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## Appendix

The Study Group of Millennium Genome Project for Hypertension:

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*Original Article*

# Relationship between Metabolic Syndrome and Trp64Arg Polymorphism of the β3-Adrenergic Receptor Gene in a General Sample: The Shigaraki Study

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**It has been reported that the β3-adrenergic receptor gene (*ADRB3*) is associated with abnormal metabolic risk factors. Therefore, we examined whether the Trp64Arg polymorphism of *ADRB3* affects the occurrence of metabolic syndrome (MS). The participants were 2,395 subjects who underwent a medical examination in Shigaraki in Shiga, Japan. Among them, 1,416 subjects who gave informed consent for genetic analysis and were not receiving treatment for hypertension, diabetes, or hyperlipidemia were enrolled in this study. MS was diagnosed in 86 (16.0%) of 537 men, and 8 (0.9%) of 879 women. There was no significant relationship between *ADRB3* polymorphism and the frequency of MS. Multiple logistic regression analysis including smoking, sex, and age as confounding factors showed no interaction between MS and *ADRB3* polymorphism (odds ratio: 0.94; 95% confidence interval: 0.59–1.49; $p=0.78$). Subjects were also analyzed according to differences in the number of abnormal metabolic risk factors. However, there was no significant relationship between *ADRB3* polymorphism and the number of such factors. In conclusion, in a general sample, the frequency of MS was 16.0% in men, and 0.9% in women. There was no relationship between *ADRB3* polymorphism and MS. (*Hypertens Res* 2006; 29: 891–896)**

*Key Words*: **β3-adrenergic receptor gene, Trp64Arg polymorphism, metabolic syndrome, hypertension**

## Introduction

The β3-adrenergic receptor is expressed in visceral adipose tissue and is thought to contribute to lipolysis, energy metabolism and regulation of the metabolic rate (*1–3*). A missense mutation of *ADRB3* (Trp64Arg) in the first intracellular loop of the receptor has been reported. This mutation has been reported to be associated with diabetes mellitus (*4, 5*), abdominal obesity (*5*), obesity (*6*), insulin resistance (*5*) and the

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basal metabolic rate (*4*).

In Japan, Kadowaki *et al.* (*7*) reported that the frequency of the mutant allele in obese subjects was significantly higher than that in non-obese subjects. The presence of this mutation was also accompanied by significantly higher fasting and 2-h serum insulin levels during an oral glucose tolerance test (OGTT). Thus, they suggested that *ADRB3* might contribute to obesity and hyperinsulinemia/insulin resistance in Japanese subjects.

On the other hand, there have been several reports that the Trp64Arg mutation of *ADRB3* was not associated with glucose metabolism, non–insulin-dependent diabetes mellitus (NIDDM) (*8*), insulin resistance syndrome (*8*), obesity (*9*) or insulin sensitivity (*10*). We also reported that the Trp64Arg polymorphism of *ADRB3* did not appear to have any pathophysiological significance in Japanese samples (*11*).

People with metabolic syndrome (MS) are at high risk of developing type 2 diabetes (*12*) and cardiovascular disease (*13*). The Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (NCEP-ATP III) proposed a new definition of MS (*14*). In 2005, the International Diabetes Federation (IDF) (the IDF consensus worldwide definition of the metabolic syndrome; available from: http://www.idf.org/home/index.cfm?unode=1120071E-AAC E-41D2-9FA0-BAB6E25BA072) and a group of eight Japanese societies (*15*) announced new and separate diagnostic criteria for MS.

In our previous study employing a group of hypertensive patients, the prevalence of MS by the new Japanese criteria was 44.0% in men and 23.3% in women (*16*).

However, there have been no reports about the correlation between the new Japanese criteria of MS and *ADRB3* polymorphism. Therefore, in this study, we evaluated the relationship between the new Japanese criteria of MS and the Trp64Arg polymorphism of *ADRB3* in Japanese residents in a rural community.

## Methods

### Study Population

The Shigaraki Study was based on a medical examination undertaken in 1999 in Shigaraki, a farming community near Kyoto, western Japan (*17–22*). A total of 2,902 subjects underwent the examination, of whom 2,395 were enrolled in this genetic study after receiving a full explanation and providing informed consent. Of these subjects, 403 were excluded for the following reasons: undetermined genotype, $n=127$; a serum aspartate aminotransferase (AST [GOT]) or alanine aminotransferase (ALT [GPT]) level of over 100 IU/l, $n=13$; and/or a history of transient ischemic attack, stroke, angina pectoris, or myocardial infarction, $n=263$. Moreover, 576 (221 men and 355 women) were excluded because they were receiving treatment for hypertension, diabetes or hyperlipidemia. The remaining 1,416 subjects (537 men and 879 women) were analyzed. Subjects were between the ages of 30 and 79. This study was approved by the Institutional Review Board of Shiga University of Medical Science (Nos. 11-15, 1999).

