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ORIGINAL PAPER

Association of TRB3 gene Q84R polymorphism with type 2 diabetes mellitus in Chinese population

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Abstract *Background* TRB3, a human homolog of Drosophila Tribbles, has been shown as a critical negative regulator of Akt (also known as protein kinase B), which is a key component in insulin signaling. In addition, TRB3 is another PPAR-target gene and functions as an important link between glucose and lipid metabolism. The Q84R polymorphic variant of TRB3 has been linked to insulin resistance and related clinical outcomes. However, it is unclear whether this polymorphism is associated with type 2 diabetes mellitus (T2DM) in the Chinese population. *Methods* In this study, we genotyped Q84R polymorphism in 177 patients with T2DM and 245 control subjects in Chinese population by using the polymerase chain reaction/ligase detection reaction (PCR/LDR) assay. *Results* No significant difference in the Q84R genotype frequency was observed between T2DM patients and controls ($P = 0.642$). In T2DM group, the Q84R variant in cases was associated with higher FINS, higher HOMA-IR, and lower LnISI ($P = 0.003$, 0.001, and 0.001, respectively). However, the changes in HOMA-IR and LnISI were not significant in controls (the P value is 0.098 and 0.203, respectively). In addition, FINS levels were also significantly increased from Q84Q to R84 in controls ($P = 0.036$). *Conclusion* Our data indicate that the TRB3 Q84R polymorphism is not associated with T2DM in Chinese population. However, the Q84R variant is associated with insulin resistance among T2DM patients in Chinese population.

Keywords Type 2 diabetes · TRB3 · Mutation · Insulin resistance · LDR

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Introduction

The metabolic syndrome is a clustering of risk factors known to increase the risk for the development of diabetes mellitus and cardiovascular disease [1], and insulin resistance (IR) is implicated as a hallmark and physiopathologic foundation of metabolic syndrome. Akt/PKB, a serine/threonine protein kinase, plays a critical role in insulin receptor-coupled phosphatidylinositol 3-kinase-mediated signaling [2].

TRB3 is located on the 20p13 human chromosome region that has been associated with T2DM [3, 4], which attracts many researchers. TRB3 (Drosophila Tribbles-homologue proteins), which belongs to a newly identified family of proteins, disrupts insulin signaling by binding directly to Akt and blocking activation of the kinase [5, 6] and may be involved in metabolic syndrome by inhibiting activation of PKB [7]. Du et al. [5] found that amounts of TRB3 RNA and protein were increased in livers of db/db diabetic mice compared with those in wild-type mice, and that hepatic overexpression of TRB3 in amounts comparable to those in db/db mice promoted hyperglycemia and glucose intolerance. Therefore, they indicated that TRB3 contributed to IR in individuals with susceptibility to T2DM by interfering with Akt activation. Prudente et al. [8] have first indicated that the Q84R polymorphism was associated with IR and related clinical outcomes.

We therefore assessed the hypothesis that the Q84R mutation might be a risk factor for T2DM and IR.

Materials and methods

Study subjects

Consecutive 177 (107 males and 70 females aged 35–79) unrelated diabetes patients (case group) were recruited from the inpatients who were admitted to Gansu Provincial People's Hospital in 2007 and 2008. Identification of T2DM was based on the World Health Organization Definition (WHO) 1999 definition [9]. The non-diabetic subjects (control group) consisted of 245 health volunteers (125 males and 120 females aged 35–79) who were selected randomly during the same period in Medical Examination Center (MEC) of Gansu Provincial People's Hospital. There were 422 subjects participating in the study conducted according to the principles of the Declaration of Helsinki. Written informed consent was received from each study subject for genotyping and the oral glucose tolerance test (OGTT) together with the measurement of autonomic functions. The study subjects were asked not to use any alcohol-containing drinks or tobacco products over 24 h preceding the study. At the visit, anthropometric measurements (height, weight, waist circumference, and hip circumference) and blood pressure measurements were carried out. Also basic laboratory measurements were performed (total cholesterol, low-density lipoprotein and high-density lipoprotein cholesterol concentrations, triglycerides, HbA_{1c}, fasting plasma glucose, alanine transferase, blood urea nitrogen, and creatinine concentrations) by standard methods in Gansu Provincial People's Hospital. All subjects were Chinese people residing in Gansu Province and had no family history of diabetes and no history of significant concomitant diseases. Exclusion criteria were ethanol abuse, acute or chronic hepatopathy and nephropathy, abnormal laboratory test results, and abnormal clinical signs or symptoms. Ethanol should be particularly stressed, because ethanol intake is positively associated with HDL-cholesterol concentration and inversely associated with insulin concentrations [10], and is a factor leading to IR [11]. In addition, ethanol induces TRB3 and prevents plasma membrane association of Akt, Akt-Thr308 phosphorylation, and subsequent Akt-mediated signaling [12]. The baseline characteristics of diabetic subject and control subjects are shown in Table 1.

