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ORIGINAL PAPER

Evaluation of the association between retinal binding protein 4 polymorphisms and type 2 diabetes in Chinese by DHPLC

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Abstract Serum retinal binding protein 4 (RBP4) was recently described as a new liver- and adipocyte-derived signal that may contribute to Type 2 diabetes mellitus (T_2DM) and insulin resistance. The aim of this study was to test whether the *RBP4* gene could be used as a genetic marker to predict the development of T_2DM amongst the Chinese population of Han. For this study, a normal control group of 115 healthy subjects and an experimental group of 107 patients with T_2DM were examined. A combined method of denaturing high-performance liquid chromatography (DHPLC) and sequencing was applied to the detection of the RBP4 gene variants. Two SNPs, rs17484721 and rs36035572, were analyzed. Phenotypes and biochemical indicators related to the metabolism of glucose and lipid were measured. We found that there are significant differences between the control group and the patients group in terms of their respective distributions of genotype and allele frequency. The TG levels of the TT and II genotype was significantly higher than that of the TC + CC and ID + DD, respectively, in both patient group and control group. These findings suggest that the variations in the RBP4 gene may be associated with T_2DM and serum triglyeride levels in the Han Chinese.

Keywords Retinal binding protein 4 · Single nucleotide polymorphism · Denaturing high-performance liquid chromatography · Type 2 diabetes Mellitus · Linkage disequilibrium

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Introduction

Type 2 diabetes mellitus (T_2DM) is a common glucose homeostasis disorder. The growing prevalence of the disease is a trend seen even in developing countries [1]. Insulin resistance and progressive β-cell failure are key factors in the pathogenesis of T_2DM [2]. Genome scans and association studies suggest a complex genetic etiology for diabetes susceptibility [3].

The current study showed that adipocytes secrete a plethora of cytokine-like products that are called adipokines (including tumor necrosis factor α, resistin, leptin, interleukin6, and adiponectin), which bear a close relationship to glucose homeostasis and the metabolic actions of insulin [4]. Recent studies have suggested an even broader role for adipocytes in the control of whole body glucose metabolism. Retinal binding protein 4 (RBP4) was recently described as a new adipokine that is related to T_2DM [5]. Over-expression of serum RBP4 can be driven in murine adipocytes enforced to diminish the expression of GLUT4, and the resultant increased serum RBP4

induces hepatic expression of gluconeogenic enzymes and impaired insulin signaling in muscle. It should be added that RBP4 may be a key factor in regulating peripheral tissue response to insulin-stimulated GLUT4 action [6]. Recent studies [7, 8] have demonstrated that serum RBP4 levels correlate with both the magnitude of insulin resistance in diabetes and the parameters of metabolic syndrome (MetS). This was also demonstrated by Graham et al [9]. A study aiming at the Chinese people indicated that elevated RBP4 levels may contribute to the development of insulin resistance [10]. The evidence of serum RBP4 level correlating with both insulin resistance and T_2DM is enough but the mechanisms are unclear. Ost et al. [11] found that RBP4 contribute to insulin resistance by acting locally to inhibit phosphorylation of IRS1 at serine (307), a phosphorylation site that may integrate nutrient sensing with insulin signaling. These studies suggested that genetic variations in RBP4 might alter the risk for T_2DM [12].

The chromosomal location of RBP4 is in 10q23.33 and between the *GPR120* (G protein coupled receptor 120) gene and the *PDE6C* (phosphodiesterase 6C) gene. This district has been linked to an increased risk for T_2DM in different populations [13, 14]. The total length of the RBP4 gene, which is composed of five exons and six introns, is about 10 kb[1]. A previous study showed that the noncoding SNPs may increase diabetes susceptibility in Caucasians and may contribute to insulin resistance and reduced insulin secretion [15]. Another study aiming at Mongolians indicated that the *RBP4* gene is associated with T_2DM [16]. There has been strong evidence that type 2 diabetes has genetic determinants, which may influence the disease rates among different peoples [17–19]. The metabolic basis of diabetes also varies among various ethnic groups [20–22]. Not much is known about the relationship between *RBP4* gene polymorphisms and T_2DM for the Chinese people. Hence, the genomic region of RBP4 was genetically surveyed in China.

