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PCR and restriction enzyme analysis based diagnostic system for thermogenesis regulation genes

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Abstract

Since obesity is major risk factor of life style related diseases such as type II diabetes mellitus and dyslipidemia, prevention and treatment of obesity is a most essential issue from the public health perspective. Recently it has been shown that the polymorphisms of energy metabolism related genes such as β3-adrenergic receptor (β3-AR) are closely related to obesity, insulin resistance and type II diabetes mellitus. In the case that a person carries plural polymorphisms of these genes, it is expected that the effects of gene polymorphism will be more pronounced than in the case of single polymorphism or normal type. The relationship of plural polymorphism of these genes to obesity and insulin resistance should be examined in detail. Whereas usual DNA analysis is complicated and takes much time, in this paper we examined a DNA analysis system using © FTA Matrix cards and PCR and restriction enzyme analysis for the energy metabolism related genes that takes less time. We also investigated the association of the simultaneous occurrence of the polymorphisms in β3-AR, UCP1 and UCP3 with obesity and insulin resistance.

Using our system, we successfully analyzed the gene variation related to the mechanisms of thermogenesis and lipolysis. This analysis needs only a few drops of whole blood using the © FTA Matrix card. The required time for analysis is about 5 hours, much less than analysis by previous methods. There was no significant difference found in physical and biochemical characteristics between the normal group and the groups with gene variance. This system could enable epidemiological study of the plural polymorphisms of energy metabolism related genes, that are associated with obesity and insulin resistance, and could be useful for personalized medical care in the future.

Introduction

Prevention and treatment of obesity is a most essential issue from the public health perspective because the prevalence of obesity is gradually increasing in Japan[1)]. Obesity, especially abdominal obesity, is associated with several major life style related diseases such as type II diabetes mellitus and dyslipidemia[2,3)]. Obesity is prescribed by balance of energy intake and expenditure, genetic and environmental factors[4)].

It is thought that the β3-adrenergic receptor (β3-AR) plays an important role in thermogenesis[5)]. Some studies reported that the T → C polymorphism at nucleotide position 190 base pairs (bp) of β3-AR gene is associated with visceral obesity and an earlier onset of type II diabetes[6-8)]. The uncoupling protein 1 (UCP1) is another factor that influences thermogenesis[9)]. A → G polymorphism at position −3826 bp of UCP1 was positively associated with BMI[10)]. C → T polymorphism at position −55 bp of uncoupling protein 3

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(UCP3) is associated with increased BMI[11]. Furthermore, polymorphism of UCP3, expressed predominantly in skeletal muscle[12,13], has drawn much attention.

These three thermogenesis-related genes were investigated alone and in combination in many subjects. However, there is no study that investigates these three genes at the same time. Therefore, an easy and speedy technique of DNA analysis should be established for the appropriate prescription of individuals.

In this paper, we reported on a comparative DNA analysis system using PCR and restriction enzyme analysis for energy metabolism related genes. We also investigated the association of the simultaneous occurrence of polymorphisms in β3-AR, UCP1 and UCP3 with obesity and insulin resistance.

SUBJECTS AND METHODS

Subjects and Ethics

Present study was authorized by the ethics committees of Tokyo Medical University. Written informed consent was obtained from all subjects before participation in this study. Subjects were 30 healthy Japanese (10 men and 20 women ; mean age, 31.0±4.3 years) who had no history of type II diabetes mellitus, hypertension or dyslipidemia.

Blood samples

Peripheral blood samples were taken from subjects in the morning after an overnight fast.

For DNA analyses, 500 μℓof whole blood was spotted on © FTA Matrix card (FTA Classic Card : Whatman BioScience, Newton, MA, USA), and the card was allowed to air dry for one hour at room temperature until completely dry[14]. The cards were stocked in desiccators per the manufacture's instruction.

For biochemical analyses, sera were separated immediately and stored at −80°C until needed.

BMI analysis

Measurements of physique were body height, body weight, body mass index (BMI ; the weight in kilograms divided by the square to the height in meters), waist circumference at the umbilicus, maximum hip circumference and the waist to hip (W/H) ratio (the ratio of the waist circumference in centimeters to the hip circumference in centimeters).

Biochemical analysis

Serum triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and immunoreactive insulin (IRI) of serum samples and glucose of plasma sample were analyzed using an enzymatic technique (TG, TC, LDL-C and glucose), direct technique (HDL-C), and enzyme immunoassay technique (IRI) performed at SRL, Inc (Tokyo, Japan).

