RESEARCH NOTE

Genetic variants of retinol-binding protein 4 in adolescents are associated with liver function and inflammatory markers but not with obesity and insulin resistance

CHIN-JUNG LIN[1], NAIN-FENG CHU[2,3], YI-JEN HUNG[1], DEE PEI[4], CHIEN-HSING LEE[1], FONE-CHING HSIAO[1], CHIEH-HUA LU[1] and CHANG-HSUN HSIEH[1]*

[1]*Division of Endocrinology and Metabolism, Department of Internal Medicine, Tri-Service General Hospital, 114 Taipei, Taiwan, Republic of China*
[2]*School of Public Health, National Defense Medical Center, Taipei, Taiwan, Republic of China*
[3]*Department of Health, Taitung Hospital, 950 Taitung, Taiwan, Republic of China*
[4]*Division of Endocrinology and Metabolism, Cardinal Tien Hospital, 235 New Taipei City, Taiwan, Republic of China*

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Introduction

Obesity is a global health problem not only in adults but also in adolescents. It increases the risk of metabolic disorders such as type 2 diabetes (T2D), hypertension, insulin resistance (IR) and dyslipidemia. The adipose tissues which store energy is also an endocrine organ which influences insulin sensitivity by releasing multiple cytokines, including retinol-binding protein 4 (RBP4) (Rosen and Spiegelman 2006). RBP4 is a novel adipokine that has been suggested to be related to IR, dyslipidemia and proinflammatory status.

The expression of RBP4 has been reported to be elevated in the adipose tissue of adipose-Glut4 knockout mice, and injections of recombinant RBP4 in normal mice have shown to cause IR. In addition, an increase in serum RBP4 has been reported to diminish the expression of glucose transporter 4 in adipocytes, induce the hepatic expression of the gluconeogenic enzyme and impair insulin signalling in muscles (Yang *et al.* 2005).

The *RBP4* gene maps on chromosome 10q23-q24, and the genetic variants in this region have been linked to elevated fasting blood glucose levels in white Europeans (Meigs *et al.* 2002) and an increase risk of T2D in Mexican Americans (Duggirala *et al.* 1999). Several studies have confirmed the positive association of *RBP4* genetic variants with IR or T2D. However, different results have been observed in different ethnic populations. In Mongolians, genetic polymorphisms (−803, G > A; +5169, C > T; +6969,G > C; +7542, T > del) have been associated with an increased risk of T2D (Munkhtulga *et al.* 2007) and another study suggested that genetic variants were associated with a reduction in insulin secretion in Caucasians (Craig *et al.* 2007). In addition, the single-nucleotide polymorphisms (SNPs) rs17484721 and rs36035572 were reported to be possibly associated with T2D and serum triglyceride levels in another study of a Chinese population (Liu *et al.* 2008). However, no positive correlations were found between SNP and IR or T2D in African Americans (Craig *et al.* 2007), Newfoundlanders (Shea *et al.* 2010) and south Indians (Nair *et al.* 2010). However, these studies mostly included adults and the data published on adolescents are limited. In this study, we explored the association of IR, metabolic syndrome (MetS), obesity, inflammatory markers and liver function abnormalities with gene SNPs of *RBP4* in Taiwanese adolescents.

Materials and methods

Study design and sampling

The Taipei Children Heart Study-II is an epidemiologic study that evaluated obesity and cardiovascular disease risk factors among school children in Taipei during 2003. To obtain a representative distribution of demographic and lifestyle characteristics, we conducted a cross-sectional survey among junior high school students in Taipei. After a multistage sampling of 85 junior high schools, we randomly selected 1500 school adolescents for this survey. The sampling method and results are described elsewhere (Chu *et al.* 1998). After considering the power of the study and excluding those with missing data, a total of 1086 adolescents (524 boys and 562

*For correspondence. E-mail: 10324peter@gmail.com.

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girls) with a mean age of 13.7 years (range 13–15 years) were included in the final analysis. The prevalence of the obesity is 12% among schoolchildren in Taiwan (Chu and Pan 2007), whereas it is 16% in our study. The power is 96.7% of such a study assuming that we conduct a two-sided test with $\alpha = 0.05$.