Result

Analysis of the result of denaturing high-performance liquid chromatography analysis (DHPLC)

Two SNPs (rs17484721 and rs36035572) were identified in Chinese Han population by DHPLC among 237 blood samples. Each SNP has two allele (T/C of rs17484721, I/D of rs36035572) and three genotypes (TT/TC/CC of rs17484721, II/ID/DD of rs36035572). The two SNPs had minor allele frequencies >5%. We obtained three chromatogram of DHPLC: 147 homozygous wild type (Fig. 1a), 71 heterozygous mutants (Fig. 1c) and 4 homozygous mutants (Fig. 1b). We confirmed them by direct sequencing. DNA sequencing analysis showed that the variations in the two SNPs always took place simultaneously. Whenever a T allele was detected at rs17484721, a I allele was always detected at rs36035572; whenever a C allele was detected at rs17484721, a D allele was always detected at rs36035572 due to mutation. The result of DNA sequencing analysis is shown in Fig. 2. We assessed their linkage disequilibrium (LD) and found that they were in strong pairwise LD ($D' > 0.9$, $r^2 > 0.9$).

Case–control study

We tested the genetic variation in all the subjects and found that the T and I allele frequencies were 82.2% and the C and D allele frequencies were 17.8%. The observed genotype frequencies of the two SNPs did not deviate from Hardy–Weinberg quilibrium. For the two SNPs, the frequency of the TT and II genetype were higher in the case group compared with that of the control group (dominant model, $P = 0.021$). The frequency of allel T and allel I were higher and the allel C and allel D were lower in T_2DM patients compared to case–controls (the allele frequency, $P = 0.026$). The data of genotypes and allele frequencies were in Table 1.

Haplotype case–control study

The haplotypes (either T/T-I/I or C/C-D/D) were constructed by the two SNPs. The T/T-I/I haplotype was higher and the C/C-D/D haplotype was lower in T_2DM patients compared to case–controls ($P = 0.024$). Table 2 showed the frequencies of haplotypes.

Analysis clinical characteristics of the genotypes

To assess the genotype–phenotype correlation (see Table 3), we evaluated biochemical indicators of all the subjects. We found an association between genotype and serum triglycerides (TG). The TG serum levels of the TT and II genotype was significantly higher than that of the TC + CC and ID + DD, respectively, in both patient group ($P = 0.029$) and control group ($P = 0.013$).

Discussion

We screened parts of intron 5 of the *RBP4* gene by DHPLC. We only found two SNPs, rs17484721 and rs36035572, which had been listed in Genebank. No novel SNPs were found. We also found that they were in a very

[1] http://www.ncbi.nlm.nih.gov/

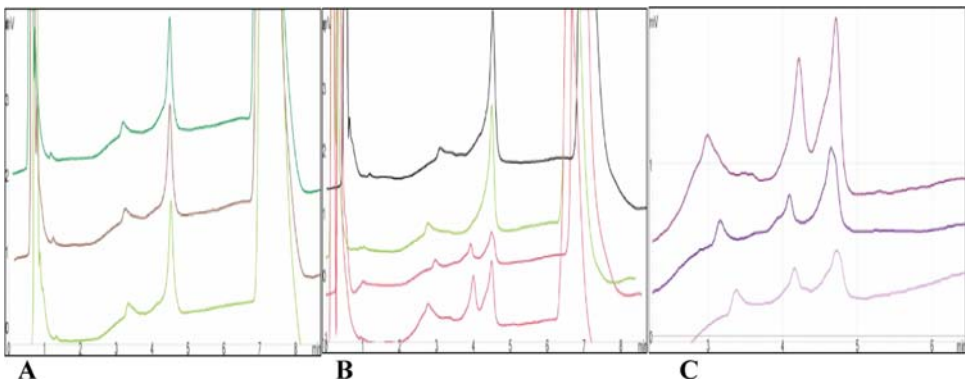

Fig. 1 Chromaotgraphic WAVE pattern by DHPLC for the RBP4 PCR products. Note: **a** DHPLC chromatogram of the carriers without mutation; **b** DHPLC chromatogram of the carriers with homozygous mutation; **c** DHPLC chromatogram of the carriers with heterozygous mutation

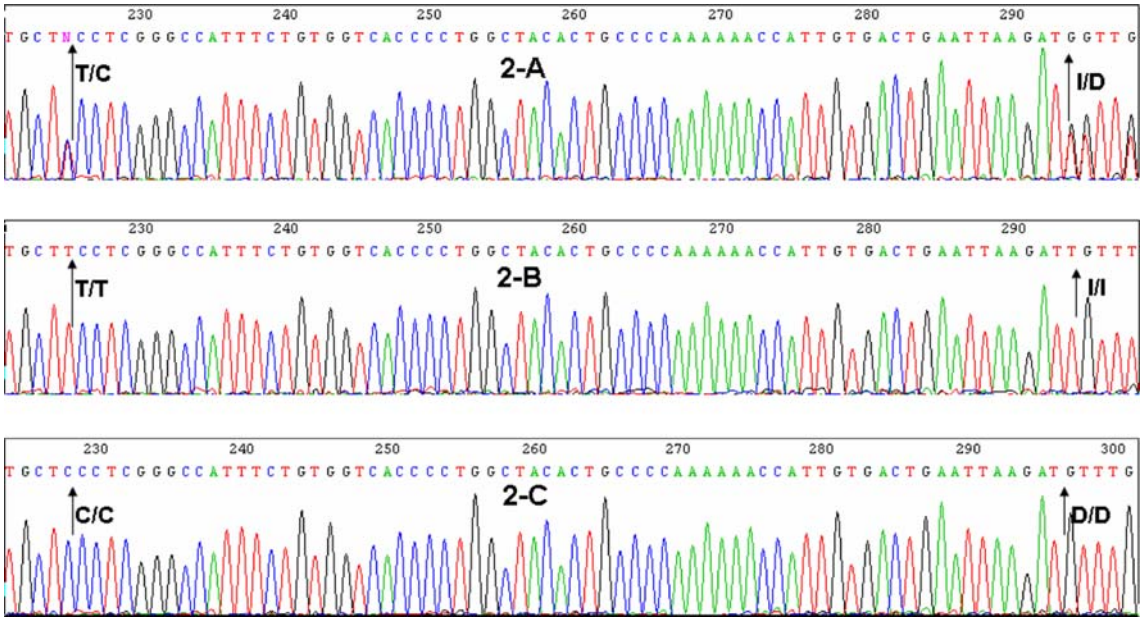

Fig. 2 The result of DNA sequence analysis. Note: On the left is rs17484721; on the right is rs36035572. The distance between the two SNPs is 68 bp. I represents the inserted variation of T; D represents T at rs36035572 that was lost due to mutation. 2-A: heterozygous variant with T/C and I/D; 2-B: the sequence without any mutation; 2-C: homozygous variant with C/C and D/D

tight LD block (D′ >0.9, $r^2 > 0.9$). Munkhtulga et al [16] reported that the tight LD block, where rs17484721 and rs36035572 are located, continued until the 3′end of the downstream gene, *GPR120* gene, which is the receptor for unsaturated long-chain free fatty acid (FAA). It could promote the secretion of glucagon-like peptide-1 (GLP-1) from the gastro-intestinal tract [23]. Because GLP-1 is an important factor for insulin secretion during hyperglycemia [24], *GPR120* gene is indeed a potential candidate gene to be resolved by further genetic association study with diabetes. The present investigation revealed that there are significant differences between the control group and the patient group regarding the genotype frequencies and allele frequency of the two SNPs. Furthermore, the results from the present study demonstrated convincingly the TT and II genotype contributes to the susceptibility to T_2DM. This report is the first one to show that the two SNPs of the RBP4 gene are correlated with T_2DM in Chinese Han population.

The loci of rs17484721 and rs36035572 are in the non-coding region of RBP4 gene. Our result indicated that they were associated with diabetes. The mechanisms by which non-coding RBP4 variants could increase diabetes risk are unclear. One possible explanation is that both tested SNPs have impact on alternative splicing or are in strong LD with variants that may have a functional role in RBP4 gene function or gene expression. We have not measured serum RBP4 levels because RBP4 is expressed widely, with the highest level in the liver, but with significant expression also in pancreatic islets based on expressed sequence tags (ESTs). Hence, the gene may have non-endocrine effects [15]. Another possible explanation why the detected SNPs

Table 1 Genotypes and allele frequencies of RBP4 SNPs and diabetes susceptibility in Chinese people

	rs17484721	rs36035572
Control		
Major homo	68	68
Minor homo	44	44
Hetero	3	3
Total	115	115
Major allele	0.783	0.783
Minor allele	0.217	0.217
HWE(*P*-value)	0.662	0.662
DM		
Major homo	79	79
Minor homo	27	27
Hetero	1	1
Total	107	107
Major allele	0.865	0.865
Minor allele	0.134	0.134
HWE(*P*-value)	0.962	0.962
Chi-square *P*-value[OR (95% CI)]		
Recessive model	0.349[0.35(0.04–3.44)]	0.349[0.35(0.04–3.44)]
Dominant model	0.021[a][0.51(0.29–0.91)]	0.021[a][0.51(0.29–0.91)]
Genotype model	0.051	0.051
Allele frequency	0.024[a][0.56(0.34–0.93)]	0.024[a][0.56(0.34–0.93)]

Odds ratio (OR) with 95% confidence interval (CI) is given just for comparison with two groups

[a] Statistically significant after Bonfferroni correction

Table 2 Haplotype case–control study in Chinese Han population

Haplotype	Control	Diabetes	Chi-square	*P*-value
T/T-I/I	0.783	0.865	5.084	0.0242
C/C-D/D	0.217	0.134	5.084	0.0242

could increase diabetes risk is that the RBP4 gene was in a tight LD block, and the SNPs in the LD block showed significant association with diabetes [16].

This study also found that the TG serum levels of the TT and II genotype was significantly higher than that of the other genotypes in both the patient group and the control group. This observation confirmed that the TT and II genotype is correlated with serum triglyeride levels in Chinese Han population. The mechanism of this association is unclear. Because rs17484721 and rs36035572 loci are located in a tight LD block, which was illuminated above, the possible reason for this association may result from a simple LD between the two SNPs and unknown genes affecting levels of TG.

In conclusion, we believe that the polymorphisms of rs17484721 and rs36035572 contribute to the risk of T_2DM and that their variations correlate with serum triglyeride levels in Chinese Han population. The present study is the first to find genetic variants at the two SNPs of *RBP4* gene in Chinese Han population. Further study is required to unravel the exact mechanism how the *RBP4* gene contributed to the risk of T_2DM.

Materials and methods

Subjects

This study was approved by the Ethics Committee of Lanzhou University. After providing informed consent, 107 diabetic patients were recruited from the People's Hospital of Gansu Province. All patients, who are Northwestern of Chinese Han population, were diagnosed according to the 1999 revised criteria for T_2DM established by WHO. Type 1 diabetes, liver diseases, renal diseases and heart diseases were excluded by clinical and laboratory examination because these possibly affected insulin sensitivity and insulin secretion. The 115 subjects in the control group were unrelated healthy subjects recruited from individuals presenting for check-up in the hospital. They had no known family history of diabetes and had either a normal 75 g oral glucose tolerance test or a fasting glucose level below 6.0 mmol/l (108 mg/dl). The healthy subjects and the patients were matched by age and sex. The clinical data profile is shown in Table 4.

PCR amplification

Genomic DNA was extracted from EDTA (ethylenediamine tetraacetate)-anticoagulated peripheral blood according to standard protein K digestion and phenol/chloroform extraction method [25]. The reference sequence

Table 3 Biochemical indicator comparison between case and control groups in Chinese people ($\bar{x} \pm s$)

Item genotype	Control TT/II	TC + CC/ID + DD	Case TT/II	TC + CC/ID + DD
Years	52.44 ± 8.95	51.21 ± 9.05	53.96 ± 9.89	53.33 ± 9.57
BMI(kg/m^2)	24.38 ± 3.54	22.73 ± 3.12*	24.56 ± 2.83	24.15 ± 3.19
WHR	0.84 ± 0.07	0.82 ± 0.07	0.88 ± 0.08	0.89 ± 0.08
FPG(mmol/l)	4.79 ± 0.67	4.62 ± 0.73	12.13 ± 6.10	11.69 ± 5.67
FPI(mU/l)	13.91 ± 12.17	12.39 ± 9.82	15.05 ± 12.09	16.32 ± 16.66
HOMA-IR	2.55 ± 2.36	2.15 ± 2.13	8.77 ± 10.35	7.93 ± 7.62
SBP(mmHg)	124.42 ± 16.41	125.00 ± 20.08	135.67 ± 22.30	133.75 ± 15.78
DBP (mmHg)	80.79 ± 9.8	78.15 ± 11.32	82.43 ± 10.72	82.64 ± 11.14
TC(mmol/l)	4.82 ± 1.14	4.70 ± 1.16	4.90 ± 1.39	5.08 ± 1.31
TG(mmol/l)	1.68 ± 1.07	1.15 ± 0.46*	2.41 ± 1.28	1.73 ± 0.70*
HDL-C(mmol/l)	0.86 ± 0.16	0.90 ± 0.24	0.93 ± 0.35	1.02 ± 0.34
LDL-C(mmol/l)	1.87 ± 0.60	1.87 ± 0.65	2.17 ± 0.77	2.33 ± 0.87