Insulin resistance was assessed by homeostasis model assessment (HOMA)[15]. The arteriosclerosis index (AI) was calculated with the following formula : TC (mg/dl)-HDL-C (mg/dl)/HDL-C (mg/dl).

DNA Analyses

(1) DNA preparation

A disk of diameter 2.0 mm was removed from within the middle of the dried stain and transferred to a PCR tube. The disk was washed two times each with © FTA Purification Reagent and followed by TE (10 mM Tris-HCl pH8.0, 0.1 mM EDTA pH8.0). After washing, the disk was dried completely and used as a PCR template matrix.

(2) PCR amplification

The targeted DNA sequence was amplified by PCR with the template matrix disk and 50 μℓ of PCR mixture containing, 25 μℓ of Premix Taq (ExTaq version ; TAKARA, Ohtsu, Japan), 1 μℓ each of primers (Table 1), and 23 μℓ of ultra pure water (Milli-Q Synthesis A10, Millipore, Tokyo, Japan).

(3) Restriction Enzyme analyses

The PCR was performed with denaturation at 94°C for 5 minutes, followed by 35 cycles (β3-AR and UCP3) or 30 cycles (UCP1) of denaturation at 94°C for 30 seconds, annealing at 61°C for 30 seconds, extension at 72°C for 30 seconds, with a final extension at 72°C for 10 minutes.

Seventeen μℓ of products were digested in a final volume of 20μℓ of mixture containing of 2μℓ 10×buffer and 1μℓ restriction enzyme. The following restriction enzyme were used to distinguish the point mutation in each genes : T → C polymorphism of β3-AR, *Eco*R II(TAKARA, Ohtsu, Japan) ; A → G polymorphism of UCP1, *Fba* I (TAKARA, Ohtsu, Japan) ; C → T polymorphism of UCP3, *Hae* III (TAKARA, Ohtsu, Japan). Mixtures were incubated at 37°C for one hour.

(4) Agarose gel electrophoresis and observation

PCR products and digested samples were separated on 3 percent Metaphor agarose gel (FMC Bio Products) and visualized under UV illumination with ethidium bromide staining.

Table 1 List of primers

	Forward	Reverse
beta 3-AR[7]	5′-CGC CCA ATA CCG CCA ACA C	5′-CCA CCA GGA GTC CCA TCA CC
UCP1[16]	5′-CCA GTG GTG GCT AAT GAG AGA A	5′-GCA CAA AGA AGA AGC AGA GAG G
UCP3[17]	5′-GGA TAA GGT TTC AGG TCA GGC	5′-AAG GGA TGA GGG AGG AGA AA

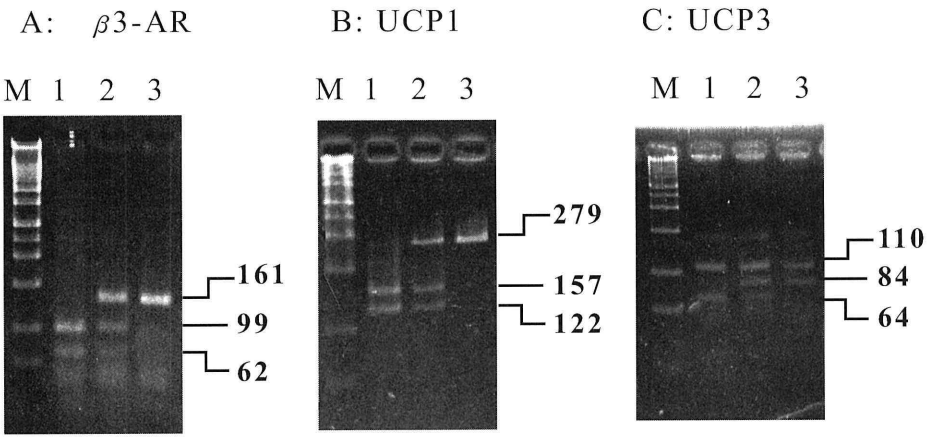

Fig. 1 Detection of Polymorphism by PCR and Analysis of Restriction-Fragment-Length Polymorphism
In all panels, lane M showed the molecular marker (Hi-Lo™).
In the panel A, the PCR products of the β3-adrenergic receptor (210 bp) were digested with the restriction enzyme *Eco*R II and visualized by staining with ethidium bromide. Lane 1 : normal homozygote (TT), lane 2 : heterozygote (TC), lane 3 : homozygote with variances (CC).
In the panel B, the PCR products of UCP1 (279 bp) were digested with the restriction enzyme *Fba* I and visualized by staining with ethidium bromide. Lane 1 : normal homozygote (AA), lane 2 : heterozygote (AG), lane 3 : homozygote with variances (GG).
In the panel C, the PCR products of UCP3 (194 bp) were digested with the restriction enzyme *Hae* III and visualized by staining with ethidium bromide. Lane 1 : normal homozygote (CC), lane 2 : heterozygote (CT), lane 3 : homozygote with variances (TT).