### Measurement of Waist Circumference

The waist was measured at the level of the umbilicus using a tape measure; subjects wore an undergarment and stood during the measurement. Waist measurements were taken by the same person. Each measurement was performed in triplicate and the average value was used.

### Blood Pressure and Biochemical Examinations

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured twice using a standard sphygmomanometer on the right arm while the subject was seated after having rested for at least 5 min. Korotkov's first and fifth points were regarded as the SBP and DBP, respectively, and the blood pressure (BP) was measured by a well-trained nurse. The mean of 2 measurements from each subject was used for data analysis. The body mass index (BMI) was calculated as the weight (kg) divided by the square of the height (m). Non-fasting blood was drawn and serum total cholesterol (TC) and high density lipoprotein–cholesterol (HDL-C) levels were determined in one laboratory (Medic, Shiga, Japan). The measurement precision and accuracy of these serum lipids were certified through the lipid standardization program by Osaka Medical Center for Cancer and Cardiovascular Disease, which is a member of the Cholesterol Reference Method Laboratory Network (CRMLN) controlled by the centers for Disease Control and Prevention (Atlanta, USA) (*23*).

### Criteria of Metabolic Syndrome

We used the 2005 Japanese criteria of MS (*15*). Briefly, men with an abdominal circumference of ≥85 cm and women with an abdominal circumference of ≥90 cm were defined as abdominally obese. MS is defined as abdominal obesity plus at least 2 of the following abnormalities: hypertriglyceridemia (triglyceride [TG] ≥150 mg/dl), low HDL-C (HDL-C <40 mg/dl), hypertension (SBP ≥130 mmHg and/or DBP ≥85 mmHg), and impaired fasting glucose (fasting blood glucose level ≥110 mg/dl).

### Determination of the Trp64Arg Polymorphism of *ADRB3*

DNA was isolated from peripheral leukocytes and *ADRB3* genotypes were determined as previously reported (*1*). Genotypes, determined by the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method for 75 random samples consisting of 25 PCR products for each

**Table 1. Characteristics of the Study Population According to the Trp64Arg Polymorphism of *ADRB3* in Men and Women, Shigaraki Study in 1999 (Not Receiving Treatment for Hypertension, Diabetes or Hyperlipidemia)**

| Risk characteristics | Polymorphism of *ADRB3* | | | | | |
|---|---|---|---|---|---|---|
| | Men (537) | | | Women (879) | | |
| | Trp/Trp | Trp/Arg+Arg/Arg | *p*-value | Trp/Trp | Trp/Arg+Arg/Arg | *p*-value |
| *N* (1,416) | 368 | 157+12 | | 585 | 257+37 | |
| Age (years) | 55.5±15.5 | 53.6±16.1 | 0.1921 | 51.3±15.2 | 53.3±15.9 | 0.0789 |
| BMI (kg/m$^2$) | 22.4±2.8 | 22.2±3.0 | 0.3827 | 21.9±3.0 | 22.1±3.0 | 0.4327 |
| Waist (cm) | 80.2±8.3 | 79.4±9.0 | 0.3100 | 70.5±7.5 | 71.6±8.3 | 0.0404 |
| Abdominally obese (men waist ≥85 cm, women waist ≥90 cm) | 109/259 (29.6%) | 33+5/124+7 (22.5%) | 0.0851 | 6/579 (1.0%) | 6+0/251+37 (2.0%) | 0.2211 |
| Alcohol (P/N) | 282/86 | 115+9/42+3 | 0.4143 | 157/428 | 75+10/182+27 | 0.5160 |
| Smoking (P/N) | 279/89 | 119+10/38+2 | 0.8966 | 48/537 | 24+3/233+34 | 0.6242 |
| 130/85 (P/N) | 167/201 | 73+7/84+5 | 0.6726 | 110/475 | 51+5/206+32 | 0.9305 |
| TG/HDL (P/N) | 145/223 | 63+5/94+7 | 0.8543 | 205/380 | 93+15/164+22 | 0.6211 |
| TG (mg/dl) | 145.3±99.8 | 140.3±94.3 | 0.5815 | 106.2±62.0 | 108.6±65.9 | 0.6041 |
| HDL (mg/dl) | 52.8±14.3 | 55.0±15.1 | 0.1059 | 61.9±14.1 | 60.4±13.8 | 0.1276 |
| BG ≥110 (P/N) | 97/271 | 38+6/119+6 | 0.9370 | 118/467 | 58+6/199+31 | 0.5812 |
| MS (P/N) | 61/307 (16.6%) | 20+5/137+7 (14.8%) | 0.6008 | 4/581 (0.7%) | 4+0/253+37 (1.4%) | 0.3188 |

