ORIGINAL

Association between accumulation of visceral fat and the combination of $\beta 3$ adrenergic receptor Trp64Arg, $\beta 2$ adrenergic receptor Arg16Gly and uncoupling protein 1 -3826A>G polymorphisms detected by Smart Amplification Process 2

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Abstract. $\beta 2$ and $\beta 3$ adrenergic receptors ($\beta 2AR$, $\beta 3AR$) and uncoupling protein 1 ($UCP1$) have been considered as candidate genes for obesity. Although each polymorphism of $\beta 3AR$ Trp64Arg, $\beta 2AR$ Arg16Gly and $UCP1$ -3826A>G is known to be associated with obesity, the interaction among these polymorphisms is not fully understood. We analyzed $\beta 3AR$ Trp64Arg, $\beta 2AR$ Arg16Gly and $UCP1$ -3826A>G polymorphisms by the Smart Amplification Process 2 in 222 Japanese subjects without the medication of hypertension, dyslipidemia or diabetes, and investigated the association between the physical and metabolic characteristics and the combination of these polymorphisms. In analysis of the genotypes combination, only the carriers of both $\beta 2AR$ Arg/Arg and $UCP1$ G/G genotypes had significantly higher waist to hip ratio ($p=0.014$). In analysis of the alleles combination, a significant difference was observed in waist to hip ratio among the groups stratified by the carrying number of the alleles of $\beta 3AR$ Arg, $\beta 2AR$ Arg and $UCP1$ G ($p=0.026$), and the waist to hip ratio was significantly higher in the carriers of four and five risk alleles than in the carriers from zero to three risk alleles ($p=0.005$). The present study demonstrated the interaction among $\beta 3AR$ Trp64Arg, $\beta 2AR$ Arg16Gly and $UCP1$ -3826A>G for the accumulation of visceral fat.

Key words: Visceral fat, Smart Amplification Process (SmartAmp), $\beta 2$ adrenergic receptor, $\beta 3$ adrenergic receptor, Uncoupling protein 1

OBESITY is determined by the interaction between genetic and environmental factors [1]. The $\beta 2$ and $\beta 3$ adrenergic receptors ($\beta 2AR$, $\beta 3AR$) and uncoupling protein 1 ($UCP1$) have been considered as candidate genes for obesity because of their roles in the regulation of lipolysis and thermogenesis. The Trp64Arg polymorphism of $\beta 3AR$ was first reported to be associated with early onset of diabetes mellitus, acceler-

ated weight gain, abdominal obesity and insulin resistance [2, 3, 4]. In Japanese, this polymorphism was also associated with higher body mass index (BMI) [5], difficulty in weight loss and lower basal metabolic rate [6]. The Arg16Gly polymorphism of $\beta 2AR$ was found to be associated with altered receptor function because Gly16 carriers showed a five-fold increase in agonist sensitivity, although the polymorphism was not associated with obesity in Swedish women [7]. On the other hand, the Gln27Glu polymorphism of $\beta 2AR$ was associated with higher BMI and larger fat cell size in the same study. In the subsequent study, the Arg16 allele was reported to be associated with high frequency of obesity [8], difficulty in weight reduction [9]

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and higher BMI [10]. The -3826A>G polymorphism of *UCP1* was reported to be associated with obesity in Canada [11]. Interaction between $\beta 3AR$ Trp64Arg and *UCP1* -3826A>G was reportedly related to weight gain [12], resistance to weight loss [13] and lower BMR [14]. Interaction between $\beta 3AR$ Trp64Arg and $\beta 2AR$ Arg16Gly was reported to be associated with changes in fat mass and abdominal adiposity to training [15]. Despite the reports on these interactions, the relationship among three polymorphisms of $\beta 3AR$ Trp64Arg, $\beta 2AR$ Arg16Gly and *UCP1* -3826A>G for obesity remains to be elucidated. It is important to study the possible associations among multiple polymorphisms of these candidate genes in the pathogenesis of obesity.

