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The haplotype identified in *LEPR* gene is associated with type 2 diabetes mellitus in Northern Chinese

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ABSTRACT

Leptin receptor (LEPR) plays an important physiological role in energy metabolism. The study addressed the relationship between leptin receptor gene variations and type 2 diabetes mellitus (T2DM). Three single nucleotide polymorphisms (SNPs) of *LEPR* gene, Arg109Lys (A/G), Asn656Lys (C/G) and Pro1019Pro (C/T) were detected in a northern population in China. Totally, 317 patients with T2DM and 282 healthy controls were recruited randomly from urban communities in Harbin area in the Northeast of China. All polymorphisms were genotyped by Sequenom SNP detection system in both case and control groups. Linkage disequilibria analysis showed moderate linkage disequilibria between the pair-wise SNPs for all three SNPs. Then, we identified the haplotype covering the three SNPs (AGC) with higher risk of T2DM (OR = 1.69 (1.09–2.61)), and showed that there existed significant difference between cases and controls (9.8% vs. 6.0%, $P = 0.02$). We also observed significant difference in frequencies of the heterozygous haplotype combination (GGT/AGC), that is 17.0% vs. 8.2% in cases and controls, respectively ($P = 0.001$). It further supported the evidence that the haplotype (AGC) was associated with T2DM. So, AGC haplotype in *LEPR* gene could be a risk factor associated with T2DM in Northern Chinese.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a multifactor complicated syndrome; its etiology involves both environmental and genetic factors. The leptin receptor (*LEPR*) gene had been considered as one of candidate genes associated with T2DM in many population studies in last decade [1,2]. The leptin receptor is a member of cytokine receptor family, which plays a critical role in leptin signal transduction and regulation of energy balance including glucose metabolism and body

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Abbreviations: *LEPR*, leptin receptor gene; T2DM, type 2 diabetes mellitus; PCR, polymerase chain reaction; OGTT, oral glucose tolerance test; BMI, body mass index; STAT, signal transducer and activator of transcription; SNP, single nucleotide polymorphism; MALDI-TOF MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry.

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weight by activating signal transducer and activator of transcription (STAT) proteins STAT3, STAT5 and STAT6. Hekerman et al. suggested that three intracellular tyrosine at positions 985, 1077 and 1138 were responsible for the signal transduction from *LEPR* to STATs [1]. In animal models, *LEPR* gene mutation can lead to serious obesity and diabetes, and these complicated syndromes can be completely rescued by introduction of a neuron-specific *LEPR*-B transgenes in *db/db* mice [2], which confirmed the critical role of *LEPR* gene in the etiology of obesity and diabetes. It was also suggested LEPR could help recombinant leptin across the human blood–brain barrier by binding and endocytosis [3].

Since Gotoda et al. found out the DNA sequence variants on entire coding sequence of the human leptin receptor cDNA from 22 morbidly obese patients [4], some studies on association of common polymorphisms in *LEPR* gene with metabolism and obesity [5–8], as well as insulin resistance and T2DM [9,10] had been conducted in different populations.

There were also some reports that supported the association between *LEPR* polymorphisms and metabolism as well as inflammatory phenotypes. For examples, Loos et al. found that Arg109Lys polymorphism were significantly associated with respiratory quotient while walking at 4.5 km/h and Asn656Lys polymorphism was associated with resting metabolic rate in the Quebec Family Study [11]. In 630 Caucasian individuals, Zhang et al. found Pro1019Pro (T/C) polymorphism was associated with inflammatory traits including plasma fibrinogen and C reactive protein levels [12], while Lu et al. suggested this polymorphism was associated with hyperlipidemia and fat distribution in T2DM in Southern Chinese [13]. de Luis Roman et al. found that Asn656Lys polymorphism was associated with decreased leptin response and weight loss secondary to life style modification in obese patients [14].

Though some studies were conducted on the association of *LEPR* variation with obesity and T2DM, and the association for Gln223Arg polymorphism in *LEPR* gene was found in some populations. Few studies were seen to explore the association between *LEPR* Arg109Lys, Asn656Lys and Pro1019Pro polymorphisms, which changed the amino acid attribute or located adjacent to active sites in important functional domain, and the susceptibility of T2DM in Northern Chinese. Based on our knowledge, the study concerning the haplotype of the three polymorphisms in *LEPR* gene is still not clear. So, we conducted the case–control design to explore the relationship between the *LEPR* variants and T2DM in Northern Chinese.

2. Subjects and methods

2.1. Study subjects and clinical characteristics

We randomly recruited 317 patients (49.3 ± 13.7 years old) with T2DM from local resident diabetes epidemiological screening in urban communities in Harbin area in China. Diabetes was diagnosed based on the American Diabetes Association (ADA) fasting plasma criteria (2005). Subjects were defined as diabetic either through an oral glucose tolerance test (OGTT) using 75 g glucose load (dissolved in 250 ml water) or if received anti-diabetic treatment by oral hypoglycemic agents or by insulin injection. Totally, 282 local residents (45.2 ± 5.7 years old) with normal glucose tolerance by OGTT screening were involved through a routine physical examination. Height, weight and blood pressure were measured in all subjects. The plasma glucose concentration was analyzed by a glucose oxidase method [15]. Informed written consents were obtained from all subjects. The ethics committee of Beijing Hospital, Ministry of Health, P.R. China approved the study. All subjects are from unrelated Han ethnic group in North China.

2.2. Genotyping

Genomic DNA was extracted from peripheral blood leukocytes by phenol–chloroform method. The SNPs including Arg109Lys, Asn656Lys and Pro1019Pro in *LEPR* gene were genotyped by Sequenom SNP detection system based on MALDI-TOF MS technique. The primer sequences for PCR and extension were shown in supplementary Table 1. As a measure of quality control, the genotyping results were confirmed by direct sequencing of 20 subjects randomly selected from case or control. Part of genotyping and sequencing results were given in supplementary Fig. 1 as examples.

2.3. Statistical analysis

All statistical analyses were conducted by SPSS 11.5 software package. Genotype and allele frequencies in different groups were compared through Chi-square or exact Fisher's test analysis and quantitative phenotypes were analyzed with Student's *t*-test and expressed as mean ± S.D. The odds ratio and its 95% confidence interval between case and control groups were calculated. Significant threshold was set at $P \leq 0.05$ and Bonferroni correction was conducted. Haplotypes were analyzed by using HAP-haplotype resolution version 3.0 [16] and D' were calculated for assessment of linkage disequilibrium (supplementary Table 2). Hardy–Weinberg equilibrium of the genotype distribution was assessed in control population (Table 2).

3. Results

3.1. Clinical characteristics for case and control

The clinical characteristics of T2DM patients and control subjects were summarized in Table 1. There were 233 individuals in 317 patients, who report a positive family history of T2DM in first or/and secondary rank relatives. The plasma glucose levels, BMI and blood pressure in diabetic patients were significantly higher than those in control group. The range of T2DM course for patients was 0–28 years (average 6.2 ± 5.3).

3.2. Distribution of LEPR genotypes and alleles in healthy controls and type 2 diabetic patients

Overall, the alleles were determined in 599 individuals including 317 patients with T2DM and 282 healthy control subjects. The genotypes for Arg109Lys, Asn656Lys and Pro1019Pro in *LEPR* gene were accord with Hardy–Weinberg

Table 1 – The clinical characteristics of T2DM patients and control subjects

Phenotypes	Controls	Cases
Age (years)	45.2 ± 5.7	49.3 ± 13.7
Sex (M/F)[a]	156/161	170/112
DM course (years)	–	6.2 ± 5.3
BMI (kg/m^2)	23.8 ± 2.9	25.5 ± 4.1**
WHR	0.8 ± 0.1	0.9 ± 0.1
SBP (mmHg)	104.6 ± 8.0	127.1 ± 21.7**
DBP (mmHg)	80.0 ± 12.6	86.7 ± 12.8**
Glu2h (mmol/L)	4.5 ± 1.1	–
FPG (mmol/L)	4.1 ± 0.4	9.3 ± 3.9**

Note: Continuous variables are expressed as mean ± S.D. values; **$P \leq 0.01$; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Glu2h, plasma glucose at 2 h after a 75 g glucose load; FPG, fasting plasma glucose. Age and sex adjusted by statistics program, results in supplementary Table 3.

[a] The sex distribution was not markedly difference by Chi-square analysis compared between cases and controls.

equilibrium in control subjects ($P = 0.556$, 0.979 and 0.665, respectively). Between case and control groups in Northern Chinese, there were no significant differences detected in the distribution of alleles and genotypes created by three coding polymorphisms of *LEPR* gene (Table 2).

3.3. *Linkage disequilibrium and distribution of LEPR haplotypes and haplotypes combination in healthy controls and type 2 diabetic patients*

D' for measurement of linkage disequilibrium was shown in supplementary Table 2, only moderately linkage disequilibrium were revealed between Arg109Lys (G/A) and Asn656Lys (G/C) ($D' = 0.56$), Arg109Lys (G/A) and Pro1019Pro (T/C) ($D' = 0.61$), Asn656Lys (G/C) and Pro1019Pro (T/C) ($D' = 0.60$) polymorphisms.

By haplotypes counting, only six out of eight possible haplotypes were observed in our study population. There were significant differences in distribution of haplotypes constituted by the polymorphisms. The haplotype covering the three SNPs (AGC) (9.8% vs. 6.0%) was more prevalent in T2DM patients than in control subjects ($P = 0.02$) and with higher risk to T2DM, OR = 1.69 (95% CI 1.09–2.61) (Table 3). In the analysis of the haplotype combination, we found a heterozygote combination of AGC haplotype, GGT/AGC, which were more prevalent in T2DM patients than in controls (17.0% vs. 8.2%, $P = 0.001$, OR = 2.31 (95% CI 1.38–3.88)) (Table 4). After age, sex, and BMI were adjusted, the difference of the haplotype frequency between case and control was still significant ($P = 0.026$). Both haplotype and haplogenotype with AGC in case were still significantly higher than that in control by Bonferroni correction. We applied the method to evaluate the significance level for the results of our studies. Adjusted by Bonferroni correction, the significance level was 0.025 (0.05/2), a little higher than that in 3-SNP haplotypes in this study ($P = 0.02$), and more than those in our heterozygous haplotype combination (GGT/AGC) studies ($P = 0.001$). Because of the high stringency of the Bonferroni correction, we also analyzed the significance level of our datasets to correct them by the other three methods such as by the first method used from Legendre in 1988, we got the value of the significance level ($P = 0.021$) [17]. By the second method, the Bonferroni step-down (Holm) correction, we got the same result ($P = 0.02$ and 0.001) [18]. We evaluated the significance level by three methods, including the Bonferroni correction. Nearly all analysis results support our study and the evidences from studies of LEPR support that the haplotype variance is associated with T2DM.

Haplotype distribution consisting of two SNPs were done and showed that not GC, but AC and AG frequencies were

Table 3 – Haplotype frequencies of the three coding polymorphisms in *LEPR* gene inT2DM patients and control subjects

Haplotype	Controls ($n = 564$)	Cases ($n = 634$)	P, OR (95% CI)
G-G-T	444 (78.7%)	494 (77.8%)	0.74, 0.95 (0.72–1.26)
A-G-C	34 (6.0%)	62 (9.8%)	0.02, 1.69 (1.09–2.61)
A-G-T	29 (5.1%)	34 (5.4%)	0.86, 1.05 (0.63–1.74)
A-C-T	24 (4.3%)	19 (3.0%)	0.24, 0.70 (0.38–1.28)
G-G-C	23 (4.1%)	15 (2.4%)	0.09, 0.57 (0.29–1.10)
G-C-T	6 (1.1%)	10 (1.6%)	0.44, 1.49 (0.54–4.13)

The *P* value, OR and 95% CI of each haplotype relative to other haplotypes as a group are shown. The results are not shown for very rare haplotypes no more than 1% in both groups.

Table 2 – Genotype and allele frequencies of the polymorphisms in *LEPR* gene in T2DM patients and control subjects

Variations	HWE (*P*)	AA	AB	BB	A/B
Arg109Lys (G/A)					
Cases	0.829	213 (67.2%)	93 (29.3%)	11 (3.5%)	0.82/0.18
Controls	0.556	203 (72.0%)	71 (25.2%)	8 (2.8%)	0.85/0.15
Gln656Lys (G/C)					
Cases	0.665	289 (91.2%)	27 (8.5%)	1 (0.3%)	0.95/0.05
Controls	0.979	249 (88.3%)	32 (11.3%)	1 (0.4%)	0.94/0.06
Pro1019Pro (T/C)					
Cases	0.722	244 (76.9%)	69 (21.8%)	4 (1.3%)	0.88/0.12
Controls	0.665	225 (79.8%)	53 (18.8%)	4 (1.4%)	0.89/0.11

For the Arg109Lys, Asn656Lys and Pro1019Pro polymorphisms in *LEPR* gene, major allele G, G, T, respectively, was designated as A; while minor allele designated as B. The genotype and allele frequencies between case and control were compared by Pearson's Chi-square analysis ($P > 0.05$). Number and percentage were shown for genotypes while only frequency was shown for alleles. HWE, Hardy–Weinberg equilibrium.

Table 4 – Haplotype combination frequencies of three coding polymorphisms in *LEPR* gene in T2DM patients and control subjects

Haplotype combination	Controls (n = 282)	Cases (n = 317)	P, OR (95% CI)
GGT/GGT	175 (62.1%)	191 (60.3%)	0.65, 0.93 (0.67–1.29)
GGT/AGT	26 (9.2%)	26 (8.2%)	0.66, 0.88 (0.50–1.55)
GGT/AGC	23 (8.2%)	54 (17.0%)	0.001, 2.31 (1.38–3.88)
GGT/GGC	18 (6.4%)	12 (3.8%)	0.15, 0.58 (0.27–1.22)
GGT/ACT	17 (6.0%)	11 (3.5%)	0.14, 0.56 (0.26–1.22)
GGT/GCT	6 (2.1%)	9 (2.8%)	0.58, 1.34 (0.47–3.83)
GGT/GCC	4 (1.4%)	0 (0.0%)	0.03, 2.14 (1.96–2.33)
AGC/GGC	3 (1.1%)	1 (0.3%)	0.35, 0.29 (0.03–2.85)
AGC/ACT	3 (1.1%)	2 (0.6%)	0.67, 0.59 (0.10–3.56)
AGC/AGT	3 (1.1%)	1 (0.3%)	0.35, 0.29 (0.03–2.85)
AGT/ACT	0	5 (1.6%)	0.06, 1.90 (1.76–2.06)

The P value, OR and 95% CI of each haplotype combination relative to other haplotype combinations as a group are shown. The results for very rare haplotype combinations no more than 1% in both groups is not shown.

significantly higher in case group than that in control (see in supplementary Table 3). It suggested that it was possible AG haplotype associated with T2DM, as AGC haplotype contained AG. However, we failed to find any difference with phenotypic variables of AGC haplotype carriers in both case and control (supplementary Table 4).

4. Discussion

To our knowledge, the present study should be the first association study of the three coding polymorphisms in *LEPR* gene in diabetic case and control in Northern Chinese. Although we have not obtained evidence supporting the association of three coding polymorphisms in *LEPR* gene with T2DM, respectively, we identified a risk haplotype in *LEPR* gene, which can significantly increase the susceptibility to T2DM in Northern Chinese.

Similar to our study, the results in Korean population, suggested *LEPR* gene polymorphisms were not associated with T2DM, but Arg109Lys variation was marginally associated with BMI of the subjects [19]. However, there were as many studies that obtained diversified results. In the Finnish Diabetes Prevention Study, Salopuro et al. found that two polymorphisms (Lys109Arg, Gln223Arg) in the extra-cellular domain of the leptin receptor predicted the conversion to T2DM in high-risk individuals with IGT [10]. While Wauters et al. investigated the relationship between *LEPR* polymorphisms and glucose and insulin response to an OGTT and found Lys109Arg, Asn656Lys and Gln223Arg polymorphisms were associated with the insulin and glucose response after glucose load, which was closely associated with T2DM [20].

Moderate linkage disequilibrium was shown between pairwise SNPs in Northern Chinese. A haplotype associated with T2DM significantly was identified and showed that the individuals with the heterozygote haplotypes combination were in higher risk to be susceptible to T2DM. In Caucasian nuclear families, Liu et al. also found the significant linkage disequilibrium between pairs of the three polymorphisms in *LEPR* gene [8], which suggested a possible basis for synergistic interaction of gene variants in of *LEPR* gene in the etiology of obesity and T2DM (supplementary Tables 5–7). In fact, our haplotype analysis consisting of three SNPs suggested the possible susceptible locus to T2DM likely located among Arg109Lys, Gln656Lys and Pro1019Pro in *LEPR* gene (supplementary Table 2). It is possible that the DNA segment covered by the haplotype identified to hide the marker or variation responsible for the susceptible locus to T2DM and may be any polymorphism, which located among Arg109Lys, Gln656Lys and Pro1019Pro in *LEPR* gene. Chung et al. suggested certain polymorphism, which was strongly supported being associated with obesity and insulin resistance, resulted in change of charge from neutral to positive and most likely has some functional consequences. In addition, studies suggested the SNP in the leptin receptor was associated with familial combined hyperlipidemia [21]. It needs to develop further genomic and functional gene study to confirm the substantial role of the SNP in Northern Chinese.

With the haplotype combinations analysis, only GGT/AGC combination was associated with T2DM in the population (P = 0.001). However, the GGT haplotype frequencies was nearly the same (77.8% and 78.7%) in the case and control, AGC haplotype frequency was the one with significant difference existed (9.8% and 6.0%) in the case and control (P = 0.02). In fact, GGT in the haplotype combination in the population was a possible neutral haplotype frequently appeared with certain unknown biological function. It seemed to present in two ways while existing with non-AGC, it was not associated with T2DM; however, while existing with AGC, it showed stronger association with T2DM. However, how the mechanism of GGT modified the risk of AGC haplotype was not clear. The biological meaning of the combination (GGT/AGC) remains unclear presently, we deduced that it was the AGC haplotype which increased the risk of T2DM. The risk or association was further increased when it was in a heterozygous state with GGT haplotype. Maybe it was associated with the gene expression regulation of the risk haplotype.

LEPR is a single transmembrane domain receptor with two cytokine domains, which binds to leptin. Though, individually, none of these polymorphisms in *LEPR* gene were founded to be associated with T2DM. It should be noted that a small effect of these polymorphisms on susceptibility of T2DM could not be excluded, since the sample size was limited in the present study. *LEPR* is an important functional gene on energy metabolism and plays a critical role in leptin signal transduction. Several other important amino acids, for example, Met1139 and Gln1141 are determinants defining the specificity toward the different STAT factors and can also affect leptin response and signal transduction [19]. Arg109Lys variation was near to *fa* mutation that impaired leptin signal pathway in model rats and both of them occurred in the first cytokine domain, it is highly possible that this coding variation also affect the function of LEPR [7]. Chung et al. suggested the Asn656Lys polymorphism can result in positive to neutral change of charge, which may lead to modification of the structure and function of *LEPR* protein [20]. In addition, the amino acids (Lys109 and Lys656) are conserved among rat, mouse, and human species, which suggest they may play a part in protein function [21].

In summary, we identified a highly susceptible AGC haplotype covering three SNPs in *LEPR* gene. It suggested that the haplotype in *LEPR* gene were significantly associated with the susceptibility of T2DM in Northern Chinese. Further studies were still necessary to identify the real susceptible locus responsible for association and define the functional roles of the haplotype in *LEPR* gene in etiology of T2DM.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.diabres.2008.02.016.

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