

Association of $\beta 3$ Adrenergic Receptor and Peroxisome Proliferator-activated Receptor Gamma 2 Polymorphisms With Insulin Sensitivity: A Twin Study¹

TIAN-JIAO CHEN^{*}, CHENG-YE JI^{*2}, XIAO-YING ZHENG[†], AND YONG-HUA HU[§]

^{*}Institute of Child and Adolescent Health, Peking University, Beijing 100083, China; [†]Institute of Population Research, Peking University/WHO Collaborating Center on Reproductive Health and Population Science, Beijing 100083, China; [§]Department of Epidemiology and Health Statistics, School of Public Health, Peking University, Beijing 100083, China

Objective To study the effect of $\beta 3$ adrenergic receptor ($\beta 3$ AR) Trp64Arg and peroxisome proliferator activated receptor gamma 2 (PPAR $\gamma 2$) Pro12Ala polymorphisms on insulin resistance. **Methods** One hundred and eight dizygotic twin pairs were enrolled in this study. Microsatellite polymorphism was used to diagnose zygosity of twins. Insulin sensitivity was estimated with logarithm transformed homeostasis model assessment (HOMA). PCR-RFLP analysis was performed to detect the variants. As a supplement to the sib-pair method, identity by state (IBS) was used to analyze the association of polymorphisms with insulin sensitivity. **Results** The genotype frequencies of Trp64Trg, Trp64Arg, and Arg64Arg were 72.3%, 23.8%, and 3.9%, respectively, while the genotype frequencies of Pro12Pro, Pro12Ala, and Ala12Ala were 89.9%, 9.6%, and 0.5%, respectively. For $\beta 3$ AR Trp64Arg the interclass co-twin correlations of Waist-to-hip ratio (WHR), blood glucose (GLU), and insulin (INS), homeostasis model assessment insulin resistance index (HOMA-IR) of the twin pairs sharing 2 alleles of IBS were greater than those sharing 0-1 allele of IBS, and HOMA-IR had statistic significance. For PPAR $\gamma 2$ Pro12Ala most traits of twin pairs sharing 2 alleles of IBS had greater correlations and statistic significance in body mass index (BMI), WHR, percent of body fat (PBF) and GLU, but there were low correlations of either insulin or HOMA-IR of twin pairs sharing 1 or 2 alleles of IBS. The combined effects of the two variations showed less squared significant twin-pair differences of INS and HOMA-IR among twins sharing 4 alleles of IBS. **Conclusions** $\beta 3$ AR Trp64Arg and PPAR $\gamma 2$ Pro12Ala polymorphisms might be associated with insulin resistance and obesity, and there might be slight synergistic effects between this two gene loci, and further studies are necessary to confirm this finding.

Key words: Dizygotic twins; Beta-3 adrenergic receptor; Peroxisome proliferator activated receptor gamma 2; Polymorphism; Insulin resistance

INTRODUCTION

Insulin resistance means a decrease in sensitivity and response of target tissues to the action of insulin, and as a result, physiologic concentrations of insulin produce a subnormal biological response. Insulin resistance is determined usually by assessing insulin sensitivity. Insulin resistance and hyperinsulinemia, as the physiologic base of metabolic syndrome, constitute the main risk factors for atherosclerotic cardiovascular diseases and type 2 diabetes^[1]. Although these conditions are rarely seen in young individuals, the pathologic process and risk factors associated with their development may have occurred during childhood. Recent studies have revealed the

presence of components of insulin resistance syndrome in both children and adolescents^[2-4]. A direct link between obesity and insulin resistance has also been reported in young individuals. A number of studies have shown that overweight during childhood and adolescence is significantly associated with insulin resistance and abnormal lipids. The incidence of type 2 diabetes in children has increased alarmingly, emerging as a critical health problem due to obesity^[5-6].

There is evidence from family studies that insulin resistance and obesity are affected by genetic factors^[7-8]. The search for genes has been continued using the candidate gene. Linkage between obesity and polymorphism in some chromosomes has been

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²Correspondence should be addressed: Cheng-Ye JI, Institute of Child and Adolescent Health, Peking University, 38 Xueyuan Road, Beijing, China. Tel: 86-10-82802344. Fax: 86-10-82801178. E-mail: jichengye@263.net

Biographical note of the first author: Tian-Jiao CHEN, Doctor on child and adolescent health, working in the Institute of Child and Adolescent Health, Peking University Health Science Center.

demonstrated, though the responsible gene(s) remains to be elucidated. Is there any linkage or association between insulin resistance and polymorphism in some chromosomes or genes? In regards to candidate genes, recent studies have focused on the adrenergic receptor system, in particular, the beta-3-adrenoceptor subtype gene. The beta-3-receptor stimulates mobilization of lipids from white fat cells and increases thermogenesis in brown fat cells, treatment of obese animals with selective beta-3-adrenergic agonists reduces fat stores most effectively^[9]. Mutation of the gene for the beta-3-adrenergic receptor results in the replacement of tryptophane by arginine at position 64 (Trp64Arg) in the first intracellular loop of the receptor. This gene has been implicated in obesity and diabetes since it is associated with an earlier onset of noninsulin- dependent diabetes mellitus, a feature of insulin resistance and tendency to gain weight^[10-14].