The Smart Amplification Process 2 (SmartAmp2) is a unique genotyping technology that can detect a genetic mutation accurately in a single step for 30min under the isothermal condition using a drop of whole blood [16]. Therefore, the method is useful to analyze the multiple polymorphisms for many subjects with a simple procedure in the clinical setting.

In the present study, we investigated the association between physical and metabolic characteristics and the combination of $\beta 3AR$ Trp64Arg, $\beta 2AR$ Arg16Gly and *UCP1* -3826A>G polymorphisms analyzed by the SmartAmp2 method in Japanese subjects.

Material and Methods

Subjects

Participants were recruited from on going health survey for the evaluation of metabolic syndrome and glucose tolerance in the five areas (Maebashi-city, Ogo-town, Gunma-town, Nakanojo-town and Yoshioka-town) in Gunma prefecture in Japan. We selected the 222 participants (91 men, aged 26-81 years; 131 women, aged 31-83 years) without the medication of hypertension, dyslipidemia or diabetes by interviewing on lifestyle habits and medical history. Written informed consent was obtained from all participants.

This study was conducted with the approval of the ethics committee of Gunma University Graduate School of Medicine.

Physical and blood chemistry examinations

Body weight and body fat percentage were measured using MC-190 Inner Scan (Tanita, Tokyo, Japan), and body mass index (BMI) was calculated as a weight/height² (kg/m²). Waist circumference was measured

in the standing position midway between the iliac crest and the lower costal margin and hip circumference was measured at its maximum.

Venous blood samples were drawn from the participants after overnight fasting. Plasma glucose, insulin, total cholesterol, HDL cholesterol, and triglyceride were measured at the Clinical Laboratory Center of Gunma University Hospital. Insulin resistance was examined by homeostasis model assessment of insulin resistance (HOMA-IR) using the following formula: fasting plasma glucose (mg/dL) $\times$ fasting insulin (μ U/mL) 405⁻¹ [17].

Genotyping

The three polymorphisms of $\beta 3AR$ Trp64Arg, $\beta 2AR$ Arg16Gly and *UCP1* -3826A>G were genotyped by the SmartAmp2 method using $\beta 3AR$, *UCP1* and $\beta 2AR$ Typing Kit (K.K. DNAFORM, Yokohama, Japan). The detailed procedure of the SmartAmp2 method was described before [16]. In brief, templates used for the SmartAmp2 were the whole blood samples collected with the tube containing EDTA-3K. The whole blood samples were diluted three times with pretreatment solution, and were denatured at 98°C for 3 min. After chilling on ice, the denatured samples were mixed with reaction buffer containing SYBR Green I (Takara Bio Inc., Otsu, Japan), *Aac* DNA polymerase (K.K. DNAFORM, Yokohama, Japan) and the wild type or mutant primer of each polymorphism. SmartAmp2 reaction mixtures were incubated at 60°C for 40 min under isothermal condition in a 7500 Real-Time PCR Systems (Applied Biosystems, Forester City, CA, US), where changes in the fluorescence intensity of SYBR Green I dye indication DNA amplification were monitored during the reaction. We detected the genotypes of each polymorphism by the presence or absence of DNA amplification at the time of 40 min, according to the products manual.

Statistical analysis

The results of each measurement are expressed as mean $\pm$ SD. The genotype frequencies were tested for Hardy-Weinberg equilibrium by χ^2 analysis. The association between genotypes of each polymorphism and the metabolic characteristics was determined using one-way analysis of variance (ANOVA). The interaction between two polymorphisms in physical characteristics was analyzed by two-way ANOVA. The relationship among three polymorphisms was analyzed by

Table 1 Physical and metabolic characteristics among the three genotypes of $\beta 3AR$ Trp64Arg, $\beta 2AR$ Arg16Gly and $UCP1$ -3826A>G polymorphisms.