Table 1 Comparison of clinical characteristics of case and control groups ($\bar{x} \pm s$)

	Case	Control	*P* value
n	177	245	
Sex (F/M)	107/70	125/120	0.055
Age (years)	56.15 ± 11.81	55.55 ± 12.69	0.648
Height (m)	164.97 ± 8.16	164.94 ± 7.82	0.971
Weight (Kg)	66.81 ± 11.94	65.48 ± 11.42	0.246
Waist circumference (cm)	86.50 ± 10.09	83.45 ± 10.18	0.002**
Hip circumference (cm)	96.07 ± 6.70	96.69 ± 6.93	0.360
BMI (kg/m^2)	24.46 ± 3.47	24.00 ± 3.50	0.190
WHR	0.90 ± 0.06	0.86 ± 0.07	0.000**
Systolic BP (mm Hg)	132.77 ± 19.19	125.26 ± 19.11	0.000**
Diastolic BP (mm Hg)	80.65 ± 10.94	78.28 ± 10.85	0.029*
FPG (mmol/l)	11.44 ± 4.26	4.71 ± 0.71	0.000**
Fasting Insulin (mU/l)	10.58 ± 3.47	8.70 ± 5.60	0.000**
Hemoglobin A1c (%)	8.43 ± 2.02	4.83 ± 0.62	0.000**
TC (mmol/l)	4.85 ± 1.38	4.68 ± 1.07	0.514
TG (mmol/l)	2.20 ± 1.59	1.62 ± 0.82	0.000**
HDL-C(mmol/l)	1.07 ± 0.32	1.22 ± 0.45	0.001**
LDL-C(mmol/l)	2.54 ± 0.84	2.30 ± 0.74	0.002**
HOMA-IR	5.36 ± 2.58	1.85 ± 1.44	0.000**
LnISI	−4.674 ± 0.499	−3.574 ± 0.514	0.000**

Note: *BMI* body mass index, *WHR* waist to hip ratio, *BP* blood pressure, *FPG* fasting plasma glucose, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *TC* total cholesterol, *TG* triglyceride, *LnISI* logarithm of ISI

Sex was evaluated by χ^2-test. Age, BMI(normal distributed), height, weight, waist circumference, systolic BP, diastolic BP, fasting Insulin, LnISI, and LDL-C were expressed as means ± SD and compared by Student's *t* test. Hip circumference, FPG, TC, TG, HDL-C, hemoglobin A1c, and HOMA-IR were expressed as means ± SD and analyzed by Mann–Whitney *U* test. Vs Cases group: $^*P < 0.05$, $^{**}P < 0.01$

Laboratory measurements

Venous blood sample of 5 ml was drawn from all subjects into tubes containing ethylenediamine tetraacetic acid after an overnight fast. Biochemical indicators and phenotypes related to glucose and lipid metabolism were tested in Clinical Laboratory in Gansu Provincial People's Hospital. Anthropometric measurements from cases and control subjects were done in our ward and in Medical Examination Center, respectively. HOMA-IR (Homeostasis model assessment of IR), fasting glucose (mmol/l) × fasting insulin (μU/ml)/22.5 [13], was expressed as an index of IR. ISI (insulin sensitivity index), 1/FPG × FINS, was used to assess Insulin sensitivity [14].

Genotyping

DNA extraction

Blood leukocyte DNA was extracted from whole blood from study subjects by using the phenol–chloroform method.