Another important gene is peroxisome proliferators-activated receptor gamma (PPAR γ). The protein coded by this gene belongs to the family of nuclear receptors that bind to specific promoter sequences and regulate expression of other genes. Although PPAR γ is responsible for differentiation of fibroblasts to adipocytes and regulation of their function, the Pro12Ala polymorphism in the PPAR γ 2 isoform is suggested to be linked with the multifactorial form of several metabolic diseases^[15-18].

Twin is a specific population used to study the association of gene polymorphism and function since twin pairs have the same age, intra-uterus, and early living environment, consequently confounding factors can be better controlled in the study^[19]. Considering the fact that monozygotic twins have the same genetic background, this study selected the dizygotic twins as objects to explore the association of β 3AR and PPAR γ 2 polymorphisms with insulin sensitivity and obesity.

METHODS

Subjects

One hundred and eight dizygotic (DZ) twin pairs aged 5-18 (mean age, 12.1 \pm 3.5) years were recruited through the Qingdao Twin Registry. Both members of a pair received physical and laboratory examinations on the same day, and informed written consents were obtained from all the subjects and their parents before the examinations. Samples taking anti-obesity or any drugs prescribed specially to lower blood glucose or lipid levels within one month were excluded. The

study was approved by the Medical Ethics Committee of Peking University.

Variable Measurement

Short tandem repeat DNA markers were used to diagnose zygosity of twins with the reagent kit (Geneprint Fluorescent STR Multiplex GammaSTRTM) from Promega Corporation of USA. DNA was extracted from blood coagulation by phenol-chloroform method. Four highly polymorphic microsatellite loci (D16S539, D7S820, D13S317, D5S818) were amplified and the products were sized by laser detection. Zygosity was diagnosed by comparing the concordance of the genotype of the four microsatellite loci with single blind method. The probability that any twin pair was MZ if all the 4 markers were concordant was 99.6%. This work was completed in Beijing Forensic Medical Center.

Variables included height (cm), weight (kg), waist (cm), hip (cm), blood glucose (GLU, mmol/L) and insulin (mU/L). The indices included body mass index (BMI, kg/m²), waist-to-hip ratio (WHR), percentage of body fat (PBF, %) and homeostasis model assessment insulin resistance index (HOMA-IR). BMI was calculated as weight(kg)/height²(m²). PBF was calculated by the formula of Brožok (1963) using skin-fold. HOMA-IR was calculated as GLU \times insulin/22.5. Venous blood was collected and the participants were instructed to fast for at least 8 h overnight before a morning examination. Insulin was assayed with radio immunoassay kit (RIA kit, China Institute of Atomic Energy). Quality control of physical and laboratory measures was acceptable.

Genetic Analysis

Genomic DNA was extracted and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was performed to detect the polymorphism.

PCR amplification of the β 3AR gene was conducted using the following primers: 5'-CGC CCA ATA CCG CCA ACA-3' and 5'-CCA CCA GGA GTC CCA TCA-3'. The PCR conditions were as follows: incubation at 94 $^{\circ}$ C for 5 min, followed by 35 cycles of denaturation at 94 $^{\circ}$ C for 30 s, annealing at 61 $^{\circ}$ C for 30 s and extension at 72 $^{\circ}$ C for 30 s and a final extension at 72 $^{\circ}$ C for 5 min. PCR products were directly digested by adding 15 units of BstO-I enzyme (New England Biolab Incorporated) for 4 h at 60 $^{\circ}$ C, and the fragments obtained were separated on 12% nondenaturing polyacrylamide gels stained with AgNO₃. Genotypes were defined as wild-type homozygote (Trp64Trp: 99 bp, 62 bp), heterozygote

(Trp64Arg: 161 bp, 99 bp, 62 bp), and mutant homozygote (Arg64Arg: 161 bp).

PPAR γ 2 PCR amplification was carried out using the forward primer 5'-GCC AAT TCA AGC CCA GTC-3' and the reverse primer 5'-GAT ATG TTT GCA GAC AGT GTA TCA GTG AAG GAA TCG CTT TCC G-3'. The reaction mixtures were incubated at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 62°C for 40 s, extension at 72°C for 40 s and a final extension at 72°C for 7 min. PCR products were digested with the restriction enzyme, BstU-1 (NEB) at 37°C overnight. The enzyme cutting wild allele at a site introduced by the reverse primer, underlined the mismatched base. Genotypes were defined as wild-type homozygote (270 bp), heterozygote (270 bp, 227 bp, 43 bp), and mutant homozygote (227 bp, 43 bp).

Statistical Analysis

Analysis was performed in twins whose genotype distribution was in accord with Hardy-Weinberg equilibrium.

The underlying basis for linkage approach is to compare the quantitative variation between co-twins as a function of the number of marker alleles that the twin pair shares identically by descent (IBD). Since parental data were not available in this twin study, IBD was estimated from the observed identity by state (IBS) and based on the frequencies of alleles for each marker. