Association of Ob-R Gene Polymorphism and Insulin Resistance in Japanese Men

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Leptin and its receptors are known to play a role in glucose metabolism. We succeeded in cloning human Ob-R cDNA and revealed 7 single nucleotide polymorphisms (SNPs) (Lys109Arg, Arg223Gln, Ser343Ser, Ser492Thr, Lys656Asn, Ala976Asp, and Pro1019Pro) in the coding region of Ob-Rb. Although these 7 SNPs were not associated with an obese phenotype, several studies have reported that some of them were associated with impaired glucose metabolism. To clarify whether the Arg223Gln and A3057G (Pro1019Pro) polymorphisms influence glucose metabolism in Japanese, 696 Japanese men were genotyped. Individually, the Arg223Gln and the A3057G polymorphisms were not associated with the glucose metabolic parameters. No associations were found between haplotype and clinical parameters. However, in 327 subjects with normal glucose tolerance (NGT), the subjects with Arg/Gln or Gln/Gln + A/A haplotype showed significantly higher serum insulin levels and homeostasis model assessment (HOMA) index than those with Arg/Arg + A/A haplotype and Arg/Gln or Gln/Gln + A/G or G/G haplotype. The subjects with Arg/Gln or Gln/Gln + A/A haplotype showed a significantly lower fasting glucose to insulin (GI) ratio than those with Arg/Arg + A/A haplotype. These results suggest that the Ob-R gene may serve as a modifier gene for insulin resistance in Japanese men.

OBESITY IS frequently associated with the development of type 2 diabetes mellitus (DM) in many populations.[1] Although environmental factors are important in the development of both type 2 DM and obesity, it is clear that a genetic predisposition to these diseases plays a significant role.[2] The predisposition to these diseases was suggested to be conferred by a number of different genes.

Leptin is an adipocyte-derived hormone with multiple regulatory potentials. It may act primarily as a blood-borne satiety factor that decreases food intake and increases energy expenditure, thereby leading to a significant reduction in body weight. The biologic actions of leptin were suggested to be mediated largely through interactions with its cognate receptor (Ob-R) that is expressed in the hypothalamus.[3-7] Studies conducted in rodent models of obesity have demonstrated that mutations in either the Ob gene (*ob/ob* mice) or Ob-R gene (*db/db* mice, Zucker *fa/fa* rats, Koletsky rat) result in an obese phenotype with obesity-related complications, such as DM.[8,9]

Analyses of single nucleotide polymorphisms (SNPs) are useful to understand the pathogenesis of diseases.[10] We previously succeeded in cloning human Ob-R cDNA and revealed 7 SNPs (Lys109Arg, Arg223Gln, Ser343Ser, Ser492Thr, Lys656Asn, Ala976Asp, and Pro1019Pro) in the coding region of Ob-Rb, a biologically active long isoform.[11] Although we showed that these 7 SNPs were not associated with an obese phenotype,[11] a recent study in Caucasians reported that the Ob-R polymorphisms are associated with impaired glucose metabolism.[12] Another study in postmenopausal Caucasian women reported that the Arg223Gln polymorphism significantly affects the efficiency of leptin binding to the soluble form of Ob-R.[13] Another study in Nauruan males reported that Ob-R Pro1019Pro (A3057G) polymorphism is associated with serum insulin levels.[14] These results prompted us to explore the clinical significance of Ob-R gene polymorphism in glucose metabolism in Japanese.

SUBJECTS AND METHODS

Subjects

In humans, several studies report that: (1) in women circulating leptin levels are higher than those in men, even when adjusted for body mass index (BMI) and absolute fat mass[15-17]; (2) among women serum leptin levels are higher in premenopausal than in menopausal subjects[18-20]; and (3) most investigators agree that, in cycling women, circulating leptin levels are higher in the luteal phase than in the follicular phase.[20-22] These data suggest that serum leptin levels in women are more variable than those in men. Thus, we selected men for this study.

We studied 696 non-obese Japanese men who were recruited from the general health check-up center of NTT hospital West Japan and the Kyoto University Hospital between 1997 and 1999. A total of 512 people who were recruited from the general health check-up center of NTT hospital West Japan were taking no medication. All subjects were clinically examined by a physician (Dr Takashi Miyawaki, one of the authors of this manuscript) and shown to be in good health. Patients with endocrine diseases, such as Cushing's disease, were carefully excluded, in addition to patients who had liver disease. The state of glycemia was classified into 3 categories, diabetes, impaired fasting glucose (IFG), and normal glucose tolerance (NGT). Diabetes was defined when fasting plasma glucose (FPG) was 126 mg/dL or higher, and/or glycosylated hemoglobin (HbA_{1c}) was 6.5% or higher. NGT was defined when FPG was below 110 mg/dL. IFG was defined in those who belong neither to diabetes nor to NGT. Based on these criteria, the subjects were classified as the NGT group (n = 377), IFG group (n = 99), or type 2 DM group (n = 36).

A total of 184 Japanese men who were recruited from the Kyoto University Hospital were defined as type 2 DM. They were taking some medications.

All subjects gave informed consent to participate in the present study. This study was approved by the ethical committee on human research of Kyoto University Graduate School of Medicine (No.G20-1).

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Anthropometric Measurements

Height and body weight were measured, and BMI was calculated as body weight (in kilograms) divided by height (in meters) squared. Percent body fat (% fat) was assessed based on the principles of bioelectrical impedance.[23] Fat mass and fat-free mass were calculated using %fat and body weight.

Biochemical Analyses

Fasting blood samples were obtained for measurement of glucose, HbA_{1c}, and lipid levels. Serum insulin and leptin levels were measured from fasting blood samples in 327 subjects with NGT. We calculated the insulin resistance index using the homeostasis model assessment (HOMA), as the product of fasting insulin (μU/mL) and fasting glucose (mg/dL) divided by 405.[24] It correlated well with euglycemic clamp estimates of insulin resistance. We also calculated the fasting glucose (mg/dL) to insulin (μU/mL) ratio (GI ratio) as an index of insulin sensitivity.[25] Plasma total cholesterol (TC), triglyceride (TG), and high-density lipoprotein-cholesterol (HDL-C) levels were measured. Low-density lipoprotein-cholesterol (LDL-C) was calculated using Friedewald's formula.[26]

Genotyping Protocol

Genomic DNA was extracted from peripheral blood leukocytes. The Ob-R gene polymorphisms, Arg223Gln in exon 6 and A3057G in exon 20, were determined using a polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP)-based protocol as described by Matsuoka et al.[11]

Statistical Analyses

All values are expressed as means ± SE. Statistical analyses were performed with Stat-View 5.0 software (SAS Institute, Cary, NC). Qualitative traits were analyzed by contingency table chi-square tests. The differences for 4 groups were assessed by analysis of variance. For the comparison of subgroups, analysis of variance followed by Fisher's protected least significant difference (PLSD) was performed as a multiple comparison test. Significance was accepted at the level of $P < .05$.

RESULTS

The genotype frequency of the Arg223Gln in 696 Japanese men was as follows: Arg/Arg homozygotes, 72.7%; Arg/Gln heterozygotes, 25.1%; and Gln/Gln homozygotes, 2.2%. The genotype frequency of the A3057G was as follows: A/A homozygotes, 74.5%; A/G heterozygotes, 23.9%; and G/G homozygotes, 1.6%. The genotype frequencies for these polymorphisms were found to be in Hardy-Weinberg equilibrium. The frequencies of the Gln allele and G allele were determined to be 14.7% and 13.5%, respectively. Individually, the Arg223Gln and the A3057G polymorphisms were not associated with the obese or glucose metabolic parameters in these subjects (data not shown).

Table 1. Haplotype Frequency (%) in Japanese Men

Haplotype	NGT (n = 377)	IFG (n = 99)	DM (n = 220)	*P* Value
Arg/Arg, A/A	59.4	61.7	57.7	.89
Arg/Arg, A/G or G/G	14.6	12.1	12.3	
Arg/Gln or Gln/Gln, A/A	13.5	12.1	16.4	
Arg/Gln or Gln/Gln, A/G or G/G	12.5	14.1	13.6	

Abbreviations: NGT, normal glucose tolerance group; IFG, impaired fasting glucose group; DM, type 2 diabetes mellitus group.

The haplotype frequency in 696 Japanese men was as follows: Arg/Arg + A/A, 59.2% (n = 412); Arg/Arg + A/G, 12.9% (n = 90); Arg/Arg + G/G, 0.6% (n = 4); Arg/Gln + A/A, 14.2% (n = 99); Arg/Gln + A/G, 10.6% (n = 74); Arg/Gln + G/G, 0.3% (n = 2); Gln/Gln + A/A, 1.2% (n = 8); Gln/Gln + A/G, 0.3% (n = 2); and Gln/Gln + G/G, 0.7% (n = 5). Due to the low prevalence of the Arg/Arg + G/G, Arg/Gln + G/G, Gln/Gln + A/A, Gln/Gln + A/G, and Gln/Gln + G/G, the haplotype groups were regrouped as Arg/Arg + A/A (n = 412), Arg/Arg + A/G or G/G (n = 94), Arg/Gln or Gln/Gln + A/A (n = 107), and Arg/Gln or Gln/Gln + A/G or G/G (n = 83) for analysis.