PCR amplification

Amplification of TRB3 gene fragment (168 bp) was performed with a pair of primers: upstream primer 5′-GTC

TTGGGCCCTATGTCCTC-3′ and downstream primer 5′-CACCCTGGCAATCCTTTG-3′ (designed using Oligo 6 primer analysis software). The reaction mixture (20 μl) contained 2 μl of 1× PCR Buffer, 0.3 μl of *Taq* DNA polymerase, 4 μl of Q-solution (QIAGEN), 2 μl of 100× deoxynucleoside triphosphates(Promega,2 mM/each), 7.5 μl of sterile H_2O, and 2 μl of each primer(2 pM/l). PCR amplification conditions were 95°C for 15 min (1 cycle), 94°C for 30 s, 53°C for 1 min and 72°C for 1 min (35 cycles), and 72°C for 7 min (1 cycle). To evaluate the overall amplification efficiency, PCR product was electrophoresed on 3% agarose gels and visualized on an electrophoresis apparatus (FR-200A, Shanghai Furi Science & Technology Co. Ltd) (Fig. 1).

Post-PCR LDR reaction

Sequences of LDR probes were as follows: rs2295490_modify 5′-P-GGTAGGCCCGCCCGCCCTCCTCGGGTTTTTTTTTTTTTTTT-FAM-3′, rs2295490_A 5′-TTTTTTTTTTTTTTTGTGCCTGTAGGGCAGTGCAGGGCCT-3′, and rs2295490_G 5′-TTTTTTTTTTTTTTTTTTTTGTGCCTGTAGGGCAGTGCAGGGCCC-3′ (Shanghai Sangon Biological Engineering Technology & Services Co. Ltd.). In a 200-μl PCR tube, LDR was performed in a final volume of 10 μl containing 2 μl of PCR product (100 ng/μl), 1 μl Tris–HCl buffer (pH 7.6), 1 μl of LDR probe (12.5 pmol/μl), 6 μl of sterile H_2O, and 2 U of Taq DNA ligase (New England Biolabs, NEB). The reaction mixtures were heated for 2 min at 95°C, followed by 35 thermal cycles of 94°C for 30 s (denaturation) and 60°C for 2 min (annealing and ligation). Prior to polyacrylamide gel electrophoresis, the LDR products were mixed with 1 μl of Genescan-500 Tamra Size Standards (ABI), 1 μl of formamide loading buffer (90% formamide, 10 mM EDTA, PH 8.0, 0.1% xylene cyanol, 0.1% bromophenol blue) and denatured for 2 min at 95°C. The LDR products were separated by electrophoresis on 5% denaturing polyacrylamide gels (5.0 mol/l urea, 40% formamide) run at a constant temperature (40°C) and voltage (3,000 V) and were analyzed thereafter with Genescan™ 672 analysis software.

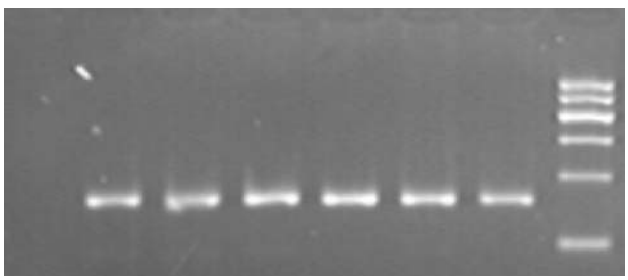

Fig. 1 Agarose gels electrophoresis after PCR. Length of PCR production was 168 bp; 100 bp DNA Marker I/DL600

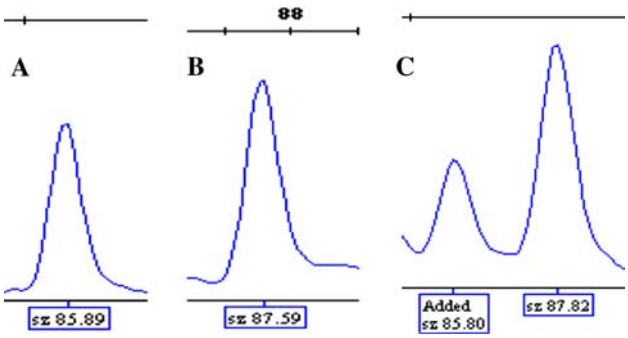

Fig. 2 The result of genotype analysis. **a** Q84Q genotype. **b** R84R genotype. **c** Q84R genotype

One microliter of each LDR amplification product was genotyped using GENEMAPPER™ V3.7 software (Fig. 2).

