not significantly so ($p=0.094$).

Discussion

The present study demonstrated that *TSC C1784T* and *ADRB3 T727C* were associated with the antihypertensive effect of TZDs in Japanese patients with EHT. A hypertensive patient with the minor homozygote or heterozygote of these two SNPs is predicted to be a non-responder to TZDs.

The TSC is present in the distal convoluted tubule, which

Table 2. Comparison of Allele Frequency between R and NR to TZD

Gene	Minor allele *vs.* common allele			
	SNP	Odds ratio	*p*	95% CI
ADDI	*Gly460Trp*	1.300	0.427	0.680–2.487
GNB3	*C825T*	1.620	0.146	0.844–3.110
TSC	*C−1991A*	1.483	0.586	0.356–6.167
	C−605T	1.483	0.586	0.356–6.167
	C−213G	1.483	0.586	0.356–6.167
	C1784T	3.816	0.013	1.253–11.627
	A1918G	0.594	0.235	0.251–1.410
	A2141T	2.378	0.102	0.821–6.886
	T18806C	0.825	0.566	0.428–1.591
	T29320A	1.459	0.790	0.090–23.770
	C29372T	1.143	0.718	0.554–2.359
	G34372A	—	0.147	—
	C39375T	0.681	0.311	0.323–1.436
MLR	*C−2G*	1.147	0.731	0.524–2.509
	G538A	0.685	0.418	0.274–1.716
WNK1	*G786A*	1.021	0.965	0.407–2.561
	C108560T	1.026	0.967	0.310–3.400
	A128177C	1.124	0.791	0.473–2.673
	C133634T	1.189	0.721	0.461–3.067
	G135642T	0.950	0.881	0.484–1.864
	C141114T	0.820	0.617	0.377–1.785
	C142763T	0.967	0.971	0.157–5.961
WNK4	*C14597T*	1.467	0.704	0.201–10.700
	C14717T	1.780	0.287	0.609–5.203
NCX1	*T−23690C*	0.849	0.721	0.346–2.084
	C−23449A	0.864	0.846	0.199–3.757
	T−23200C	0.651	0.553	0.156–2.711
	T−23181C	0.850	0.633	0.436–1.656
	A−22729C	0.914	0.861	0.334–2.505
	C−22144G	2.967	0.357	0.263–33.454
	G252581A	0.906	0.779	0.456–1.802
AGT	*A−20C*	1.265	0.540	0.596–2.687
	G−6A	0.758	0.527	0.320–1.793
ACE	*G12568C (I/D)*	0.768	0.443	0.392–1.508
AT1-R	*A1166C*	0.712	0.639	0.171–2.961
	A−153G	1.172	0.818	0.302–4.552
CYP11B2	*C−344T*	1.554	0.219	0.768–3.145
ADRB1	*G1413C*	1.724	0.228	0.707–4.204
	A393G	0.692	0.432	0.276–1.738
ADRB2	*C−47T*	1.098	0.869	0.361–3.338
	G2118A	1.531	0.203	0.793–2.956
	G2151C	1.228	0.744	0.358–4.217
ADRB3	*T727C*	4.591	0.003	1.541–13.680
ADRA1A	*T44653C*	0.630	0.412	0.207–1.913
ADRA1B	*G834A*	1.381	0.333	0.718–2.657
	G1167A	—	—	—
ADRA2A	*A3023G*	1.223	0.556	0.626–2.389

SNP, single nucleotide polymorphism; R, responder; NR, non-responder; TZD, thiazide diuretics; CI, confidence interval.

has been suggested to be the principal mediator of sodium and chloride reabsorption in this segment of the nephron. Simon *et al.* (*9*) demonstrated complete linkage of Gitleman syndrome to the genetic variants of *TSC*. The loss of function of TSC in patients with *TSC* gene variants could lead to low blood pressure, hypokalemic alkalosis, hypomagnesaemia and hypocalciuria (*29*). Melander *et al.* reported that gene polymorphism of TSC may influence EHT (*30*). It has recently been reported that TSC might interact with WNK4, which is one of the causative genes of Gordon syndrome (*31*, *32*). TZDs are generally effective in patients with Gordon syndrome. This would be due to the interaction between WNK and TSC. Four kinds of TZDs were used for the present study, and the main pharmacological mechanism of the antihypertensive effect common among those four TZDs was the blockage of TSC in the distal tubule. For these reasons, we expected that the gene polymorphisms of *TSC*, *WNK1* and *WNK4* might be related to the effect of TZDs. One SNP of *TSC* showed a significant association with the effect of the TZDs; however, there were no positive SNPs in either *WNK1* or *WNK4*.