A total of 696 Japanese men were divided into 3 groups based on glucose tolerance. When the haplotype frequency was compared in the NGT group (n = 377), IFG group (n = 99), and DM group (n = 220) using the chi-square test, no significant difference was identified (Table 1).

The association between haplotype and clinical parameters in the subjects without medical treatment (n = 512) is shown in

Table 2. Associations Between the Haplotype and Clinical Parameters in the Subjects With No Medical Treatment (n = 512)

Parameters	Arg/Arg A/A	Arg/Arg A/G or G/G	Arg/Gln or Gln/Gln A/A	Arg/Gln or Gln/Gln A/G or G/G	*P* Value
N	307	73	67	65	
Age (yr)	51.5 ± 0.2	51.3 ± 0.4	51.2 ± 0.4	51.3 ± 0.5	.89
BMI (kg/m^2)	23.4 ± 0.2	23.7 ± 0.3	24.0 ± 0.3	23.3 ± 0.3	.35
% fat (%)	18.9 ± 0.3	20.5 ± 0.6	18.5 ± 0.7	19.0 ± 0.7	.13
Fat mass (kg)	12.5 ± 0.3	14.0 ± 0.5	12.7 ± 0.6	12.5 ± 0.6	.11
Fat-free mass (kg)	52.5 ± 0.5	53.7 ± 0.9	54.4 ± 1.0	52.8 ± 1.0	.27
Systolic blood pressure (mm Hg)	123.8 ± 1.1	122.8 ± 1.7	124.1 ± 2.0	122.5 ± 2.2	.92
Diastolic blood pressure (mm Hg)	77.6 ± 0.7	77.0 ± 1.2	77.6 ± 1.3	74.8 ± 1.4	.41
Fasting plasma glucose (mg/dL)	104.0 ± 0.8	105.0 ± 1.6	102.9 ± 1.8	104.1 ± 1.7	.87
HbA_{1c} (%)	5.15 ± 0.03	5.13 ± 0.07	5.22 ± 0.06	5.22 ± 0.06	.54
Total cholesterol (mg/dL)	219.0 ± 2.0	218.2 ± 4.2	222.0 ± 3.6	213.4 ± 4.3	.53
Triglyceride (mg/dL)	146.8 ± 7.1	145.1 ± 9.7	147.8 ± 10.8	144.1 ± 10.6	1.00
HDL-C (mg/dL)	57.2 ± 0.8	54.6 ± 1.5	56.2 ± 1.8	54.0 ± 1.6	.26
LDL-C (mg/dL)	132.4 ± 2.0	134.5 ± 4.0	136.2 ± 3.8	130.6 ± 4.1	.77

NOTE. Values are expressed as means ± SE. Differences for 4 groups were assessed by analysis of variance.

Table 3. Associations Between the Haplotype and Insulin Resistance in Subjects With NGT (n = 327)

Parameters	Arg/Arg A/A	Arg/Arg A/G or G/G	Arg/Gln or Gln/Gln A/A	Arg/Gln or Gln/Gln A/G or G/G	*P* Value
N	195	53	41	38	
Age (yr)	51.2 ± 0.3	51.2 ± 0.5	51.0 ± 0.6	51.3 ± 0.6	.98
Fasting plasma glucose (mg/dL)	104.4 ± 1.0	104.1 ± 1.6	105.3 ± 2.3	105.8 ± 2.1	.92
HbA_{1c} (%)	5.13 ± 0.04	5.13 ± 0.08	5.32 ± 0.08	5.26 ± 0.07	.13
Fasting IRI (μU/mL)	6.2 ± 0.2*	7.0 ± 0.5	8.0 ± 0.7	6.2 ± 0.4†	.01
HOMA	1.6 ± 0.1*	1.8 ± 0.1	2.1 ± 0.2	1.6 ± 0.1†	.01
GI ratio	20.2 ± 0.6‡	17.8 ± 1.0	16.3 ± 1.1	19.2 ± 1.1	.02
Leptin (ng/mL)	4.3 ± 0.4	4.0 ± 0.4	5.0 ± 0.4	4.1 ± 0.3	.76

NOTE. Values are expressed as means ± SE. Differences for 4 groups were assessed by analysis of variance. For the comparison of subgroups, analysis of variance followed by Fisher's PLSD was performed as a multiple comparison test.

