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Association between polymorphisms in *RAPGEF1*, *TP53*, *NRF1* and type 2 diabetes in Chinese Han population

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ABSTRACT

Type 2 diabetes is a common complex disorder with environmental and genetic components. The aim of the present study was to investigate the association between the polymorphisms of *RAPGEF1*, *TP53* and *NRF1* and the risk of type 2 diabetes in the Chinese Han population. We genotyped rs11243444 (*RAPGEF1*), rs1042522 (*TP53*) and rs1882095 (*NRF1*) in a case-control study, including 273 type 2 diabetes and 247 healthy controls. A significant association was found in a variant of *TP53* (rs1042522, odd ratio (OR) = 1.28, 95% confidence interval (CI) = 1.00–1.64; $P = 0.046$), whereas polymorphisms in *RAPGEF1*, *NRF1* were not associated with the risk of type 2 diabetes. Furthermore, a potential gene–gene interaction showed the odds of being affected with type 2 diabetes was 2.54 times greater in subjects with the *TP53* (rs1042522) and *RAPGEF1* (rs11243444) risk alleles than those without either (95% CI = 1.34–4.81; $P = 0.004$) and the *NRF1* gene polymorphism reached significance when paired with *TP53*:(OR = 3.87, 95% CI = 1.87–8.40; $P = 0.0006$). We demonstrated that the polymorphism in *TP53* (rs1042522) was associated with type 2 diabetes, and that potential interaction of *TP53* (rs1042522) and *RAPGEF1* (rs11243444), or *NRF1* (rs1882095) increased the risk of type 2 diabetes.

1. Introduction

Type 2 diabetes is a major health problem worldwide [1], which represents the leading cause of chronic kidney disease, diabetic retinopathy, limb amputation and a major risk factor for myocardial infarction and stroke. To date, more than 200 million individuals worldwide are affected by type 2 diabetes. Moreover, as obesity increases and the population become older, the morbidity and the mortality are still progressively increasing in many developing countries, especially in China. The prevalence of this disease is expected to double by 2025, which will certainly increase the personal and social burden [2].

Type 2 diabetes is characterized by insulin resistance and pancreatic B cell dysfunction but the underlying molecular mechanisms have not been elucidated yet. The combination of multiple genetic and environment factors play important roles in the pathogenesis of type 2 diabetes. It has been proposed that the genetic background is able to heavily modulate the individual response to environmental factors so that up to 30–70% of type 2 diabetes risk can be attributed to genetics [3]. For a long time, investigators have worked to

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unravel the role of genetics in type 2 diabetes through epidemiological studies of candidate genes and genetic linkage in families. Candidate gene studies have identified lots of genes which are related with insulin resistance or pancreatic B cell dysfunction [4]. While this has provided important insights into some rare monogenic forms of diabetes, understanding the genetics of common type 2 diabetes remains a major challenge. Recently, the genome-wide association (GWA) studies using high density SNPs across the genome and complex genetic analyses have provided convincing evidence that several genes with previously unknown function are associated with the risk of diabetes [5–9]. These have not only uncovered a number of new genetic loci associated with diabetes and provided new targets for mechanistic investigation, but are also forcing us to reconsider the degree of genetic heterogeneity and possibly even the role of genetics itself in the pathogenesis of type 2 diabetes [10].

As a complementary approach to GWA studies, using a candidate gene approach, Gaulton et al. [11] reported that polymorphisms in *RAPGEF1* (rs4740813), *TP53* (rs1042522) and *NRF1* (rs1882095) increased the risk of type 2 diabetes. Recently, Hong et al. [12] discovered that the polymorphism in *RAPGEF1* (rs11243444) was also associated with type 2 diabetes in Korean population. However, it has been reported that there are significant differences in the contribution of known SNPs among some ethnic populations. It is not clear that whether these polymorphisms in *RAPGEF1* (rs11243444), *TP53* (rs1042522) and *NRF1* (rs1882095) were associated with type 2 diabetes in the Chinese population, moreover, the occurrence of type 2 diabetes was associated with environment and genetic polymorphism or the gene–gene interaction, and whether the potential interactions were associated with occurrence of type 2 diabetes remains unclear. In the present study, we confirmed whether these polymorphisms [*RAPGEF1* (rs11243444), *TP53* (rs1042522), *NRF1* (rs1882095)] and the interaction between these polymorphisms were the risk of type 2 diabetes in Chinese Han subjects.