TSC consists of 26 exons and is located on 16q13. We performed direct sequencing for *TSC*, including the promoter region, and detected 52 SNPs. We screened 11 SNPs that showed a minor allele frequency of greater than 5% for TZD sensitivity (Fig. 1). As a consequence, only *TSC C1784T* showed a significant correlation with the effect of TZDs. Since *TSC C1784T* is located in intron 1, as shown in Fig. 1, this SNP itself may not influence TSC function. Although we could not find functional polymorphisms linked with *C1784T*, there may be functional polymorphisms in much further upstream regions of the promoter or genes adjacent to *TSC*.

Administration of a TZD often induces activation of the RAAS and/or SNS as a result of circulating volume reduction. Thus, we investigated the participation of gene polymorphisms of the RAAS and SNS. The gene polymorphisms were selected from previous studies investigating the correlation between BP regulation and gene polymorphisms (*33*). Although Sciarrone *et al.* (*34*). reported that Caucasian hypertensive patients with the *I* allele of *ACE I/D* were more sensitive for hydrochrolthiazide than those with *DD*, no polymorphisms of the RAAS-related genes, including *ACE I/D*, showed a significant correlation with the effect of TZDs in the present study. Furthermore, neither *C825T* of *GNB3* (*5*) nor *Gly460Trp* of *ADD1* (*6*, *34*), which have previously been reported to influence the sensitivity to TZDs, showed a significant correlation with the effect of TZDs in the present study. It is suggested that the reason for the difference between the present findings and previous findings on the participation of the RAAS genes, *GNB3* and *ADD1*, in the effects of TZDs might be the ethnicity of the study subjects. Most studies (*5*, *34*) investigated the participation of the RAAS genes, *GNB3* and *ADD1*, in the effect of TZDs in Caucasians. In contrast, all subjects in the present study

Table 3. TZD- Sensitive Gene Polymorphisms of *TSC C1784T* and *ADRB3 T727C*

SNP	Sex	Genotype	R	NR	χ^2	*p*
TSC C1784T	M+F	CC	40	20	6.052	0.049
		CT	5	9		
		TT	0	1		
		CC	40	20	5.556	0.037
		TT+CT	5	10		
	Odds ratio=4.000, 95% CI=1.204–13.284					
		C allele	85	49	6.168	0.016
		T allele	5	11		
	Odds ratio=3.816, 95% CI=1.253–11.627					
ADRB3 T727C	M+F	CC	1	1	10.649	0.005
		CT	3	11		
		TT	40	18		
		TT	40	18	10.056	0.003
		CC+CT	4	12		
	Odds ratio=6.667, 95% CI=1.889–23.525					
		C allele	5	13	8.533	0.005
		T allele	83	47		
	Odds ratio=4.591, 95% CI=1.541–13.680					

TZD, thiazide diuretics; R, responder; NR, non-responder; SNP, single nucleotide polymorphism; M, male; F, female; CI, confidence interval.