		$\beta 3AR$ Trp64Arg				$\beta 2AR$ Arg16Gly				$UCP1$ -3826A>G			
		Trp / Trp	Trp / Arg	Arg / Arg	<i>p</i>	Arg / Arg	Arg / Gly	Gly / Gly	<i>p</i>	A / A	A / G	G / G	<i>p</i>
Men/Women		52 / 90	35 / 35	4 / 6		29 / 24	45 / 70	17 / 37		15 / 34	54 / 68	22 / 29	
Age	years	63.1±10.6	63.6±9.6	65.3±8.8		63.6±10.9	63.4±10.6	63.2±8.8		64.1±7.9	62.9±11.4	63.8±9.2	
Weight	kg	56.4±9.5	56.8±9.3	57.0±6.3	0.682	56.8±10.2	56.6±9.2	56.2±8.6	0.482	56.0±6.8	56.6±10.1	57.1±9.3	0.622
BMI	kg/m ²	22.9±2.8	22.5±2.6	22.4±2.5	0.433	22.6±2.7	22.8±2.9	22.7±2.3	0.791	22.9±2.2	22.7±2.9	22.8±2.6	0.869
Waist circumference	cm	82.7±8.4	83.5±7.5	85.0±9.9	0.737	82.7±7.6	83.5±8.3	82.4±8.6	0.530	83.5±6.9	82.4±8.9	84.1±7.5	0.343
Waist to hip ratio		0.89±0.07	0.90±0.06	0.90±0.08	0.567	0.90±0.07	0.90±0.07	0.89±0.07	0.779	0.90±0.06	0.89±0.07	0.91±0.06	0.095
Body fat	%	25.5±7.8	24.3±7.7	25.4±8.9	0.996	22.3±7.4	25.9±8.0	26.3±7.1	0.141	25.9±8.2	24.8±8.1	25.1±6.6	0.940
Systolic blood pressure	mmHg	134.6±21.4	134.0±19.3	127.1±20.9	0.437	135.4±22.0	132.7±20.8	135.6±19.2	0.581	134.9±20.9	132.6±20.6	136.6±20.7	0.526
Diastolic blood pressure	mmHg	76.2±12.5	76.3±11.6	72.8±14.1	0.676	76.6±13.8	74.8±11.7	78.4±11.6	0.162	76.1±12.4	75.3±12.8	78.1±10.7	0.351
Total cholesterol	mg/dL	210.7±34.0	218.7±33.6	217.3±22.6	0.086	212.1±33.2	214.2±33.9	213.5±33.7	0.907	216.2±36.2	212.1±32.6	214.4±33.5	0.901
HDL cholesterol	mg/dL	62.0±16.0	58.9±13.7	66.7±14.0	0.329	62.3±14.8	61.0±16.5	60.6±12.9	0.293	61.2±15.7	62.3±16.1	58.7±12.5	0.295
Triglyceride	mg/dL	103.0±56.6	117.7±71.4	92.7±42.2	0.292	96.3±48.2	110.6±63.4	110.5±67.9	0.171	103.7±44.4	107.3±63.8	110.1±69.8	0.937
Plasma glucose	mg/dL	101.7±12.3	104.3±18.4	102.1±7.6	0.711	103.0±15.2	101.4±13.5	104.4±15.2	0.281	102.0±12.4	101.9±14.5	104.6±15.8	0.522
Insulin	μU/mL	4.72±2.54	4.03±2.36	3.36±2.16	0.057	4.06±2.35	4.47±2.76	4.74±1.96	0.307	4.36±2.26	4.37±2.66	4.68±2.31	0.738
HOMA-IR		1.20±0.68	1.07±0.80	0.86±0.62	0.196	1.06±0.72	1.14±0.80	1.22±0.53	0.404	1.11±0.63	1.12±0.77	1.23±0.68	0.617

Data are expressed as mean ± SD. *p* are analyzed after adjusting for age and sex by one-way ANOVA. HOMA-IR, homeostasis model assessment of insulin resistance

one-way ANOVA comparing the physical characteristics among the groups stratified by the carrying number of risk alleles. Comparisons between two groups were tested by unpaired Student's *t* test or Mann-Whitney U test as appropriate. Mean values, after adjusting for age and sex, were evaluated by analysis of covariance (ANCOVA).

All statistical analyses were performed using SPSS Statistics version 19.0 (SPSS, Chicago, IL, US).