Statistical analysis

All statistical analyses were conducted using the SPSS (version 13.0; SPSS, Inc.). A χ^2-test was carried out to assess whether the observed genotype frequencies were in Hardy–Weinberg equilibrium. To compare the means of the variables measured, the Student's *t* test and Mann–Whitney's *U* test were used,where appropriate. The one 84R homozygote identified was pooled among the heterozygote in the analyses. Results with *P* value less than 0.05 were considered statistically significant. Odds ratios (OR) and 95% confidence intervals (CI) were used to compare categorical variables. All the tests carried out were two-sided.

Results

In this study, we detected the Q84R polymorphic variant of TRB3 in a Chinese sample (177 cases and 245 controls). Compared with the control subjects, T2DM patients had significantly higher waist circumference, hip circumference, WHR, systolic BP, diastolic BP, FPG, TG, LDL-C, HBA1c, HOMA-IR, and lower HDL-C and ISI. However, there were no significant differences in BMI and TC levels between the two groups (Table 1).

All genotypes accorded with the Hardy–Weinberg equilibrium after adjusting for age, sex, and BMI ($P = 0.823$ in diabetes group and $P = 0.642$ in non-diabetes group, respectively). The frequencies of the polymorphisms Q84Q, Q84R, and R84R were 67.8%, 29.4%, and 2.8% in the diabetes cases and 66.5%, 30.6%, and 2.9% in controls, respectively. The OR for those with the QR and RR genotypes compared with those with the QQ genotype was 1.045(95% CI 0.731–1.495) ($P > 0.05$). The SNP showed no significant difference in both genotype and allele frequencies between the cases and controls (Table 2).

Table 2 Genotype and allele distribution of the Q84R polymorphism in case and control groups

SNP	Genotype(frequency)			HWE p	▲P	Allele(frequency)		*P*	OR(95%CI)
Rs2295490	Q84Q	Q84R	R84R			Q	R		
Case	120(67.8%)	52(29.4%)	5(2.8%)	0.823	0.962	292(82.5%)	62(17.5%)	0.808	1.045(0.731,1.495)
Control	163(66.5%)	75(30.6%)	7(2.9%)	0.642		401(81.8%)	89(18.2%)		

Note: ▲*P*: QQ vs QR + RR

Table 3 The baseline characteristics of the study subjects according to TRB3 genotype

Characteristics	Case			Control		
	Q84Q	R84	*P* value	Q84Q	R84	*P* value
Sex (F/M) (*n*)	72/48	37/20	0.530	90/73	36/46	0.095
Age(years)	55.2 ± 11.8	58.2 ± 11.7	0.120▼	55.5 ± 12.7	54.0 ± 12.0	0.364▽
FPG (mmol/l)▼	11.03 ± 4.25	12.30 ± 4.17	0.053	4.76 ± 0.79	4.60 ± 0.51	0.429
Fasting insulin (mU/l)▼	10.09 ± 3.39	11.61 ± 3.44	0.003**	8.27 ± 5.28	9.55 ± 6.14	0.036*
HOMA-IR▼	4.90 ± 2.38	6.32 ± 2.74	0.001**	1.78 ± 1.43	1.97 ± 1.47	0.098
LnISI▽	−4.586 ± 0.495	−4.861 ± 0.458	0.001**	−3.544 ± 0.499	−3.633 ± 0.542	0.203

Note: Sex was evaluated by χ^2 test; ▽Student's *t* test, ▼Mann–Whitney's *U* test. Vs Q84Q; *$P < 0.05$, **$P < 0.01$ after adjusting for age, sex, and BMI between the two groups

R84: Because of the relatively low occurrence of subjects with R84R genotype, the R84 allele carriers (Q84R and R84R genotype) were ascribed to a single group

The baseline characteristics of the study subjects according to TRB3 genotype are shown in Table 3. In the analysis of Student's *t* test and Mann–Whitney's *U* test, we found that the R allele carriers had higher FINS, HOMA-IR and lower LnISI than the Q84Q carriers in the cases ($P = 0.003$, 0.001, and 0.001, respectively) (Table 3). However, only FINS among the three above-mentioned indicators in control subjects with R84 genotype was higher than that in those with Q84Q genotype and likewise was statistically significant ($P = 0.036$).

Discussion

In this study, we first conducted a hospital-based case–control study to assess the potential association between the TRB3 Q84R polymorphism and the presence of T2DM in Chinese population by post-PCR LDR assay.