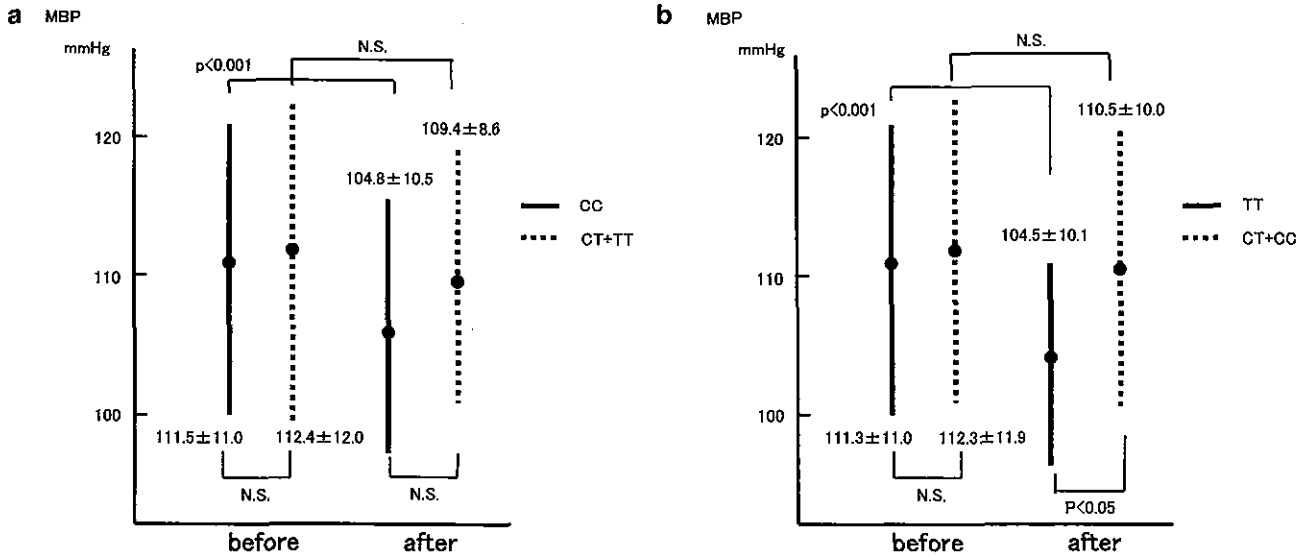

Fig. 3. *a: Comparison of the MBP changes before and after TZD therapy between homozygotes of the major allele CC and the minor homo- and heterozygote TT+CT of* TSC C1784T. *NS, not significant. b: Comparison of MBP changes before and after TZD administration between the homozygotes of the major allele TT and the minor homo- and heterozygote CC+CT of* ADRB3 T727C. *NS, not significant.*

were Japanese. The amount of salt intake in the Japanese population is generally greater than that in Caucasians (*35*). Thus, there is a possibility that Japanese may be more sensitive to TZDs than Caucasians.

The present study revealed a significant correlation between *T727C-Trp64Arg ADRB3* and the effect of TZDs. The β3 adrenergic receptors are mainly distributed in adipose tissues and involved in the regulation of lipolysis and thermogenesis. Regarding the *ADRB3* polymorphism *Trp64Arg*, it has been correlated with obesity (*36*), insulin resistance (*37*) and diabetes (*38*). In contrast, the relation between *Trp64Arg ADRB3* and hypertension is controversial (*24, 39, 40*). Re-

Table 4. Haplotype Distribution in R and NR in Two SNPs of *TSC*

Haplotype		R (n=90)	NR (n=60)	p value
H1	CC	81	47	0.622
H2	CT	5	9	0.094
H3	GC	4	2	>0.999
H4	GT	0	2	0.165

R, responder for thiazide diuretics: NR, non-responder for thiazide diuretics; SNPs, single nucleotide polymorphisms. Haplotypes were shown as combined alleles of genotyped 2 SNPs (*C−213G*, *C1784T*) of *TSC*.

garding the SNS activity, Shihara *et al.* (*41*) reported that subjects with the homo- and heterozygote of *64Arg ADRB3* had higher responses of the autonomic nerve activities after postural change than those with the wild-type gene. This suggests that *Trp64Arg ADRB3* may play an important role in the autonomic nervous system activities, including the activities of the SNS. To date, however, there has been no evidence that β3 adrenergic receptors exist and function physiologically on sympathetic nerves or the renal tubular system. Therefore, the mechanisms intervening between *ADRB3* gene polymorphism and the effectiveness of TZDs are unclear. Further investigations, including studies on the reflective activation of SNS and the worsening in insulin resistance after TZD administration, will be needed to clarify this interaction.

There is a question as to whether the contributions of *TSC C1784T* and *ADRB3 T727C* to the effects of TZDs are a TZD-specific finding. We tried to investigate the relationship between these 2 SNPs and the antihypertensive effects of an ACEI in 98 patients with EHT by the same study protocol; however, these 2 SNPs did not show any significant correlation to the effect of ACEI (unpublished data). Although investigation of other antihypertensive drugs is necessary, we speculate that the contribution of these 2 SNPs to the effect of TZDs is in fact a TZD-specific finding.

The study limitations include the retrospective design and the small sample size. The study subjects included not only patients receiving monotherapy with TZDs but also those receiving combined therapy with TZDs and other antihypertensive drugs. This issue is not considered to have much influence on the relationship between the 2 SNPs, *TSC C1784T-ADRB3 T727C*, and the effect of TZDs, because the prevalence of patients with monotherapy using TZD and the variation of the kinds of TZDs were not significantly different in between each allele of the two SNPs. However, a prospective and large-scale controlled study using TZDs is needed to confirm the importance of *TSC C1784T* and *ADRB3 T727C* on the antihypertensive effect of TZDs.

Furthermore, the BP level at pretreatment is considered an important factor in the effect of antihypertensive drugs. In the present study, BP before TZD administration was significantly higher in R than in NR. However, the BP level before TZD administration was not significantly different between TZD-sensitive and -insensitive genotypes in both *TSC C1784T* and *ADRB3 T727C-Trp64Arg*, as shown in Fig. 3. It might be possible that the BP response to TZDs was modified by the placebo effect. However, it is unlikely that *TSC C1784T* or *ADRB3 T727C* is involved in the placebo effect. A placebo-controlled prospective trial or ambulatory BP monitoring would help to confirm the significance of these SNPs in the BP-lowering effect of TZDs.