Data collection

All of the adolescents who participated completed a structured questionnaire detailing their gender, age, puberty development and lifestyle characteristics, including cigarette smoking and alcohol consumption. Research technicians measured body weight to an accuracy of 0.1 kg using a standard beam balance scale with the subjects barefoot and wearing light indoor clothing. Body height was recorded to the nearest 0.5 cm using a ruler attached to the scale. Waist circumference was measured to the nearest 0.1 cm at the level of the midpoint between the inferior margin of the last rib and the iliac crest. Hip circumference was measured at its widest point to the nearest 0.1 cm. Body mass index (BMI) was calculated as body weight (kg) divided by the square of their height (m). Blood pressure was measured in sitting position, on the right arm using an appropriate cuff size; the first and fifth Korotkoff sounds were recorded as systolic blood pressure and diastolic blood pressure, respectively. We measured the blood pressure again after a 5 min rest, and the average was used in the analysis. We categorized the study subjects on the basis of their BMI standard deviation score values (Z score) into four groups as underweight, normal weight, overweight and obese. Further, the study subjects were divided into subgroups according to the number of MetS components (0, 1, 2 and $\geq$3). The Ethical Committee of the Scientific Institute of Tri-Service General Hospital approved this study, and informed consent was obtained from the parents and children.

Prevalence of the obesity is 12% among schoolchildren in Taiwan (Chu and Pan 2007), whereas it is 16% in our study. A total sample size of 1086 adolescents will ensure that a two-sided test with $\alpha = 0.05$ has 96.7% power.

Laboratory measurements

After a 10-h overnight fast, blood samples were collected to determine plasma glucose, insulin, lipid profiles, complete blood count, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase (GPT), high-sensitivity CRP (hs-CRP) and RBP4 concentrations. Fasting plasma insulin (FPI) was measured by a commercial solid phase radioimmunoassay kit (Coat-A-Count Insulin Kit, Diagnostic Products Corporation, Los Angeles, USA). The intra-assay and inter-assay coefficients of variance for insulin were 3.3 and 2.5%, respectively. Fasting plasma glucose (FGP) was measured using the glucose oxidase method (YSI 203 glucose analyzer, Scientific Division, Yellow Spring Instrument Company, Yellow Spring, USA). Both serum levels of total cholesterol and triglycerides were measured using the dry, multilayer analytical slide method in a Fuji Dri-Chem 3000 analyzer (Fuji Photo Film Corporation, Minato-Ku, Tokyo, Japan). Serum levels of high-density lipoprotein cholesterol were determined by an enzymatic cholesterol assay after dextran sulphate precipitation. RBP4 concentrations were measured using an enzyme immunoassay kit (EIA, Phoenix Pharmaceuticals, Burlingame, USA). The serum RBP4 concentrations were measured in duplicate. The intra-assay and inter-assay coefficients of variance were less than 5 and 14%, respectively. The diagnosis of MetS was made according to the criteria of the International Diabetes Federation (Zimmet *et al.* 2007). Insulin resistance was assessed using the homeostasis model assessment as described by Matthews *et al.* (1985). The quick insulin sensitivity check index and an index of insulin sensitivity was calculated as 1/(log FPI + log FPG) (Katz *et al.* 2000).

Gene SNP selection and genotyping

Genotyping was performed using commercially available TaqMan® Genotyping Assays (Applied Biosystems) according to the manufacturer's standard PCR protocol. Briefly, 20 ng genomic DNA was mixed with 2×TaqMan Universal PCR Master Mix No AmpErase UNG and 20×TaqMan Assay Mix to a final volume of 5 μL in a 384-well plate. Each sample underwent 40 amplification cycles on a GeneAmp® PCR System 9700 (Applied Biosystems). Fluorescent signals of the two probes were analysed, and end-point fluorescent data were obtained on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems). Genotyping was determined automatically by Sequence Detection Software (Applied Biosystems). Four SNPs, rs3758538, rs3758539, rs10882273 and rs12265684 of the *RBP4* gene were selected for validation based on previous studies.

Statistical analysis

All analyses were performed using SPSS 16.0 statistical software (Chicago, USA). Clinical and anthropometric characteristics between genders were compared using a *t*-test for continuous variables and the chi-square test for categorical variables. Both allele and genotype frequencies of SNP markers were tested between genders as well as the severity of adiposity and MetS. Partial Pearson correlation was performed to determine the association between the RBP4 genetic variants and various clinical parameters of the MetS, IR and inflammatory markers after adjusting for age and gender. All values are expressed as mean ± SD. Statistical significance was defined as a *P* value less than 0.05.