Results

Physical data and metabolic characteristics with the genotypes of $\beta 3AR$ Trp64Arg, $\beta 2AR$ Arg16Gly and $UCP1$ -3826A>G

In the present study, we analyzed $\beta 3AR$ Trp64Arg, $\beta 2AR$ Arg16Gly and $UCP1$ -3826A>C polymorphisms using the SmartAmp2 method. In the 222 participants, 142 individuals (64.0%) were carrying the Trp/Trp, 70 (31.5%) the Trp/Arg, and 10 (4.5%) the Arg/Arg for $\beta 3AR$ genotype. Fifty-three individuals (23.9%) were carrying the Arg/Arg, 115 (51.8%) the Arg/Gly, and 54 (24.3%) the Gly/Gly for $\beta 2AR$ genotype. Forty-nine individuals (22.0%) were carrying the A/A, 122

(55.0%) the A/G, and 51 (23.0%) the G/G for $UCP1$ genotype. The distributions of these polymorphisms were in Hardy-Weinberg equilibrium.

The physical and biochemical characteristics of the carriers with the three genotypes of $\beta 3AR$ Trp64Arg, $\beta 2AR$ Arg16Gly and $UCP1$ -3826A>C polymorphisms are shown in Table 1. The physical characteristics of BMI, waist circumference, waist to hip ratio, body fat and systolic and diastolic blood pressure and the metabolic characteristics of total cholesterol, HDL cholesterol, triglyceride, plasma glucose and HOMA-IR were not significantly different among the carriers of the three genotypes of each polymorphism.

Association between the physical characteristics and the risk genotypes combination of $\beta 3AR$ Arg/Arg, $\beta 2AR$ Arg/Arg and $UCP1$ G/G

We investigated the association between the metabolic characteristics and the polymorphisms of the $\beta 3AR$ Trp64Arg, the $\beta 2AR$ Arg16Gly and the $UCP1$ -3826A>G, comparing the characteristics among the carriers of risk genotypes combination of $\beta 3AR$ Arg/Arg, $\beta 2AR$ Arg/Arg and $UCP1$ G/G. In the present study, there were no carriers of all three risk geno-

Fig. 1 Association between physical characteristics and the combination of the risk genotypes of $\beta 3AR$ Arg/Arg, $\beta 2AR$ Arg/Arg and $UCP1$ G/G. Each bar represents the mean $\pm$ SD. The number of subjects is written in parentheses above the graph bars. $p\ddagger$ written above each graph represent the interaction between two risk genotypes and are analyzed after adjusting for age and sex by two-way ANOVA. A. Physical characteristics of BMI, waist circumference and waist to hip ratio among the four groups stratified with or without the $\beta 3AR$ Arg/Arg and the $\beta 2AR$ Arg/Arg. B. Physical characteristics among the four groups stratified with or without the $\beta 3AR$ Arg/Arg and the $UCP1$ G/G genotype. C. Physical characteristics among the four groups stratified with or without the $\beta 2AR$ Arg/Arg and the $UCP1$ G/G genotype. $p\ddagger$ represent the comparison between each group and the carriers of both $\beta 2AR$ Arg/Arg and $UCP1$ G/G genotypes after adjusting for age and sex.

types simultaneously. Twenty subjects were carrying two risk genotypes, three were carrying $\beta 3AR$ Arg/Arg and $\beta 2AR$ Arg/Arg, three carrying $\beta 3AR$ Arg/Arg and $UCP1$ G/G, and fourteen carrying $\beta 2AR$ Arg/Arg and $UCP1$ G/G.