Finally, regarding the statistical approach, the Bonferroni method was not performed in this study even though multiple SNPs were investigated. The criterion for significance is $p<0.001$ (=0.05/48 SNPs) according to the Bonferroni method; however, *TSC C1784T* and *ADRB3 T727C* were associated with the antihypertensive effect of TZDs at p= 0.016–0.049 and 0.003–0.005, respectively (Table 3). Although this might be considered a weak correlation for this kind of genetic research, we consider these two SNPs as prominent candidates relating to the effectiveness of TZDs, because both *TSC* and *ADRB3* were suggested to play an important role in the effectiveness of TZDs in patients with EHT, as we mentioned above.

In conclusion, *TSC C1784T* and *ADRB3 T727C-Trp64Arg*, may be gene polymorphisms susceptible to the antihypertensive effect of TZDs in patients with EHT. Thus, the prediction of BP reduction by TZDs may be possible by evaluating these two SNPs. Since the publication of the JNC 7, TZDs are becoming increasingly important as first-line drugs (*1*). The prediction of the TZD sensitivity of patients may lead to the realization of individualized therapy for hypertension based on genetic background.

Acknowledgements

We are grateful to Yoko Tokunaga and Chiyoko Imai for their excellent technical assistance and Erumu Hayase, Yoko Yamakawa, Kanako Hoshino and Chikako Oku for their excellent secretarial work.

Appendix

The sequences of the allele-specific probes and PCR primers for the genotyping are shown in Table A1 and SNPs of *TSC*, *MLR*, *WNK1*, *WNK4* and *NCX1* are shown in Table A2.

References

1. Chobanian AV, Bakris GL, Black HR, *et al*: The seventh report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure: the JNC 7 Report. *JAMA* 2003; **289**: 2560–2572.
2. Guideline Subcommittee: 1999 World Health Organization-International Society of Hypertension guidelines for the management of hypertension. *J Hypertens* 1999; **17**:

Table A1. List of Genotyping Conditions for TaqMan PCR Method

Gene name	SNP	Primer	Final conc. (nmol/l)	Probe	Final conc. (nmol/l)	96-well annealing temp. and cycle no.	384-well annealing temp. and cycle no.
ADD1	*Gly460Trp*	CACACCTTAGTCTTCGACTTGGG	800	Fam-TTCTGCCCTTCCTC-MGB	200		58°C
	(G29071T)	ACAAGATGGCTGAACTCTGGC	800	Vic-TTCTGCCATTCCTC-MGB	200		40
GNB3	*C825T*	CAGACCAGGAGCTGATCTGCTT	800	Fam-CATCACGTCCGTGGC-MGB	200		60°C
		TTGCAGTTGAAGTCGTCGTAGC	800	Vic-ATCACGTCTGTGGCCT-MGB	200		40
TSC	*C−1991A*	CCCTGACAGCTCAAATTTCCAC	800	Fam-CTGCCTCCCTGCAA-MGB	200		58°C
		CTTGTTACCAGAGGTGCCTAAGC	800	Vic-CTGCCTCACTGCAA-MGB	200		40
	C−605T	GCAGAAATGAAATCCACAAGCA	800	FAM-TTTGAAAATCCCTGTCCTG-MGB	228	62°C	58°C
		CATGCACCGATCATTAGATTGG	800	VIC-CTTTGAAAATTCCTGTCCTG-MGB	223	40	40
	C−213G	GGCAGAACACCATTTGATTGTG	800	FAM-CTGGCCCAAAGCCAGCCACTC-TAMRA	256	62°C	60°C
		GAAGAGCCACTCCAGGACTCA	800	VIC-CTGGCCCAAACCCAGCCACTC-TAMRA	282	35	40
	C1784T	CGCAGTGGTGCAGGTCAGT	800	Fam-CAGAGACGCCGTCC-MGB	200		58°C
		AGGTGTCTGCCTTCCTGCTG	800	Vic-TGCAGAGATGCCGTC-MGB	200		40
	A1918G	CTCACCATCACCCCCTTGAC	800	Fam-CTGGTGCCTGCCTCGCCC-TAMRA	200		60°C
		CAGCAGGAAGGCAGACACCT	800	Vic-TGGTGCCCGCCTCGCC-TAMRA	200		40
	A2141T	GCTTCAGTTTCCCCATCTGTACA	800	Fam-AATAGATTAAAGCCTGCCGG-MGB	200		58°C
		GGTGGCTTTTTAGGGAAACACA	800	Vic-AATAGATTAATGCCTGCCGG-MGB	200		40
	C4527A	GATGAACGTAGGTCGCATGGT	800	FAM-TGTCGGTCACGGTGA-MGB	336	60°C	58°C
		GATGGCTGAGATGGAGAGGC	800	VIC-TGTCGGTCAAGGTG-MGB	297	40	40
	T18806C	AGCAGCTCTGGCCTAGAAAGAG	800	FAM-TGGTGCCCTTGGCCCAGG-TAMRA	330	62°C	62°C
		ACGGAGATGATAGCCCCAAAC	800	VIC-CTGGTGCCCTCGGCCCAG-TAMRA	290	35	40
	T29320A	TCACATAGTGCTCTGTCCTGAGTG	800	FAM-TCCCTATCTCCTTGGC-MGB	242	62°C	60°C
		GATCTTGCATTTGCTCCACCTC	800	VIC-CCTATCACCTTGGCC-MGB	201	40	40
	C29372T	GCAAGAGGAGGTGGAGCAAAT	800	FAM-TTCGTAGGCGGCCAG-MGB	117	60°C	58°C
		CCCTCCACACTTACGCCTTTC	800	VIC-TCGTAGGTGGCCAGAT-MGB	254	40	40
	G34372A	GGGATTCCATGAAGTCCACATC	800	FAM-AACCCTCGGGCTGA-MGB	337	62°C	—
		CTGGAAGCCCCAAAACAGAAC	800	VIC-AGAACCCTCAGGCTG-MGB	329	40	—
	C39375T	GAAGCAGAAGGGCCAAAGTTC	800	FAM-ATAGCCCTGGCGATT-MGB	267	58°C	58°C
		GATGCCTGGGACACGTGAG	800	VIC-TAGCCCTGGTGATTC-MGB	84	40	40
MLR	*C−2G*	TTGTGGCTTAGCAAATGCAATT	800	Fam-TTTGTTAGCGATGGAGAC-MGB	602	62°C	
		CAGGGAGACTGTGGTAGCCTTT	800	Vic-ATTTGTTAGGGATGGAGAC-MGB	224	40	
	G538A	GGGCTTTTCTCATGACACATGATA	800	Fam-CTTTTAACAATGGCGCGC-MGB	189		60°C
		CGCCCTTGAGATCATTTATGTCT	800	Vic-TTTAACAACGGCGCGCA-MGB	361		40
NCX1	*T−23690C*	CTCTCCCCACAGGTCATTCTG	800	Fam-ATTTAACTTATAGCAAGGAA-MGB	200		58°C
		GCAGGAATCGTTCTTGCCTAA	800	Vic-TTAACTTACAGCAAGGAA-MGB	200		40
	C−23449A	GAATCTGCAATCCCCATGTGAT	800	Fam-CTCACATTCATGTTTGAG-MGB	200		56°C
		AGAACCACTGCTCTAGGCCAAT	800	Vic-ACTCACATTAATGTTTGAGG-MGB	200		40
	T−23200C	TTCTGAGGTGCAAGGAGGGTT	800	Fam-CCCCCTTTTTGTTGC-MGB	100		56°C
		GGCAGTCACCACGACTGATAGA	800	Vic-CCCCCTCTTTGTTG-MGB	100		40
	T−23181C	GGCAGTCACCACGACTGATAGA	800	Fam-TCCAGGAACCTCAGTTT-MGB	200		56°C
		AGGCTATTTCTTCCATTCCGC	800	Vic-CCAGGAACCTCGGTTT-MGB	200		40
	A−22729C	GCCTGGTGCAGTGTTCCTTTA	800	Fam-ATTATGAGGAAAGTGATTTA-MGB	200		58°C
		GCCCTTTCCAAGAGAAGCATTA	800	Vic-TATGAGGACAGTGATTTA-MGB	200		40
	C−22144G	AAAAGAAAAGTTGCAGCGCCT	800	Fam-CCACAACGCACTGC-MGB	200		56°C
		TTTTTCGATTTCCTGCCGG	800	Vic-CACAAGGCACTGCG-MGB	100		40
	G252581A	AAACAAAGACATACCAGCGAGAAA	800	Fam-CTCTCTCCGTGTCTC-MGB	200		58°C
		AAATTGCTAAAGCTTCAAAGGCA	800	Vic-TCTCTCCATGTCTCC-MGB	200		40
WNK1	*G786A*	GAACTGCAGGTAAAGCCCCAC	800	Fam-TTTGACGGTCCTTTG-MGB	200		58°C
		GAACTCGATCAACTGGCTTCG	800	Vic-TTTATTTGACAGTCCTTTG-MGB	200		40
	C108560T	CTGATGGGACGGTTGACAGTG	800	Fam-TCTTCACAGAATCTCGA-MGB	200		58°C