2. Materials and methods

2.1. Subjects

According to the OR and the distribution of the alleles of the three genes [11,12], we made an estimation about the sample size by using EpiCalc 2000 software. In this study, we enrolled 273 type 2 diabetic patients (128 men and 145 women; age 55.39 ± 8.83 years, BMI $24.45 \pm 3.61\ kg/m^2$) and age and sex matched 247 healthy controls (136 men and 111 women; age 54.88 ± 8.34 years, BMI $24.50 \pm 3.10\ kg/m^2$). The diabetic patients were recruited from Nanjing First Hospital Affiliated to Nanjing Medical University according to 1999 World Health Organization criteria [13] between January 2009 and March 2010. Healthy control subjects were also recruited from the same hospital which came to do physical examination and they had no history of diabetes and with fasting plasma glucose level less than 5.7 mmol/L. Anthropometric measurements including body weight and height were performed on subjects in light clothing without shoes. Body mass index was calculated according to the Quetelet equation. Both the patients and controls came from the same area. There were no significant differences in all the data (including dietary habits, smoking and alcohol) collected in the questionnaire between the two groups. In particular, the study was conducted under the supervision of the Institutional Review Board of Nanjing First Hospital Affiliated to Nanjing Medical University and all participants provided written informed consent.

2.2. Laboratory methods

A 4-mL whole blood sample was drawn into a disodium EDTA tube (after the subjects had fasted for at least 10 h), stored at room temperature and centrifuged within 4 h. All measurements were performed at Central Laboratory of Nanjing First Hospital Affiliated to Nanjing Medical University. The quality of biological measures was assessed within the frame work of the project. Plasma total cholesterol, triglyceride levels, plasma high-density lipoprotein (HDL) cholesterol and plasma low-density lipoprotein (LDL) cholesterol were measured using enzyme assays and plasma glucose was measured using the standard glucose hexokinase method (BIOSINO, Beijing, China) (OLYMPUS AU2700, Japan). The HbA1c values were determined by high-performance liquid chromatography (BIO-RAD D-10, USA).

2.3. DNA isolation

Genomic DNA was extracted from the peripheral blood mononuclear cells with a Easy Nucleic Acid Isolation SE Blood DNA Kit (Omega, USA) according to the manufacturer's instructions. In brief, cells were lysed by cell lysis solution, and then the RNA in the sample was digested by RNase A solution. The protein was precipitated by protein precipitation solution. Finally, isopropanol was used to precipitate the genomic DNA, followed by washing with 70% ethanol.

2.4. Genotyping

The polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) was used to detect the polymorphisms in the *RAPGEF1*, *TP53* and *NRF1* gene.

The PCR amplification of *RAPGEF1*, *TP53* and *NRF1* gene were performed in a 25 μL reaction system consisting of 0.5 mmol/L of each primer (Table 1), 4 mmol/L of $MgCl_2$, 2.5 μL of AmpliTaq Polymerase (Takara, Dalian, China) and PCR buffer (10 mmol/L of Tris–HCl, pH 8.3; 50 mmol/L of KCl). The PCR cycle conditions were 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 59 °C for 30 s (*TP53*) or 56 °C for 30 s (*RAPGEF1*, *NRF1*), and 72 °C for 30 s, and then incubated at 72 °C for 7 min. After PCR amplification, 10 μL of PCR product was digested by 10 U of the restriction enzyme *Bcu* I (*RAPGEF1*) (MBI, Vilnius, Lithuania), *Hha* I (*TP53*) (MBI, Vilnius, Lithuania) or *Bsel* I (*NRF1*)(MBI, Vilnius, Lithuania) with buffer G (Tango; MBI) in a final volume of 30 μL at 37 °C (*RAPGEF1*, *TP53*) or 55 °C (*NRF1*) overnight. Fragments of DNA were electrophoresed on 4% agarose gels stained with ethidium bromide. To validate the genotyping results the genotyping experiments were

Table 1 – Sequences of primers and restriction enzymes used in PCR–RFLP analysis and expected size of products of each *RAPGEF1*, *TP53* and *NRF1* genotype.