The relationships between two polymorphisms of the $\beta 3AR$ Arg/Arg, the $\beta 2AR$ Arg/Arg and the $UCP1$ G/G in physical characteristics are shown in Fig. 1. Between the $\beta 2AR$ Arg/Arg and the $UCP1$ G/G, a significant interaction was observed in waist to hip ratio analyzed after adjusting for age and sex by two-way ANOVA ($p=0.014$), although the significant interactions were not observed in BMI ($p=0.590$) and waist circumference ($p=0.092$). The waist to hip ratio in the carriers of the $\beta 2AR$ Arg/Arg and the $UCP1$ G/G geno-

types was significantly higher than that in the carriers of only $\beta 2AR$ Arg/Arg genotypes ($p=0.006$), the carriers of only $UCP1$ G/G genotypes ($p=0.011$) and the non-carriers of these genotypes ($p=0.009$). Thus the carriers of the risk genotypes of both $\beta 2AR$ Arg16Gly and $UCP1$ -3826A>G had significantly higher waist to hip ratio. When the comparison was made separately for men and women, this interactions in waist to hip ratio was not significant.

The significant interactions between the $\beta 3AR$ Trp64Arg and the $\beta 2AR$ Arg16Gly and between the $\beta 3AR$ Trp64Arg and the $UCP1$ -3826A>G were not observed in the physical characteristics, although the carriers of $\beta 3AR$ Arg/Arg and $\beta 2AR$ Arg/Arg genotypes tended to have low waist circumference and waist to

Fig. 2 Association between physical characteristics and the combination of the risk alleles of $\beta 3AR$ Arg, $\beta 2AR$ Arg and $UCP1$ G. A. Physical characteristics of BMI, waist circumference and waist to hip ratio among stratified to the carrying number of the risk alleles of the $\beta 3AR$ Arg, the $\beta 2AR$ Arg and the $UCP1$ G. B. Physical characteristics between the non-carriers and the carriers of one, two and three risk alleles (0-3) and the carriers of four and five risk alleles (4+5). The number of subjects is written in parentheses above the graph bars. p are analyzed after adjusting for age and sex.

hip ratio. In the physical characteristics of systolic and diastolic blood pressure and the metabolic characteristics of total cholesterol, HDL cholesterol, triglyceride, plasma glucose and HOMA-IR, there were no significant interactions between two polymorphisms of the $\beta 3AR$ Trp64Arg, the $\beta 2AR$ Arg16Gly and the $UCP1$ -3826A>G (data not shown).

Association between the physical characteristics and the risk alleles combination of $\beta 3AR$ Arg, $\beta 2AR$ Arg and $UCP1$ G

To investigate the relationship among the risk alleles of the $\beta 3AR$ Arg, the $\beta 2AR$ Arg and the $UCP1$ G, we compared the physical characteristics of BMI, waist circumference and waist to hip ratio among the groups

stratified by the carrying number of these risk alleles (Fig. 2). Although there was no difference in BMI ($p=0.659$) and waist circumference ($p=0.165$), a significant difference was observed in waist to hip ratio among the six groups ($p=0.026$). Particularly, the carriers of four and five alleles had significantly higher waist to hip ratio than the non-carriers and the carriers of one, two and three alleles ($p=0.005$). These differences of the waist to hip ratio were significant only in women among the groups stratified by the carrying number of these alleles ($p=0.015$) and between the carriers of four and five alleles and the carriers from zero to three alleles ($p=0.008$). In the metabolic characteristics, there were no differences among carrying number of risk genotypes and alleles (data not shown).

Discussion

In the present study, we investigated the relationship between obesity and polymorphisms of $\beta 3AR$ Trp64Arg, $\beta 2AR$ Arg16Gly and $UCP1$ -3826A>G, compared with the combination of risk genotypes and risk alleles in Japanese subjects. According to the analysis of risk genotypes combination, only the carriers of both $\beta 2AR$ Arg/Arg and $UCP1$ G/G genotypes had significantly higher waist to hip ratio. To our knowledge, this is the first report showing a significant difference between the $\beta 2AR$ Arg/Arg and the $UCP1$ G/G in waist to hip ratio as the accumulation of visceral fat. In contrast, the relationships between the $\beta 3AR$ Trp64Arg and the $\beta 2AR$ Arg16Gly and between the $\beta 3AR$ Trp64Arg and the $UCP1$ -3826A>G were not observed in the physical characteristics. Synergic effect of $\beta 3AR$ Trp64Arg and $UCP1$ -3826A>G was demonstrated in the studies showing both $\beta 3AR$ Arg allele and $UCP1$ G allele were related to weight gain [12], resistance to weight loss [13] and lower BMR [14]. In regard to the interaction between $\beta 3AR$ Trp64Arg and $\beta 2AR$ Arg16Gly, both $\beta 3AR$ Arg allele and $\beta 2AR$ Arg allele carriers in Black subjects made the total fat mass and total abdominal fat significantly lower after training in HERITAGE study, suggesting that the carriers of $\beta 3AR$ Arg and $\beta 2AR$ Arg alleles might reduce abdominal fat easily [15]. The carriers of $\beta 3AR$ Arg/Arg and $\beta 2AR$ Arg/Arg genotypes in the present study also tended to have low waist circumference and waist to hip ratio. The carriers of $\beta 3AR$ Arg/Arg and $UCP1$ G/G genotypes did not show significant changes in the physical characteristics. These results may be, at least in part, due to the presence of few carriers of $\beta 3AR$ Arg/Arg genotype in the present study.