Table A1. (Continued)

Gene name	SNP	Primer	Final conc. (nmol/l)	Probe	Final conc. (nmol/l)	96-well annealing temp. and cycle no.	384-well annealing temp. and cycle no.
		CCTGTTCATGTTGGGAACCATA	800	Vic-TCTTCATAGAATCTCG-MGB	200		40
	A128177C	GTTGCTCCTGCAGAGCCAGT	800	Fam-AGTAGCACAGACCCAA-MGB	200		58°C
		TCTACAGAGGAAGCCAAAGTGGT	800	Vic-AGTAGCACAGCCCCA-MGB	200		40
	C133634T	TTGATTTGCTCTTCAGTACGCAG	800	Fam-AGCGTCTCACGGACT-MGB	200		58°C
		GCACCTACAGACAACAAAGGGAA	800	Vic-AGCGTCTCATGGACT-MGB	200		40
	G135642T	AAAACTTACACCAACCGCAGAAG	800	Fam-CTGTGATCATCTCTG-MGB	200		58°C
		ATTCAGTCCCAGCAACCTCTAGA	800	Vic-ACTGTGATAATCTCTG-MGB	200		40
	C141114T	TGGGACGATTTCAGGTAAGACAG	800	Fam-ATTCCTTCCTTTGGAGGA-MGB	200		58°C
		TTGTGTCCCAAATAGGTAGGCA	800	Vic-ATTCCTTCTTTTGGAGGAG-MGB	200		40
	C142763T	ACGACCCACTTTGTTTGCTGTA	800	Fam-CTGAAAACGTCCAACCT-MGB	200		58°C
		GTCAGACACTGGGCAGCCTAC	800	Vic-CCTGAAAACATCCAACCT-MGB	200		40
WNK4	*C14597T*	CTGGCTGTGATGACTGTGGC	800	Fam-TCCCCTCCCTAGCCT-MGB	200		58°C
		TGAAGGGCTTTCCTGGCC	800	Vic-TCCCCTCTCTAGCCTG-MGB	200		40
	C14717T	CACAGCTGAGGTGGAGAGTGAG	800	Fam-CTCCACTCTGCACTC-MGB	200		58°C
		GGAGGTGGTGAGGCCTAGAAA	800	Vic-ACTCCATTCTGCACTC-MGB	200		40
AGT	*A(−20)C**	CTTCTGGCATCTGTCCTTCTGG	250	Direct sequence			64°C
		CTGGTCTTATGAGAGGGGAGAGG	250				35
	*G(−6)A**	Same as *A(−20)C*		Direct sequence			
ACE	*G12568C*	AGCAGAGGTGAGCTAAGGGCT	667	Fam-CTCAAGGCATTCAA-MGB	200		58°C
	(I/D)	GGCCATCACATTCGTCAGATCT	667	Vic-CTCAAGCCATTCAA-MGB	200		40
AT1	*A(−153)G*	AACGCTGATCTGATAGTTGACACG	800	Fam-CCGTCAATATCCCGAG-MGB	200		60°C
		CTCTGTTTTGCATTCCCTCCTC	800	Vic-CCGTCAGTATCCCGA-MGB	200		40
	A1166C	AGAGAACATTCCTCTGCAGCACT	800	Fam-CAAATGAGCATTAGCT-MGB	200		60°C
		CGGTTCAGTCCACATAATGCAT	800	Vic-CAAATGAGCCTTAGCT-MGB	200		40
CYP11B2	*C(−344)T*	TGGACATTTTCTGCAGTTTTTGA	800	Fam-ATCCAAGGCTCCCTCT-MGB	100		56°C
		TCCTTTCTCCAGGGCTGAGA	800	Vic-CAAGGCCCCCTCT-MGB	100		40
ADRB1	*G1413C*	TTCTTCAACTGGCTGGGCTAC	800	Fam-CCTTCCAGGGACTGC-MGB	200		58°C
		GTCTCCGTGGGTCGCGT	800	Vic-CTTCCAGCGACTGCT-MGB	200		40
	A393G	CCGGTAACCTGTCGTCGG	800	Fam-CAGCGAAAGCCCCGA-MGB	200		58°C
		GATCACCAGCACATTGCCC	800	Vic-AGCGAAGGCCCCGAG-MGB	100		40
ADRB2	*C(−47)T*	CATTGGGTGCCAGCAAGAA	800	Fam-CGCCTCAGCGGGCGGA-TAMRA	100		56°C
		GAATGAGGCTTCCAGGCGT	800	Vic-CGCCTCAGCAGGCGGACC-TAMRA	100		40
	G2118A	CGCTGAATGAGGCTTCCAG	800	Fam-ACCCAATGGAAGCC-MGB	100		58°C
		CTGCGTGACGTCGTGGTC	800	Vic-ACCCAATAGAAGCCA-MGB	100		40
	G2151C	CCAGGACGATGAGAGACATGAC	800	Fam-TCCCTTTCCTGCGTGA-MGB	200		58°C
		CCTTCTTGCTGGCACCCA	800	Vic-TCCCTTTGCTGCGTG-MGB	200		40
ADRB3	*T727C*	CACGTTGGTCATGGTCTGGA	800	Fam-CGGAGTCCAGGCGA-MGB	200		58°C
		GAGGCAACCTGCTGGTCATC	800	Vic-TCGGAGTCCGGGCG-MGB	200		40
ADRA1A	*T44653C*	TCCAGCCAAGAGTTCAAAAAGG	800	Fam-CAGTGTCTCTGCAGAA-MGB	100		56°C
		CCAGGGCATGTTTGGAAGACT	800	AGTGTCTCCGCAGAA-MGB	200		40
ADRA1B	*G834A*	CGCACTCCTTGTCATCGTTG	800	Fam-TCCTTCCACCCAAGGA-MGB	200		58°C
		GTCTTGTCCACCGTCATCTCC	800	Vic-CTCCTTCCATCCAAGGA-MGB	200		40
	G1167A	CAAGATGAACATACCGACCACAA	800	Fam-CCCAACGTCTTAGCT-MGB	200		60°C
		CAACCCCAGGAGTTCCATAGC	800	Vic-CCCAACGTTTTAGCT-MGB	200		40
ADRA2A	*A3023G*	TCCCCTTCCATTCCCAACTC	800	Fam-TCTCTTTTTAAAGAAAAAT-MGB	200		56°C
		TTCAACATCAAAACCAAGGCC	800	Vic-TCTTTTTGAAGAAAAAT-MGB	100		40