Statistical analyses

All statistics were performed by StatView version 5.0. Comparison of physical and biochemical data between groups divided by genotype were tested by the Mann-Whitney U test. The results are presented as means± SD.

Results

Restriction-Fragment-Length Polymorphism

The PCR fragment of the β3-AR was 210 bp long and was digested with the restriction enzyme *Eco*R II. The T allele showed four fragments (99, 62, 30 and 12 bp). The C allele showed three fragments (161, 30 and 12 bp) (Figure 1 A).

The PCR fragment of the UCP1 was 279 bp long and was digested with the restriction enzyme *Fba* I. The A allele showed two fragments (157 and 122 bp). The G allele showed only 279 bp (Figure 1 B).

The PCR fragment of the UCP3 was 194 bp long and was digested with the restriction enzyme *Hae* III. The C allele showed three fragments of 110, 64 and 20 bp). The T allele showed two fragments (110 and 84 bp) (Figure 1C).

A result of one case (31 years old, woman : BMI : 21. 0 kg/m², W/H ratio : 0.74, TC : 199 mg/dl, HDL-C : 68 mg/dl, LDL-C : 107 mg/dl, TG : 51 mg/dl, glucose : 108 mg/dl, IRI : 11.9 μU/ml, AI : 1.9, HOMA : 3.17) is shown in Figure 2. In this case, the subject had normal homozygous β3-AR and heterozygous UCP1 and UCP3 genes.

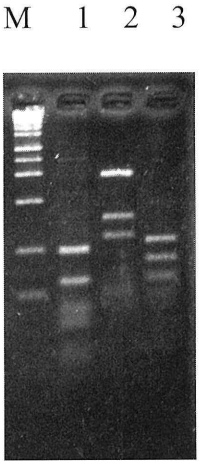

Fig. 2 Analysis of Restriction-Fragment-Length of one case
Lane M : molecular marker, Lane 1 : normalβ3-AR homozygote (TT), lane 2 : UCP1 heterozygote (AG), lane 3 : UCP3 heterozygote (CT)

Allele frequencies

The distribution of the genotypes defined by the T → C polymorphism of the β3-AR gene, the A → G polymorphism of the UCP1 gene and the C → T polymorphism of the UCP3 gene is presented in Table 2.

In the T → C polymorphism of β3-AR gene, 20 (66.7%) subjects were T homozygous (TT), 9 (30.0%) subjects were TC heterozygous (TC), and 1 (3.3%) subject was C homozygous (CC). The frequency of the C allele was 0.18.

Table 2 Distribution of genotypes defined by T → C polymorphism of the beta3-AR gene, A → G polymorphism of the UCP1 promoter gene and C → T polymorphism of the UCP3 promoter gene among 30 healthy Japanese

	Normal Homozygous	With variance	
		Heterozygous	Homozygous
beta 3-AR	20 (66.7%)	9 (30.0%)	1 (3.3%)
UCP1	8 (26.7%)	20 (66.7%)	2 (6.7%)
UCP3	15 (50.0%)	13 (43.3%)	2 (6.7%)

Table 3 Numbers of subjects of each genotype combination among β 3-AR, UCP1 and UCP3

		Beta 3-AR								
		TT			TC			CC		
UCP1		AA	AG	GG	AA	AG	GG	AA	AG	GG
UCP3	CC	4 (13.3%)	4 (13.3%)	2 (6.7%)	0	4 (13.3%)	0	1 (3.3%)	0	0
	CT	2 (6.7%)	7 (23.3%)	0	0	4 (13.3%)	0	0	0	0
	TT	1 (3.3%)	0	0	0	1 (3.3%)	0	0	0	0

(subject no. of each gene type/all no. of study %)