*P = .002, †P = .02, ‡P = .005 *v* Arg/Gln or Gln/Gln, A/A.

Table 2. No significant associations were found between haplotype and clinical parameters in these subjects. We measured serum insulin levels in 327 subjects with NGT. The subjects with Arg/Gln or Gln/Gln + A/A haplotype had significantly higher serum insulin levels and HOMA index than those with Arg/Arg + A/A haplotype and Arg/Gln or Gln/Gln + A/G or G/G haplotype. The subjects with Arg/Gln or Gln/Gln + A/A haplotype had a significantly lower GI ratio than those with Arg/Arg + A/A haplotype (Table 3). We also measured serum leptin levels in 327 subjects with NGT. No associations were found between haplotype and serum leptin levels. However, serum leptin levels in subjects with Arg/Gln or Gln/Gln + A/A haplotype tended to be higher than in those with the other haplotype (Table 3).

DISCUSSION

The present results suggested that individually the Arg223Gln and the A3057G polymorphisms were not associated with insulin resistance in non-obese Japanese men. However, among 4 haplotype groups of the Arg223Gln and the A3057G, the differences in the metabolic parameters, including fasting serum insulin levels, HOMA index, and GI ratio, were significant.

Leptin and its receptors are known to play a role in glucose metabolism. Leptin treatments given to *ob/ob* mice[27] and diet-induced obese rats dramatically improve glucose metabolism and insulin-sensitivity.[28,29] Leptin replacement therapy improves glucose and lipid metabolism in patients with lipodystrophy, indicating that leptin deficiency contributes to the insulin resistance and other metabolic abnormalities associated with severe lipodystrophy.[30] Leptin has been shown to exert insulin and glucose-lowering effects by enhancing peripheral insulin sensitivity and glucose uptake.[31] In accordance with the notion that Ob-Rs are present on pancreatic β cells, which could offer one mechanism by which leptin could modulate glucose-induced insulin secretion,[32] it is tempting to suggest that impaired leptin receptor signaling could contribute, at least in part, to impairment in glucose homeostasis.

In addition to its central effects on glucose metabolism, leptin has been shown to act on peripheral tissues. Consistent with this, Ob-R is broadly expressed in central and peripheral sites. Ob-R is alternatively spliced, and some different forms of the receptor are generated. All Ob-R isoforms contain the Arg223Gln polymorphism. In the Ob-R gene, codon 223 is located closely to the cytokine motif (WSXWS) sequences in the extracellular domain, which are crucial for ligand binding.[11] Recently, in postmenopausal Caucasian women, lower leptin binding to the soluble form of Ob-R was shown in carriers of the Arg allele of the Arg223Gln polymorphism of the Ob-R.[13] Another study in Pima Indians reported that subjects with the Arg/Arg homozygotes have significantly lower 24-hour energy expenditure and physical activity level, but have larger subcutaneous abdominal adipocyte size than those with the Arg/Gln heterozygotes and Gln/Gln homozygotes.[33] These data suggest that the Arg223Gln substitution may cause impairment in obesity-related metabolism through the modification of leptin binding. On the other hand, A3057G polymorphism exists only in the Ob-Rb isoform. A3057G variant which caused silent mutation (Pro1019Pro) is located in the intracellular domain which is involved in the interactions with Janus-activated kinase (JAKs) and signal transducers and activators of transcription (STATs).[34] Accordingly, the A3057G variant might interfere with leptin receptor signaling. The present study demonstrated that although, individually, the Arg223Gln and the A3057G polymorphisms were not significantly associated with insulin resistance, the haplotype of these polymorphisms were associated with insulin resistance. Taken together, the additive effect of the Arg223Gln and A3057G substitutions may cause impairment in glucose metabolism through leptin binding and signaling.