* The genotyping for *AGT A(−20)C* and *G(−6)* polymorphisms was performed by the direct sequence method. A pair of the PCR primers was 5′-CTTCTGGCATCTGTCCTTCTGG-3′ and 5′-CTGGTCTTATGAGAGGGGAGAGG-3′.

Table A2. List of 130 Polymorphisms and Their Allele Frequency in *TSC*, *MLR*, *WNK1*, *WNK4* and *NCX1* Genes Identified by the Direct Sequence

Gene name	Allele 1/Allele 2 SNPs	Amino acid change	Region	Allele 1 Homo	Hetero	Allele 2 Homo	Total	Allele frequency Allele 1	Allele frequency Allele 2	Flanking sequence	dbSNP ID
TSC	*C−1991A*		promoter	38	0	10	48	0.792	0.208	caccactgcctc[c/a]ctgcaatggctt	
	A−950G		promoter	1	19	21	41	0.256	0.744	tttaatagagac[a/g]gggtttcaccat	
	C−704T		promoter	46	1	0	47	0.989	0.011	cagacagcccgg[c/t]gccacaccctgg	
	C−605T		promoter	37	10	0	47	0.894	0.106	cactttgaaaat[c/t]cctgtcctgttt	
	C−553T		promoter	26	1	0	27	0.981	0.019	agcccccagtca[c/t]gtacccccgct	
	−544delT		promoter	47	1	0	48	0.990	0.010	tcacgtaccccc[t/-]gcttgctcaatc	
	C−213G		promoter	35	8	0	43	0.907	0.093	gggagtggctgg[c/g]tttgggccagcc	
	C−142T		promoter	1	20	22	43	0.256	0.744	gtgttctgcctc[c/t]ggccctgtccgg	
	G−141C		promoter	28	15	0	43	0.826	0.174	tgttctgcctcc[g/c]gccctgtccggg	
	C1784T		intron1	30	17	1	48	0.802	0.198	tggatgcagaga[c/t]gccgtccctagc	
	A1918G	Ala122Ala	exon2	31	17	0	48	0.823	0.177	ggagggcgaggc[a/g]ggcaccagcagc	rs2304479
	A2141T		intron2	0	8	40	48	0.083	0.917	acaatagattaa[a/t]gcctgccgggga	rs2304480
	G2971A		intron2	47	1	0	48	0.990	0.010	tagggcctaggt[g/a]ctcgataccctg	
	C4527A	Thr180Lys	exon4	43	2	0	45	0.978	0.022	tgctgtcggtca[c/a]ggtgacctccat	
	C7479T	Phe341Phe	exon8	38	2	0	40	0.975	0.025	tggcaccttctt[c/t]ggaatgttctcc	
	C14272T		intron10	26	18	3	47	0.745	0.255	ctggctcagccc[c/t]caccgtggagtc	rs3816119
	G14277A		intron10	46	1	0	47	0.989	0.011	tcagcccccacc[g/a]tggagtccctga	
	C14363A	Ala464Ala	exon11	45	2	0	47	0.979	0.021	catcttcggggc[c/a]accctctcctct	
	C14366T	Thr465Thr	exon11	46	1	0	47	0.989	0.011	cttcggggccac[c/t]ctctcctctgcc	rs5801
	G17337A		intron13	44	1	0	45	0.989	0.011	ggggtgggagtg[g/a]gaggcatgggtg	
	T18806C[b]		intron13	6	24	18	48	0.375	0.625	gactggtgccct[t/c]ggcccagggtgg	rs2304483
	C18850T	Ala569Val	exon14	46	2	0	48	0.979	0.021	acaacaagtggg[c/t]ggcgctgtttgg	