According to the comparison of risk alleles combination, there was a significant difference in waist to hip ratio among the groups stratified by the carrying number of $\beta 3AR$ Arg, $\beta 2AR$ Arg and $UCP1$ G alleles. Notably, the waist to hip ratio was significantly higher in the carriers of four and five risk alleles than in the carriers from zero to three risk alleles. The present study demonstrated that waist to hip ratio increased steeply at four risk alleles as the cut off number. To date, there have been no reports regarding the association between obesity and the combination of the multiple risk alleles. Because obesity is occurred by the multiple genetic factors, it is crucial to investigate the association between obesity and the combination of risk alleles or genotypes

as performed in the present study.

The association between waist to hip ratio and the carrying number of risk alleles was observed only in women in the present study. In addition, the significant change was not observed for the combination of both risk alleles and genotypes in the physical characteristics of blood pressure and the metabolic characteristics such as glucose and lipid. We collected the subjects without the medication of hypertension, dyslipidemia or diabetes, because the physical characteristics were supposed to be affected by the medical treatment. Thus, the number of subjects was small especially for men, and the few subjects showed extremely outlier in the metabolic characteristics. Because the sample size was relatively small for the enough statistical power in the present study, it is necessary to evaluate the association between the combination of these polymorphisms and each physical and metabolic characteristic in further studies with larger sample size.

While $\beta 3AR$ is expressed in white adipocyte and brown adipocyte with the role of lipolysis and thermogenesis respectively, $\beta 2AR$ is expressed only in white adipocyte with the role of lipolysis and $UCP1$ is expressed only in brown adipocyte with the role of thermogenesis. $\beta 2AR$ and $UCP1$ are not able to act directly with each other because of their expression in different types of adipocytes. However, in the present study, the waist to hip ratio as the accumulation of visceral fat increased only in the carrier of both $\beta 2AR$ Arg/Arg and $UCP1$ G/G genotypes. These effects, therefore, might be mediated by other mechanisms related to these proteins expressed in different tissues.

In the SmartAmp2 method, all process can be performed in a single step because it is not necessary to purify the genome from blood and the polymorphisms can be directly detected by the presence or absence of DNA amplification. In addition, the accurate determination is achieved by reducing the nonspecific amplification using unique asymmetrical primer set with a mismatch binding protein or a competitive probe. Therefore, the SmartAmp2 method is utilized in clinical settings, such as highly sensitive detection of the *EGFR* mutations in lung cancer [18] and detection of polymorphisms of *CYP2C9* and *VKORC1* to adjust warfarin dose [19]. The SmartAmp2 is useful to determine multiple polymorphisms in a large number of subjects for the implementation of personalized medicine, such as prevention of disorders related to obesity.

In conclusion, the present study demonstrated that

increased waist to hip ratio was associated with the accumulation of each risk allele of $\beta 3AR$ Trp64Arg, $\beta 2AR$ Arg16Gly and $UCP1$ -3826A>G. These results suggest the interaction among $\beta 3AR$ Trp64Arg, $\beta 2AR$ Arg16Gly and $UCP1$ -3826A>G for the accumulation of visceral fat.

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