In this study, we found that the haplotype was not significantly associated with obese phenotype and serum leptin levels. Genetically obese leptin-deficient *ob/ob* mice and leptin receptor defective *db/db* mice show marked obesity and DM.[35,36] In these mice, the first metabolic defect is hyperinsulinemia.[37] At this stage, these animals have normal glucose levels with hyperinsulinemia and do not have obesity. However, at the final stage, these animals have a phenotype including severe obesity and extreme insulin resistance. Although the exogenously administered leptin ameliorates obesity and impaired glucose metabolism in obese-diabetic rodent models with a reduced amount of leptin or leptin deficiency, changes in serum glucose level precede changes in body weight.[38] Together, the data suggest that the first leptin-signaling impairment may be the impairment in glucose metabolism with the impaired insulin sensitivity, but not obesity. Among subjects studied, there were

no men with severe obesity. Therefore, no associations might be found between the haplotype and adiposity including BMI, % fat, fat mass, and fat-free mass. However, the lack of association does not rule out the possibility that the haplotype may influence body adiposity. A limitation of our study is lack of data on visceral/subcutaneous fat distribution ratio and adipocyte size. Adipocytes from *db/db* mice, which lack the leptin receptor, are 5 times as large as those from normal mice.[39] Stefan et al[33] reported that in Pima Indians, subjects with Arg/Arg homozygote had a higher subcutaneous abdominal adipocyte size compared with the other 2 genotypes. In this context, it would be interesting to address the relationship between haplotype of Arg223Gln and A3057G polymorphisms and subcutaneous abdominal adipocyte size and visceral/subcutaneous fat distribution ratio. Furthermore, in a prospective study, de Silva et al[40] reported that A3057G polymorphism was associated with longitudinal increases in body weight, fat mass, and BMI in Australian women. Lack of data on long-term overfeeding or longitudinal analysis also represents a limitation of our study. In a prospective study, an association between the haplotype and whole body adiposity may be shown.

In insulin-resistant people, a low acute insulin response (AIR) to intravenous glucose load is known to be a predictor of type 2 DM. Thompson et al[41] found that in Pima Indians, the microsatellite marker D1S198 was linked with AIR in sib pairs with NGT. However, no linkage was observed between D1S198 and type 2 DM.[41] The chromosomal assignment of human Ob-R gene is on 1p31,[42,43] and the locus has been shown to be in the vicinity of D1S198.[41] Thompson et al[41] reported that in Pima Indians with NGT, there is no association between Arg223Gln polymorphism and AIR. However, in the present study, we demonstrated that in non-obese Japanese men with NGT, the haplotype of Ob-R polymorphisms might modulate insulin resistance. Therefore, it may be possible that linkage disequilibrium to D1S198 affects serum insulin concentrations.

Previous studies of the association between the Ob-R polymorphism and glucose metabolism are inconsistent. For example, Wauters et al[12] reported an association of the Arg223Gln polymorphism was found for insulin response to a 75-g oral glucose tolerance test (OGTT) in postmenopausal, obese Caucasian women. In contrast, Silver et al[44] reported that no significant associations of the Arg223Gln polymorphism were found for fasting and 2-hour plasma glucose levels or log of fasting and 2-hour insulin levels during an OGTT in Caucasians. On the other hand, de Silva et al[14] reported that A3057G polymorphism was associated with fasting insulin levels in Nauruan men. In this study of non-obese Japanese men, individually, Ob-R polymorphism does not have a role in the pathogenesis of type 2 diabetes, which is significant enough to be determined by this kind of association study. However, a combination of Ob-R polymorphisms may have a role in the pathogenesis of insulin resistance. Although we found that haplotypes of the Arg223Gln and the A3057G were associated with fasting serum insulin concentrations, these values were very low in contrast with other population.[45] The mean fasting serum insulin values in this study are similar to those reported in normal or insulin-resistant Japanese.[46,47] It appears that the serum insulin concentrations are very low even in Japanese thought to be insulin resistant.[45-47] In addition, there is a wide difference in allele frequencies of Arg223Gln and A3057G among racial groups.[11,48] The findings of the present study demonstrated that the Gln and the G allele frequencies in Japanese males were 14.7% and 13.5%, respectively. Thompson et al[48] demonstrated that there was a significant difference in the Gln allele frequency between Caucasians and Pima Indians (55% and 25%, respectively). Gotoda et al[49] also demonstrated that, in obese white British males, the Gln and the G allele frequencies were 56% and 62%, respectively. Collectively, analysis of these SNPs suggests the importance of association studies in the different ethnic groups.

In summary, the present results suggest that individually, the polymorphisms of the Ob-R are not major risk factors for type 2 DM. However, a combination of the Ob-R polymorphisms may contribute, at least in part, to insulin resistance in humans. The present data do not allow for a determination of whether the observed associations are due to direct functional consequences of the haplotype or whether it is in linkage disequilibrium with genetic markers in this genetic region. Further studies will be required to clarify the role of the haplotype combining Ob-R polymorphisms on leptin action in human glucose metabolism.

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