	T20072C	Leu623Pro	exon15	46	1	0	47	0.989	0.011	gctcctacaacc[t/c]ggccctcagcta	
	G20088A	Ser628Ser	exon15	46	1	0	47	0.989	0.011	cctcagctactc[g/a]gtgggcctcaat	
	C20201G		intron15	46	1	0	47	0.989	0.011	gagtttccaagc[c/g]tagacctgtcac	
	G21421A		intron16	20	24	3	47	0.681	0.319	atgggggcccaa[g/a]gggatgcggagc	
	C21500T		intron16	42	2	0	44	0.977	0.023	ccctcttgctgg[c/t]ttctccccagc	
	C21566G		intron16	43	1	0	44	0.989	0.011	cactttctcccc[c/g]actccttgtgtt	
	A21586G		intron16	43	1	0	44	0.989	0.011	gtgttttcccttt[a/g]tctgggcaaaag	
	C21822T	Ala714Ala	exon17	21	21	3	45	0.700	0.300	ggatgtcattgc[c/t]gaggacctccgc	
	C22682T		intron17	46	1	0	47	0.989	0.011	tcacccctatcc[c/t]ctggcaggccgc	
	C25013T[c]		intron18	23	22	3	48	0.708	0.292	ctgggggagaag[c/t]tggacctcacct	rs3764264
	G27029A		intron20	18	25	4	47	0.649	0.351	ttttcttgtgac[g/a]gtggtgcctgag	
	C27646T[b]		intron20	6	26	15	47	0.404	0.596	aaggggcgttgg[c/t]ggggccctgggc	rs2278490
	T27681C[b]*		intron20	5	23	18	47	0.351	0.628	tggatgcgcggc[t/c]gctggctctgct	rs2278489
	*A27681C**			0	1	—		0.011	—	tggatgcgcggc[a/c]gctggctctgct	
	*T27681A**			—	0	—		—	—	tggatgcgcggc[t/a]gctggctctgct	
	T29320A	Leu849His	exon22	367	5	0	372	0.993	0.007	tcattccctatc[t/a]ccttggccgcaa	
	C29372T[c]	Gly866Gly	exon22	23	22	3	48	0.708	0.292	tgtgttcgtagg[c/t]ggccagattaac	rs5804
	G34262A		intron22	44	1	3	48	0.927	0.073	tctcaagaaaaa[g/a]taataacaataa	
	G34372A[d]	Arg904Gln	exon23	45	3	0	48	0.969	0.031	accagaaccctc[g/a]ggctgagcagta	
	C34588T		intron23	41	3	4	48	0.885	0.115	cacagggcaagg[c/t]ggctgcagcccc	
	T37125C		intron23	46	1	0	47	0.989	0.011	cctcaacccact[t/c]tctcgtccccag	
	C37210T	Asn931Asn	exon24	46	1	0	47	0.989	0.011	ggccactgtcaa[c/t]gagatgcggcgg	
	A37311G[e]		intron24	23	21	3	47	0.713	0.287	acgcgacacatc[a/g]ctgggtcaggga	rs2289117
	G39097A		intron24	29	1	0	30	0.983	0.017	gaggccatagac[g/a]tggtgaaggatt	
	C39119T		intron24	29	1	0	30	0.983	0.017	attgagtgacct[c/t]gatgatatggga	
	C39142T		intron24	40	7	0	47	0.926	0.074	gaagtgaccact[c/t]ggctttctcccg	rs3816118
	G39143A[d]		intron24	44	3	0	47	0.968	0.032	aagtgaccactc[g/a]gctttctcccgc	rs2289116
	C39203T	Ser967Phe	exon25	46	1	0	47	0.989	0.011	tgctggattact[c/t]ccgagacgctgc	