SNP	Primers (5′–3′)	Restriction enzyme	PCR–RFLP products(bp)	Genotype
RAPGEF1	CCACAGAAAAAGTTTCCCAC [a] TA	Bcu I	108	CC
rs11243444	CTAAAGATGCCAGAACTCCTGAG		108,89,19	TC
			89,19	TT
TP53	AGAATGCCAGAGGCTGCTCCG [a] C	Hha I	107	GG
rs1042522	TGGGAAGGGACAGAAGATGACAG		107,85,22	GC
			85,22	CC
NRF1	TAAGGATGGGGCAGGAGGAA	Bsel I	199	TT
rs1882095	TAAAAAATGAGGGTGAGTGATGGTG		199,154,45	CT
			154,45	CC

[a] Primers in lower case letters denote the mismatched position that is required for subsequent restriction digestion for the differentiation of the polymorphisms.

repeated, and direct sequencing was performed in 10% of the samples.

2.5. *Statistical analysis*

Clinical characteristics were expressed as mean ± S.D. *T*-test were used to test differences in means of age, BMI, total cholesterol, triglyceride, HDL, LDL between type 2 diabetes and controls. One-way analysis of variance was used to test for differences in means of phenotypic characteristics between genotypes. Chi-square analysis was used to compare categorical variables. Unconditional logistic regression analysis was used to estimate odds ratio (OR) and its 95% confidence interval (CI) as a measure of the association between genotype carrier and risk of type 2 diabetes. The statistical analysis was performed using SAS statistical software package for Windows, version 9.13. *P* values of <0.05 were considered to be statistically significant.

3. Results

In comparison with controls, the type 2 diabetes group had significantly higher levels of HDL ($P < 0.001$) than the health control group (shown in Table 2). However, there was no significance in age, body mass index (BMI), total cholesterol, triglyceride, LDL in both groups. The effects of these three SNPs on those clinical features were analyzed subsequently. There were no significant associations with any characteristics for these three SNPs ($P > 0.05$). (Data not show). There were also no significant associations between HbA1C and genotypes of the three genes in type 2 diabetes (data not show).

The genotype and allele frequencies of the three SNPs, rs11243444 (T/C), rs1042522 (C/G) and rs1882095 (T/C) in the *RAPGEF1*, *TP53* and *NRF1* gene locus are listed in Table 3. Among the three SNPs, rs1042522 in *TP53* locus was significantly associated with type 2 diabetes. The risk allele G of rs1042522 was more frequent in the type 2 diabetic patients than in the control group (OR = 1.28, 95% CI = 1.00–1.64; *P* = 0.046). Heterozygous and homozygous carriers of the risk allele had higher OR of 1.66 (95% CI = 1.03–2.69; *P* = 0.038) and 1.92 (95% CI = 1.12–3.28; *P* = 0.017) relative to wild genotype, respectively. However, the rs11243444 in *RAPGEF1* locus and the rs1882095 in *NRF1* locus did not show significant differences between type 2 diabetic patients and control subjects.