Results

The demographic and biochemical characteristics of the study subjects are provided in table 1, and the associations

Table 1. Demographic and biochemical characteristics of study subjects.

	Boys ($n=524$)	Girls ($n=562$)	*P* value
Age (year/old)	13.7±0.9	13.7±0.9	0.23
Waist (cm)	78.6±10.7	73.9±8.0	<0.001
SBP (mmHg)	118.2±14.0	111.6±12.3	<0.001
DBP (mmHg)	69.2±10.5	69.8±9.5	0.27
BMI (kg/m^2)	21.6±4.2	20.4±3.4	<0.001
FPG (mg/dL)	93.8±6.6	91.7±7.0	<0.001
TG (mg/dL)	70.5±37.6	71.3±30.3	0.70
HDL-C (mg/dL)	47.4±11.2	51.7±11.2	<0.001
FPI (UIU/ML)	14.4±10.1	14.2±7.7	0.76
HOMA-IR	3.4±2.5	3.3±1.9	0.35
QUICKI	0.3±0.03	0.3±0.02	0.43
WBC (∗1000)	6.3±1.8	6.5±1.7	0.068
CRP (mg/L)	0.83±1.34	0.59±1.02	<0.001
GOT (U/L)	22.0±6.9	19.2±3.9	<0.001
GPT (U/L)	13.5±8.4	10.6±3.9	0.001
RBP4 (μg/mL)	10.4±1.8	10.1±1.6	<0.01

Data are presented as mean ± standard deviation. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; FPG, fasting plasma glucose; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; FPI, fasting plasma insulin; HOMA-IR, homeostasis model assessment-insulin resistance; QUICKI, quick insulin sensitivity check index; WBC, white blood cells; CRP, C-reactive protein; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; RBP4, retinol-binding protein 4.

Table 2. Association of *RBP4* SNP genotypes and different obesity degrees.

	Underweight ($n=123$)	Normal ($n=572$)	Overweight ($n=129$)	Obese ($n=161$)
	n (%)	*n* (%)	*n* (%)	*n* (%)
rs3758538				
GG	1 (0.8)	2 (0.4)	0	2 (1.2)
GT	11 (8.9)	54 (9.5)	17 (13.3)	13 (8.1)
TT	111 (90.2)	513 (90.2)	111 (86.7)	146 (90.7)
rs3758539				
CC	100 (81.3)	466 (82.0)	102 (79.1)	128 (80.0)
CT	22 (17.9)	98 (17.3)	27 (20.9)	31 (19.4)
TT	1 (0.8)	4 (0.7)	0	1 (0.6)
rs10882273				
CC	2 (1.6)	8 (1.4)	0	3 (1.9)
CT	29 (23.6)	103 (18.1)	34 (26.4)	38 (23.8)
TT	92 (74.8)	458 (80.5)	95 (73.6)	119 (74.4)
rs12265684				
CC	96 (78.1)	464 (81.6)	100 (77.5)	122 (75.6)
CG	25 (20.3)	101 (17.8)	29 (22.5)	36 (22.4)
GG	2 (1.6)	4 (0.7)	0	3 (1.9)

between the RBP4 SNP genotypes and different degrees of obesity are provided in table 2. The frequencies of the genotypes were not different in all of the *RBP4* polymorphisms. Table 3 presents the significance level of association of the four SNPs in the *RBP4* gene and various clinical parameters of the MetS, IR, biochemical characteristics and inflammatory markers. There were positive associations between the genetic polymorphisms and hs-CRP and GPT in rs3758539 and rs0882273 ($P=0.024$ and 0.031 in hs-CRP; $P=0.028$ and 0.03 in GPT, respectively), and a borderline association in rs12265684 ($P=0.053$ and 0.058, respectively). The positive associations remained after adjusting for confounding factors. Further, positive correlations between the genetic polymorphisms and WBC were observed in rs10882273 and rs12265684 ($P=0.03$ and 0.046, respectively). Similarly, the positive correlations remained after adjusting for confounding factors.

Table 3. The significance level of association of four SNPs in the *RBP4* gene and various clinical parameters of the metabolic syndrome, insulin resistance and inflammatory markers.