*N*: number of subjects. Values are the means±SD. *ADRB3*, β3 adrenergic receptor gene; BMI, body mass index; Waist, waist circumference; Alcohol, alcohol drinking habit; P/N, positive/negative; Smoking, smoking habit; 130/85, systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg; TG/HDL, triglyceride ≥150 mg/dl and/or high density lipoprotein cholesterol <40 mg/dl; BG ≥110, fasting blood glucose level ≥110 mg/dl; MS, frequency of metabolic syndrome.

genotype, were confirmed by direct sequencing. Briefly, after fractionation of PCR-RFLP products on 1% agarose gels (Nippon Gene, Toyama, Japan), the desired DNA bands were excised, and DNA was purified using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, USA), amplified with the above 5′ primer, and analyzed with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Norwalk, USA).

### Statistical Analysis

Data are expressed as the mean±SD. All statistical analyses were performed using the SAS statistical package. Two-way ANOVA and the $\chi^2$ test were used to assess differences among subjects with different genotypes. Since few subjects carried Arg/Arg genotypes, we combined the Trp/Arg and Arg/Arg genotypes for assessment.

To examine the independent contribution of *ADRB3* polymorphism to the risk of MS, while adjusting for the effects of other clinical characteristics, logistic analysis was used. For the risk of MS, a logistic model was developed with the following variables as covariates: sex, age, smoking, alcohol, and *ADRB3* polymorphisms.

## Results

Table 1 summarizes the characteristics of subjects according to the genotype of *ADRB3*. The Trp64Arg polymorphism of *ADRB3* was not significantly associated with age, BMI, waist, drinking and smoking habits, tendency toward hypertension, high TG and/or low HDL-C, blood glucose (BG) over 110 mg/dl, or the frequency of MS. The waist circumference of subjects with the Arg64 allele of Trp64Arg was significantly larger than that of subjects without the Arg64 allele ($p$=0.0404). In the assessment of genotypes not associated with the Trp64Arg polymorphism, the mean waist circumferences of subjects were 70.5±7.5 cm for subjects with Trp/Trp, 71.8±8.4 for subjects with Trp/Arg, and 70.6±7.6 for subjects with Arg/Arg ($p$=0.0835). There was no significant association between the waist circumference and *ADRB3* polymorphism.

The frequency of MS was 16.0% in men and 0.9% in women. There was no significant relationship between *ADRB3* polymorphism and the frequency of MS.

Multiple logistic regression analysis including smoking, sex, and age as confounding factors showed that each of these factors was associated with MS; however, there was no interaction between MS and *ADRB3* polymorphism (odds ratio: 0.94; 95% confidence interval: 0.59–1.49; $p$=0.78).

Tables 2 and 3 summarize the correlation between the Trp64Arg polymorphism of *ADRB3* and the number of abnormal metabolic risk factors in men and women, respectively. There was no relationship between *ADRB3* polymorphism and the number of abnormal metabolic factors in either sex ($p$=0.925 for men; $p$=0.765 for women).

## Discussion

Kadowaki *et al.* (*7*) and Yoshida *et al.* (*24*) reported that the

**Table 2. Relationship between the Trp64Arg Polymorphism of *ADRB3* and the Number of Abnormal Metabolic Factors in Men (Not Receiving Treatment for Hypertension, Diabetes or Hyperlipidemia)**

| Number of abnormal risk factors | Polymorphism of *ADRB3* | | *N* | |
|---|---|---|---|---|
| | Trp/Trp | Trp/Arg+Arg/Arg | | |
| 0 | 102 (68.0%) | 44+4 (32.0%) | 150 | $p=0.925$ |
| 1 | 150 (70.1%) | 63+1 (29.9%) | 214 | |
| 2 | 89 (67.4%) | 39+4 (32.6%) | 132 | |
| 3 | 27 (65.9%) | 11+3 (34.1%) | 41 | |
| | 368 | 157+12 | 537 | |

*N*: number of subjects. Abnormal metabolic factors: hypertriglyceridemia: triglycerides ≥150 mg/dl and/or high density lipoprotein cholesterol <40 mg/dl; hypertension: systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg; and impaired fasting glucose: fasting blood glucose level ≥110 mg/dl.