The locus–locus interaction between *RAPGEF1* and *TP53*, *RAPGEF1* and *NRF1*, *TP53* and *NRF1* are shown in Table 4. The risk of a diagnosis for type 2 diabetes is greatest in subjects with the *RAPGEF1* and *TP53* SNP risk alleles versus those with neither risk allele (adjusted OR = 2.54, 95% CI = 1.34–4.81; *P* = 0.004). There is no or minimal increased risk for type 2 diabetes at these two loci polymorphisms if only one risk allele is present: adjusted OR = 2.14(95% CI = 1.15–3.97; *P* = 0.016) for the presence of the *TP53* risk allele but not the *RAPGEF1* risk allele, and adjusted OR = 1.97(95% CI = 0.82–4.74; *P* = 0.132) for the presence of the *RAPGEF1* risk allele but not *TP53* risk allele. For *TP53* with *NRF1*, if only the *TP53* risk allele but not the *NRF1* risk allele is present: adjusted OR = 4.29(95% CI = 1.97–9.37; *P* = 0.0003) and if only the *NRF1* risk allele but not the *TP53* risk allele, is present: adjusted OR = 4.70(95% CI = 1.76–12.56; *P* = 0.002), while if both the *TP53* risk allele and the *NRF1* risk allele are present: adjusted OR = 3.87(95% CI = 1.87–8.40; *P* = 0.0006). There was no evidence for an increased risk for

Table 2 – Characteristics of type 2 diabetic cases and controls (Mean ± SD).

Variables	Age (years)	Sex (M/F)	BMI (kg/m2)	Total cholesterol (mmol/L)	Triglyceride (mmol/L)	HDL (mmol/L)	LDL (mmol/L)
T2DM	55.39 ± 8.83	128/145	24.45 ± 3.61	4.51 ± 0.97	1.62 ± 1.62	0.95 ± 0.24	2.99 ± 1.15
Control	54.88 ± 8.34	136/111	24.50 ± 3.10	4.59 ± 1.06	1.57 ± 1.36	1.33 ± 0.36	2.89 ± 1.01
P-value	0.500	0.076	0.856	0.373	0.731	<0.001*	0.290

* $P < 0.05$

Table 3 – Genotype and allele frequencies of three SNPs in the type 2 diabetic patients and the control subjects.

Genotype (allele)	T2DM, *n* (%)	Control, *n* (%)	OR (95% CI)[a]	P-value[b]	OR (95% CI)[c]	P-value[b]	P-value[d]
RAPGEF1							
TT	146(53.48)	146(59.11)	Reference		Reference		0.331
CT	109(39.93)	90(36.44)	1.21(0.84–1.74)	0.299	1.22(0.85–1.76)	0.284	
CC	18(6.59)	11(4.45)	1.64(0.75–3.59)	0.219	1.62(0.74–3.56)	0.231	
T	401(73.4)	382(77.3)	Reference				0.147
C	145(26.6)	112(22.7)	1.23(0.93–1.64)				
TP53							
CC	38(13.92)	54(21.86)	Reference		Reference		0.056
GC	148(54.21)	125(50.61)	1.68(1.04–2.72)	0.033	1.66(1.03–2.69)	0.038	
GG	87(31.87)	68(27.53)	1.82(1.08–3.07)	0.025	1.92(1.12–3.28)	0.017	
C	224(41.03)	233(47.17)	Reference				0.046
G	322(58.97)	261(52.83)	1.28(1.00–1.64)				
NRF1							
TT	132(48.35)	133(53.85)	Reference		Reference		0.316
CT	124(45.42)	96(38.87)	1.30(0.91–1.86)	0.151	1.29(0.90–1.86)	0.166	
CC	17(6.23)	18(7.29)	0.95(0.47–1.93)	0.890	0.87(0.42–1.78)	0.694	
T	388(71.06)	362(73.28)	Reference				0.426
C	158(28.94)	132(26.72)	1.12(0.85–1.47)				

[a] Unadjusted.
[b] *P* values were calculated using the analysis of maximum likelihood χ^2 test.
[c] Odds ratios were calculated using logistic regression to measure the ORs adjusted for age and sex.
[d] *P* values were calculated using the Pearson χ^2 test.

Table 4 – Locus–locus interaction between *RAPGEF1* and *TP53*, *RAPGEF1* and *NRF1*, *TP53* and *NRF1* on type 2 diabetes risk.