SNP ID variables	*P* value			
	rs3758538	rs3758539	rs10882273	rs12265684
FPI	0.099	0.717	0.265	0.388
HOMR-IR	0.132	0.689	0.218	0.350
QUICK	0.383	0.956	0.515	0.633
WBC	0.414	0.181	0.03*	0.046*
CRP	0.163	0.024*	0.031*	0.053
GOT	0.593	0.302	0.198	0.362
GPT	0.306	0.028*	0.03*	0.058
BMI	0.926	0.832	0.482	0.391
WC	0.495	0.983	0.755	0.068
FPG	0.556	0.509	0.084	0.224
HDL-C	0.452	0.508	0.753	0.729
TG	0.851	0.104	0.253	0.157
SBP	0.523	0.68	0.544	0.081
DBP	0.132	0.464	0.893	0.817
RBP4 levels	0.821	0.111	0.089	0.105

*$P < 0.05$.

Discussion

In this study, we investigated the associations of *RBP4* genetic polymorphisms with IR, obesity and inflammation in Taiwanese adolescents. Our results showed that none of the *RBP4* SNPs were related to obesity or IR, although some genetic variants of *RBP4* were associated with inflammatory markers and abnormal liver function. This may imply a genetic role in the pathophysiological processes of these clinical disorders.

The role of RBP4 in obesity and IR is controversial (Kotnik *et al.* 2011). The genetic role of RBP4 variants has been reported previously with inconsistent results, and the results of the current study were also inconsistent with previous studies. Ethnicity may play a role in the relationship between RBP4 and IR. Most studies on adults have suggested a positive correlation between RBP genetic variants and obesity, IR or MetS components (Craig *et al.* 2007; Munkhtulga *et al.* 2007), however, other studies did not find this correlation (Kanaka-Gantenbein *et al.* 2008; Santoro *et al.* 2009). In a study based on children, Friebe *et al.* (2011) tried to identify

an association between the −803 G > A promoter polymorphism (rs3758539) in the *RBP4* gene and obesity. They genotyped this polymorphism in 304 lean and 283 obese Caucasian children, however, they did not detect an association between the polymorphism and adiposity parameters, or with parameters of glucose and lipid metabolism or blood pressure in quantitative analyses. We also did not find an association between the RBP4 polymorphism and obesity, insulin resistance or severity of the MetS.

Obesity has been linked to chronic inflammation, higher waist circumference and waist–hip ratio, and higher levels of CRP, RBP4 and interleukin-6 in healthy young adults (Hermsdorff *et al.* 2011). We also found that the rs3758539 and rs10882273 SNPs were significantly, and rs12265684 borderline significantly correlated with CRP level, and that the rs10882273 and rs12265684 SNPs were positively correlated with white cell count. A higher level of hs-CRP was noted with the CC genotype in rs3758539 (0.76 ± 1.28 mg/L). A previous study showed that macrophages express RBP4, and that RBP4 can be inhibited by tumour necrosis factor-alpha and lipopolysaccharides (Broch *et al.* 2010).

We also found that the SNPs rs3758539 and rs10882273 were significantly, and rs12265684 borderline significantly associated with GPT, and a higher level of GPT was noted in the CC and TT genotypes in rs3758539 and rs10882273 (12.1 ± 6.5, 12.1 ± 6.5 U/L), respectively which may suggest that RBP4 plays a role in liver disorders. Recent studies have reported that RBP4 plays a role in nonalcoholic fatty liver disease (NAFLD). Huang and Yang (2012) enrolled 748 schoolchildren aged between 6 and 12 years, and they also found that higher RBP4 and GPT levels were associated with pediatric NAFLD. Our results suggest a possible genetic role of RBP4 in the pathological mechanism of liver disorders.

There are some limitations to this study. First, we only genotyped four SNPs of the RBP4 gene, and we do not know whether other genetic polymorphisms have the same association. Second, although we found an association with abnormal liver function, we did not have sonographic images of the liver to confirm a fatty liver. Third, we used only one definition of MetS, and we do not know whether the results will be the same if other criteria of MetS are used.

In conclusion, we did not find an association between genetic polymorphisms in the *RBP4* gene with obesity or IR in Taiwanese adolescents. However, further a large scale studies on the genetic role of *RBP4* in the inflammatory process or NAFLD are needed to validate our findings.

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