Table 4 Characteristics according to genotypes

	Normal type	Only beta3-AR variant	Only UCP1 variant	Only UCP3 variant	Beta3-AR/ UCP1 variants	UCP1/ UCP3 variants	All variants
No. (mean/women)	3/1	0/1	3/3	0/3	0/4	1/6	3/2
BMI (kg/m²)	22.0±1.1	19.7	23.1±4.0	23.2±2.2	21.8±3.3	21.9±4.2	22.3±3.8
Waist/hip ratio	0.80±0.07	0.76	0.82±0.06	0.81±0.01	0.76±0.04	0.85±0.12	0.83±0.05
Cholesterol (mg/dl)	191±11.3	162	194±31.0	203±19.2	199±30.1	210±23.9	181±16.3
HDL cholesterol (mg/dl)	70.5±10.4	72.0	67.7±8.3	68.3±11.0	70.0±19.1	77.1±16.8	61.6±8.7
LDL cholesterol (mg/dl)	105±13.1	77.0	108±27.1	115±21.9	111±6.6	116±35.6	103±10.2
Triglyceride (mg/dl)	48.5±9.9	42.0	72.8±35.9	66.0±4.0	59.5±12.4	63.3±15.4	64.2±23.9
Glucose (mg/dl)	94.0±8.8	107.0	98.8±13.5	86.0±5.6	92.8±4.1	94.6±7.9	96.8±8.0
Insulin (μU/ml)	5.4±1.9	11.1	4.3±1.4	6.1±0.8	6.3±1.4	7.6±4.7	7.5±4.1
AI	1.8±0.4	1.3	1.9±0.6	2.0±0.6	1.9±0.4	1.9±0.8	2.0±0.2
HOMA	1.3±0.5	2.9	1.1±0.5	1.3±0.2	1.5±0.4	1.8±1.3	1.8±1.0

In the A → G polymorphism of UCP1 gene, 8 (26.7%) subjects were A homozygous (AA), 20 (66.7%) subjects were AG heterozygous (AG), and 2 (6.7%) subjects were G homozygous (GG). The frequency of the G allele was 0.40.

In the C → T polymorphism of the UCP3 gene, 15 (50.0%) subjects were C homozygous (CC), 13 (43.3%) subjects were CT heterozygous (CT), and 2 (6.7%) subjects were T homozygous (TT). The frequency of the T allele was 0.28.

Subject distribution according to polymorphism of β3-AR, UCP1 and UCP3 is presented in Table 3.

Genotype and biochemical measurements

For the comparison of physical and biological characteristics, subjects were divided into the following groups: normal type (n=4), only T → C polymorphism of the β3-AR gene (the subject was a homozygous carrier; n=1), only A → G polymorphism of the UCP1 gene (subjects were heterozygous and homozygous carriers; n=6), only C → T polymorphism of the UCP3 gene (subjects were heterozygous and homozygous carriers; n=3), both β3-AR and the UCP1 gene variants (all were heterozygous carriers; n=4), both UCP1 and the UCP3 gene variants (all were heterozygous carriers; n=7), and all variants (n=5). No one belonged to both the β3-AR gene and UCP3 gene variants group (Table 4). There was no significant difference in physical and biochemical characteristics

between the normal group and the groups with gene variance.

Discussion

Using the system described in this study, we successfully analyzed the gene variation related to the mechanism of thermogenesis and lipolysis. This analysis needs only a few drops of whole blood using an © FTA Matrix card. It is inferred that a disk of diameter 2.0 mm contained about 20 $\mu\ell$ of whole blood, ca. 1.2 $\sim 1.6 \times 10^5$ of white blood cells. It is thought that this technique obtained enough volume of leukocytes to analyze the gene. Moreover, the © FTA Matrix card can be stored at room temperature for many years, and about 45 disks are available from one card.

As the UCP3 gene is found together with the β3-AR gene, it is possible to amplify both using the PCR thermal cycles of β3-AR gene, 35 cycles, and the total time can be shortened. For UCP1, 30 cycles of reaction is optimal for the same PCR thermal cycles. In the past, to sequence took a minimum of 3 days. Using the analysis in this study takes less than 5 hours.

The allele frequency of UCP3 (0.28) was found to be similar to previous studies in European subjects (0.27)[11,22]. While allele frequencies of β3-AR and UCP1 were 0.18 and 0.40 respectively, slightly lower in comparison with previous studies that reported 0.20 $\sim$0.23[18,19] and 0.46$\sim$0.49[20,21]. Possible reasons for the difference in results include the smaller number of subjects in this study and the apparent healthy state of the subjects.

Some previous studies have reported that β3-AR, UCP1 and UCP3 each contributed to thermogenesis[5,12,13]. There is some evidence that polymorphisms of the β3-AR, the UCP1 and the UCP3 gene are associated with increased BMI, hyperlipodemia and type II diabetes [10,17,23,24].

β3-AR, which is expressed in visceral fat in humans, is considered to play an important role in the increase of lipolysis and delivery of free fatty acids into the portal vein[25]. The T → C polymorphism of β3-AR carriers showed more abdominal obesity, higher insulin concentrations and more insulin resistance than normal type[26]. Polymorphism of β3-AR was implicated in abdominal obesity, insulin resistance and tendency to gain weight, and may be associated with early onset of type II diabetes[6-8].

UCP1 is mostly expressed in brown adipose tissue and is thought to act on regulated body metabolic rate[9]. The A → G polymorphism of UCP1 was associated with weight gain. There is evidence that the basal metabolic rate was lower in obese subjects with the polymorphisms in both UCP1 (A → G) and β3-AR (T → C) than in the subjects without these polymorphisms[16]. Additive effects of the polymorphism of genes have also been reported. Those with both β3-AR and UCP1 polymorphism showed resistance to weight loss[20] and insulin resistance[21] in obese Japanese subjects.

The UCP3 expressed in human skeletal muscle is considered to modulate thermogenesis by the same system as UCP1 is regulated[12,13]. The C → T polymorphism of UCP3 was related to an increased W/H ratio in female subjects[17], and associated with a higher atherogenic profile[27].

In this study, there was no significant difference in physical characteristics or lipid profiles between groups divided by genotypes. In the previous study with healthy Japanese subjects, there was no significant difference in serum lipids, BMI, and blood glucose between normal subjects and homozygous or heterozygous group[26]. The main reason for the result is thought to be that lipid profiles of subjects in this study were in normal range. Additionally, lifestyle factors such as physical activity or nutrition can influence those profiles.

In previous studies, the additive effects of the polymorphisms of both β3-AR and UCP1 have been reported in regard to increased insulin resistance[20,21]. There is no study that reports the effects of combined genotypes of variants of the β3-AR, the UCP1 and the UCP3.

According to the reports of Vidal-Puig, et al.[12] and Boss, et al.[13] the main function of UCP3 is regulation of thermogenesis in a manner similar to that of UCP1. The difference between UCP1 and UCP3 is the organ in which each gene is mainly expressed. Since UCP3 is expressed predominantly in human skeletal muscle which constitutes a large amount of the human body, energy expenditure and thermogenesis would be more influenced by a variant of UCP3 gene than by the UCP1 gene. Effects of polymorphism of the UCP3 gene on thermogenesis and liposysis in combination with both β3-AR and UCP1 should be further explored. This evidence would be useful for prescription of appropriate exercise and nutrition for the prevention of obesity. Epidemiological examination as to whether multiple polymorphisms are associated with obesity and insulin resistance remains to be performed.

Further evaluation of this system with large numbers of subjects is underway in our group.

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PCR 制限酵素解析によるエネルギー代謝に関わる遺伝子診断法

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【要旨】 肥満は、糖尿病や高脂血症などの生活習慣病のリスクファクターであり、その予防や治療は公衆衛生学的に重要な課題となっている。近年、β3-アドレナリン受容体（β3-AR）をはじめとするいくつかのエネルギー代謝に関連する遺伝子の変異が、肥満やインスリン抵抗性と関係することが報告されている。さらに、個人がこれらの遺伝子の変異を重複して有している場合は、単独で有している場合に比べその影響が大きいことが予想されるため、肥満やインスリン抵抗性との関連を詳細に検討していく必要がある。しかし従来のエネルギー代謝に関連する遺伝子の解析は、煩雑で時間がかかるという問題があった。そこで本研究では、エネルギー代謝に関連のある遺伝子であるβ3-AR、脱共役蛋白質（uncoupling protein : UCP）1および3について、比較的迅速かつ簡便に行える新たなDNA解析法を考案し、検討することを目的とした。また、それら遺伝子変異の組み合わせとBMIおよび血中脂質との関連を検討した。

その結果、本研究で用いたシステムでは、©FTA Matrix cardを用いることによりDNA抽出に必要な血液量は少量で、またPCRはβ3-ARとUCP3について同時に行うことが可能であり、従来の約1/6の5時間で解析が可能であった。遺伝子型の違いによりBMIおよび血中脂質に統計学的に有意な差はなかった。

本研究で示した遺伝子解析方法は簡便かつ迅速であることから、この方法を用いることにより、エネルギー代謝に関連した遺伝子と生活習慣病に関する比較的大規模な疫学研究を行うことが可能となり、また、今後さらに広まると考えられる個人の遺伝的状況に応じた健康づくり指導においても有用と考えられる。

〈**Key words**〉ポリメラーゼ連鎖反応、制限酵素解析、β3アドレナリン受容体、脱共役蛋白質1、脱共役蛋白質3