Genotype	Genotype	Case *n* (%)	Control *n* (%)	OR (95% CI)[a]	OR (95% CI)[b]
RAPGEF1	*TP53*				
TT	CC	19(6.96)	35(14.17)	1.00	1.00
TT	GC/GG	127(46.52)	111(44.94)	2.11(1.14–3.89)	2.14 (1.15–3.97)
CT/CC	CC	19(6.96)	19(7.69)	1.84(0.79–4.29)	1.97(0.82–4.74)
CT/CC	GC/GG	108(39.56)	82(33.20)	2.43(1.30–4.55)	2.54(1.34–4.81)
RAPGEF1	*NRF1*				
TT	TT	72(26.37)	86(34.82)	1.00	1.00
TT	CT/CC	74(27.11)	60(24.29)	1.47(0.93–2.34)	1.43(0.90–2.29)
CT/CC	TT	60(21.98)	47(19.03)	1.53(0.93–2.50)	1.53 (0.93–2.51)
CT/CC	CT/CC	67(24.54)	54(21.86)	1.48(0.92–2.39)	1.41(0.87–2.29)
NRF1	*TP53*				
TT	CC	10(3.66)	31(12.55)	1.00	1.00
CT/CC	CC	28(10.26)	23(9.31)	3.77(1.53–9.29)	4.70(1.76–12.56)
TT	GC/GG	122(44.69)	102(41.30)	3.71(1.73–7.93)	4.29(1.97–9.37)
CT/CC	GC/GG	113(41.3%)	91(36.84)	3.85(1.79–8.27)	3.87(1.87–8.40)

[a] Unadjusted.
[b] Odds ratios were calculated using logistic regression to measure the ORs adjusted for age and sex.

type 2 diabetes due to the presence of the rs1882095 (*NRF1*) risk allele interacting with the risk allele in *RAPGEF1* gene.

4. Discussion

In this case-control study, we confirmed that rs1042522 in *TP53* had a significant association with the risk of type 2 diabetes, whereas there were no significant associations between rs11243444 (*RAPGEF1*), rs1882095 (*NRF1*) and a predisposition for type 2 diabetes, and that potential interaction of *TP53* (rs1042522) and *RAPGEF1* (rs11243444), *TP53* (rs1042522) and *NRF1* (rs1882095) increased the risk of type 2 diabetes.

As we all know, *TP53*, a tumor suppressor gene, is located on the short arm of chromosome 17 and encodes the tumor suppressor protein p53. Studies showed that polymorphisms in *TP53* were associated with many cancers, such as pancreatic cancer [14], acute lymphoblastic leukemia [15] and colorectal cancer [16]. There were also reports that polymorphisms in *TP53* were associated with type 2 diabetes [11]. 95% of the currently known mutations of *TP53* have been identified in exons 5–8, with a small number found in exon 4 [17] including rs1042522. In this study, we confirmed that the polymorphism of *TP53* (rs1042522) was associated with type 2 diabetes and the similar conclusion was also deduced by Gaulton et al. [11] in Finns population, moreover, the polymorphism of rs1042522 was reportedly associated with

type 1 diabetes in Russian population [18]. The polymorphism in *TP53* (rs1042522) lead to the amino acid change Pro72Arg which has higher apoptotic potential. One mechanism underlying this greater efficiency is enhanced localization of the Arg72 variant to the mitochondria, which is known to contribute to p53-dependent apoptosis, as p53 mutants with compromised ability to localize to the mitochondria are also impaired in inducing apoptosis [19], and this function were also confirmed by Dumont et al. [20]. In this study, we found that the allele G in *TP53* (rs1042522) was a risk of type 2 diabetes, so we speculated that the risk allele G was associated with type 2 diabetes in that which was consistent with a possible link between increased pancreatic B cell apoptosis and impaired insulin secretion.