BMI, body mass index; FPG, fasting plasma glucose; FPI, fasting plasma insulin; HOMA-IR, insulin resistance index; SBP, systolic blood pressure; DBP, Diastolic blood pressure; TC, serum total cholesterol; TG, serum triglyeride; HDL-C, high density lipoprotein–cholesterol; LDL-C, low density lipoprotein–cholesterol; Vs TT/II group: * $P < 0.05$

Table 4 Anthropometric and clinical features of the subjects in the Han Chinese

	Control	Case
Age (years)	51.94 ± 8.97	53.80 ± 9.77
BMI(kg/m^2)	23.71 ± 3.46	24.44 ± 2.92
WHR	0.83 ± 0.07	0.89 ± 0.08*
FPG(mmol/l)	4.72 ± 0.69	12.01 ± 5.96*
FPI(mU/l)	13.31 ± 11.28	15.40 ± 13.39
HOMA-IR	2.40 ± 2.27	8.43 ± 9.68*
SBP(mmHg)	124.65 ± 17.76	134.75 ± 19.71*
DBP(mmHg)	79.72 ± 10.49	82.70 ± 10.95

Vs control; * $P < 0.05$

(i.e., wild type sequence) of the RBP4 gene was extracted from the NCBI GenBank (NT_030059) [see footnote 1]. Specific primer pair was designed by Primer5 software. The forward primer was 5′- AGGGTGCCTTCTGGCTC TTC-3′and the reverse primer was 5′-CTTTACTGGGCTG CTCAATC-3′.The PCR conditions were as follows. Initial denaturation was performed at 95°C for 2 min, and was followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 40 s, and final extension at 72°C for 10 min.

Denaturing high-performance liquid chromatography analysis

Mutation screening of the RBP4 gene was performed on the Wave system (Transgenomic Inc., Omaha, USA) by DHPLC. Each PCR product was denatured for 5 min at 95°C and then gradually re-annealed by decreasing sample temperatures from 95 to 56°C over a period of 30 min. The melting temperature was 60.5°C (predicted using Wavemaker version 4.0.32) and the application of sample was 5 μl. The column mobile phase consisted of a mixture of 100 mM triethylammonium acetate (pH 7.0) (buffer A) and 100 mM triethylammonium acetate (pH 7.0) with 25% (v/v) acetonitrile (buffer B). The elution profiles of heterozygous fragments were represented as multiple peaks and the homozygous fragments as single peaks. To distinguish the homozygote mutant type sequence, samples showing single peak were mixed with the reference DNA. If the two combined samples were different, i.e., homozygous mutant + homozygous wild type, the DNA fragments would elute at different mobilities and appeared as multiple peaks. It would indicate the presence of SNP (or nucleotide change) in one of the combined samples.

DNA sequencing

The variations were confirmed by direct sequencing using dye-labeled terminators (BigDye terminator v3.1) on an automated ABI PRISM 3730 Genetic Analyzer. (Shen Gong Ltd., Shanghai, China)

Statistical analysis

The data were analyzed using contingency table analysis and Student's t test. Hardy–Weinberg equilibrium was evaluated using the chi-square test. A comparison between the proportions of the prevalence of an allele in the patients and controls was also performed. Odd ratios were generated from two-by-two tables, and statistical significance was assessed using Fisher's extract. D' and r^2 values were analyzed to evaluate LD; $|D'| > 0.5$ or $r^2 > 0.5$ was

regarded as indicating tight linkage. Clinical characteristics were expressed as mean ± SD or mean ± SE. Statistical analyses were performed using SNPAlyze (version 7.0, Dynacom, Mobara, Japan) and SPSS (version 11.0; SPSS Inc., Chicago, Illinois, USA). Statistical significance was set at $P < 0.05$ level.

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