**Table 3. Relationship between the Trp64Arg Polymorphism of *ADRB3* Gene and the Number of Abnormal Metabolic Factors in Women (Not Receiving Treatment for Hypertension, Diabetes or Hyperlipidemia)**

| Number of abnormal risk factors | Polymorphism of the *ADRB3* | | *N* | |
|---|---|---|---|---|
| | Trp/Trp | Trp/Arg+Arg/Arg | | |
| 0 | 325 (67.7%) | 136+19 (32.3%) | 480 | $p=0.765$ |
| 1 | 144 (65.2%) | 65+12 (34.8%) | 221 | |
| 2 | 59 (62.8%) | 31+4 (37.2%) | 94 | |
| 3 | 57 (67.9%) | 25+2 (32.1%) | 84 | |
| | 585 | 257+37 | 879 | |

*N*: number of subjects. Abnormal metabolic factors: hypertriglyceridemia: triglycerides ≥150 mg/dl and/or high density lipoprotein cholesterol <40 mg/dl; hypertension: systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg; and impaired fasting glucose: fasting blood glucose level ≥110 mg/dl.

Trp64Arg mutation had pathological significance in Japanese populations. However, in our study of 1,416 subjects taking no medications for hypertension, diabetes or hyperlipidemia, the *ADRB3* polymorphism did not appear to have any significant influence on the various parameters associated with MS. Similarly, Yuan *et al.* (*25*) reported that *ADRB3* polymorphism was not a major genetic determinant of obesity or diabetes in the Japanese general population. Moreover, Masuo *et al.* (*26*) reported that the Trp64 allele was linked to BP elevation in a 5-year longitudinal study; however, our study indicated no significant relation between *ADRB3* polymorphism and BP. These discrepancies may be difficult to reconcile; however, it is possible that this polymorphism may have only a small effect on MS, and this may be masked by differences in the genetic background or environmental factors, including lifestyle. Another possibility is that the Trp64Arg polymorphism of *ADRB3* may not have any functional significance itself, but may be linked to an as-yet-unidentified mutation in this gene. Indeed, two previous studies have reported that expression of the Trp64Arg polymorphism of *ADRB3 in vitro* was associated with normal lipolytic function (*9*, *27*). However, Pietri-Rouxel *et al.* (*28*) reported a biochemical dysfunction induced by the Trp64Arg mutation in *ADRB3*, which could be important in pathophysiological situations pertaining to obesity. Indeed, although *ADRB3* exists on chromosome 8, Kissebah *et al.* (*29*) reported that trait loci on chromosomes 3 and 17 influence MS phenotypes. Thus the Trp64Arg polymorphism of *ADRB3* and its functional significance remain controversial.

Essential hypertension is reported to be associated with insulin resistance; however, Castellano *et al.* (*30*) reported that the Trp64Arg polymorphism of *ADRB3* has no relevant influence on BP levels or other features of insulin resistance syndrome. In Japan, Fujisawa *et al.* (*31*) reported that the Trp64Arg polymorphism of *ADRB3* did not play a major role in the susceptibility to essential hypertension or insulin resistance. In their study, as in our normal population in this study, the Trp64Arg polymorphism of *ADRB3* had no significant influence on various parameters associated with MS. Also, in their study, visceral obesity was reported to significantly affect glucose metabolism; however, the Trp64Arg polymorphism of *ADRB3* did not appear to have any significant effect on MS in this study. In the present study, it seemed that women with the Arg64 allele of Trp64Arg had a significantly larger waist circumference than those without the Arg64 allele ($p=0.0404$). However, there was no difference in waist circumference between subjects with and those without Trp64Arg polymorphism of *ADRB3* ($p=0.0835$).

In our sample, the frequency of MS was 16.0% in men and 0.9% in women. In the Tanno and Sobetsu Study, the fre-

quency of MS in a rural Japanese sample was 25.3% in men (*32*), and in a study in Okinawa, the frequency of MS was 30.2% in men and 10.3% in women (*33*). Our present data in men were comparable with the data from these previous studies, but our data in women were lower. This is possibly because the criteria for MS in the other studies were NCEP-ATP III and a waist girth over 85 cm. If we use a waist girth over 85 cm for women in this study, the frequency of MS was 5.8% in women in our sample. Compared with a previous study (*16*), the prevalence of MS in this study was much lower. This may be because our study subjects were not being treated, but the previous subjects were all being treated for hypertension. In addition, in the standard examination in this study, there was no relationship between *ADRB3* polymorphism and MS.

There are some limitations of our study. First, we only analyzed *ADRB3* as a candidate gene of MS. Although there are many other candidate genes of MS, such as the leptin, insulin receptor substrate-1, and adiponectin genes, these were not examined in this study. In particular, for adiponectin located at chromosome 3, Kissebah *et al.* (*29*) reported that trait loci on chromosomes 3 and 17 influenced MS phenotypes. Further investigation will need to be carried out using a large-scale sample. Second, not all the blood samples collected were fasting samples. If we use fasting blood collection, the frequency of MS will decrease and will not be related to MS. Third, the sample power was weak. A much larger number of subjects may be required to confirm the association.

In conclusion, in this general sample, the frequency of MS was 16.0% in men and 0.9% in women. There was no relationship between *ADRB3* polymorphism and MS.

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