The adaptor protein Rap guanine exchange factor 1 (*RAPGEF1*), also known as C3G is a component of the CAP/TC10 pathway. Under the activation of insulin receptors, the PI3-kinase dependent pathway and the CAP/TC10 pathway regulate the glucose transporter 4 (Glut4) translocation [21,22]. Defects in the *RAPGEF1* protein may contribute to insulin resistance and type 2 diabetes. In this investigation, we failed to find any significant association between the polymorphism in *RAPGEF1* (rs11243444) and type 2 diabetes (adjusted OR = 1.23, 95% CI = 0.93–1.64; $P = 0.147$). Though the effecter genes with type 2 diabetes in CAP/TC10 pathway has not been well elucidated, further study for the polymorphism in *RAPGEF1* (rs11243444) in other ethics should be explored for the potential ability to predict the risk of developing type 2 diabetes.

The *NRF1* variant, rs1882095, is located 1 kb downstream of the gene which is located on chromosome 7q32, a susceptibility locus for type 2 diabetes and insulin resistance [23]. Muscle-specific over expression of human *NRF1* gene in transgenic mice causes an increase in glucose transport capacity in skeletal muscle via upregulating Glut4 [24]. In this investigation, our data showed that there was no significant association between the polymorphism in *NRF1* (rs1882095) and type 2 diabetes, whereas the opposite conclusion was observed in Finns population [11], this controversial conclusion was partly due to the different allele frequencies of *NRF1* (rs1882095) in different ethnic groups. Accumulated data showed that the risk allele C in *NRF1* (rs1882095) was a minor allele in Asian subjects, including our group, while the allele C was revealed as a major allele in European populations [11]. These significant differences in risk allele frequencies across ethnic groups indicate that the genetic variations of type 2 diabetes susceptibility genes are diversely distributed among different populations. Therefore, replication studies for the polymorphism in *NRF1* (rs1882095) in other populations are necessary.

Type 2 diabetes, a multifactorial disease, is influenced by gene–gene and gene–environment interactions [25,26]. The interactions among *TP53*, *RAPGEF1* and *NRF1* were also investigated in this study. We observed that there were significant associations between the interaction of *TP53* and *RAPGEF1* or *NRF1* and type 2 diabetes. Although the polymorphism in *RAPGEF1* (rs11243444) was not significant association with type 2 diabetes, when *RAPGEF1* (rs11243444) was jointly analyzed with *TP53* (rs1042522), more than a two-fold increase in type 2 diabetes risk was observed for subjects with both risk alleles, furthermore, the similar affection of *NRF1* (rs1882095) in the interaction analysis may have been the result of the presence of rs1042522 (*TP53*) in the interaction models, however, we found no evidence that the polymorphism in *NRF1* (rs1882095), altered the strength of the association when paired with *RAPGEF1* (rs11243444). Therefore, another promising finding was that the *RAPGEF1* and *NRF1* gene may contribute to the etiology in type 2 diabetes in an interactive manner with *TP53*, this gene–gene interaction was also described by Culverhouse et al. [27] that variants show no independent association with the trait of interest, but when taken in concert with other variants, do provide evidence of interaction effects, furthermore, this phenomenon has also been studied and documented in animal models [28,29]. A major challenge for studies such as ours and those that investigate the interaction between thousands of variants is to decipher the complexity of the genetic networks between the loci that appear to have a statistical relationship [30], whereas, the association of effect genes with type 2 diabetes has not been well elucidated and the mechanism for such effect is still not well understood. Therefore, it would be pure speculation to try to explain the interactions found in this study on basis of known function and these interaction findings should be evidenced by large ethnically matched studies in other populations.

There were some limitations in this study. First, we did not analyze the association between the candidate SNPs and indices of beta cell secretory functions or insulin resistance due to a lack of data. Second, these findings may not be suitably generalized to other populations. Large ethnically matched studies would be necessary to know if such interaction is found in other subjects.

In conclusion, we demonstrated that there was significant association between polymorphism in the *TP53* (rs1042522) gene and type 2 diabetes in Chinese population, and there were potential interactions between *TP53* (rs1042522) and *RAPGEF1* (rs11243444) or *NRF1* (rs1882095) with the risk of type 2 diabetes.

Conflicts of interest

The authors state that they have no conflict of interest.

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