The present study demonstrated that distributions of rs2295490 genotypes and alleles were not statistically different between the T2DM and control groups in a Chinese population, which was consistent with the results reported for Caucasian populations from Italy [8]. And our data showed that the TRB3 Q84R polymorphism had no significant association with T2DM in the Chinese population.

The combination of elevated TRB3 protein and constitutive S6K1 activity results in decreased insulin signaling via the IRS-1/PI3-kinase/Akt pathway [15]. The serine/threonine protein kinase PKB (also known as Akt) is thought to be a key mediator of signal transduction processes. PKBβ is highly expressed in insulin-responsive tissues such as adipose tissue [16], and PKBβ is also an essential gene for the maintenance of normal glucose homeostasis [17]. PKB phosphorylation and inactivation of GSK3 is likely to stimulate the conversion of nutrients such as glucose and amino acids to storage macromolecules (glycogen and protein) in skeletal muscle, adipose tissue, and liver [18]. These findings indicate that PKB has close relation with glycometabolism.

TRB3 has been initially identified by Eric Wleschaus and co-worker [19] during the study on String (the homolog of CDC25). Du and co-workers [20] suggested that insulin-induced TRB3 expression function as an indicator how multiple insulin-induced signal transduction pathways are balanced. Bi et al. [7] found that TRB3 mRNA alone significantly increased by 94% in adipose tissue of fructose-fed rats compared with those in adipose tissue of the controls ($P < 0.05$), and there was significant positive correlation between TRB3 mRNA levels and HOMA-IR in fructose group.

However, not all experiments agree with the function of TRB3. For example, in Iynedjian's experiment [21], the ability of overexpressed TRB3/NIPK to inhibit PKB phosphorylation can be detected. Moreover, the stimulation of Ser-21 and Ser-9 phosphorylation of glycogen synthase kinase 3-α and -β, and the apparent phosphorylation level

of 4E-BP1 (eukaryotic initiation factor 4-binding protein 1), were similar in transduced and control hepatocytes. Okamoto et al. [22] indicated that phenotypic analysis of Trib3$^{-/-}$ mice did not detect any alteration in serum glucose, insulin, or lipid levels; glucose or insulin tolerance; or energy metabolism.

The previous studies, indeed, provided no evidence for significant correlation between Q84R polymorphism of TRB3 and T2DM. However, we detected that the T2DM cases with Q84Q genotype displayed significantly difference in FINS, LnISI, and HOMA-IR, compared with the cases with R84 genotype. This is similar to an earlier study by Prudente et al. [8]. But they reported that FINS, insulin sensitivity, and HOMA-IR in 178 non-diabetic Caucasian were significantly different across the three genotypes, while those of T2DM group had no significant change ($P > 0.05$) in spite of the existence of the changes. We found that the FINS levels in control group were of statistical difference ($P = 0.036$), which was in agreement with the study by Prudente et al. However, our data in control group showed that no significant difference in HOMA-IR and LnISI has been observed between Q84Q and R84 individuals. This difference in results may be due to racial difference and dietary habits. These results suggested the ability of the mutation (the adenine to guanine transition) in TRB3 to inhibit PKB phosphorylation may potentially be enhanced, although the precise mechanism remains unknown. Thus, the mutation strengthened the occurrence of IR. IR has been implicated as a pathogenesis of T2DM, thus the results also indicated that the mirror R-allele had a higher risk of T2DM occurrence in a Chinese population, which might be taken as a marker in the clinical diagnosis and prevention of T2DM or as a therapeutic target in the treatment of T2DM patients. Impaired glucose tolerance, increased hepatic glucose production, IR [23], and impaired ability of insulin to lower blood glucose [17] were found in mice deficient in Aktβ. Therefore, we expected some changes in FPG levels between Q84Q carriers and R84 carriers. In our study, however, we found no evidence for an association of the mirror R-allele with altered FPG levels both in T2DM cases and in controls.

In conclusion, our results suggest that the genetic Q84R polymorphism of TRB3 is not associated with the presence of type 2 diabetes mellitus in the Chinese population. Because we only detected the Q84R polymorphism, we cannot exclude a potential role of other TRB3 gene variants in the development of T2DM. However, our data provide evidence of a statistically significant association of Q84R polymorphism with HOMA-IR and LnISI in the Chinese population with T2DM, and suggest individuals with wild-type Q84Q are less prone to IR. Further studies are needed to test the functional role of TRB3 variants on the IRS-1/PI3-kinase/Akt pathway.

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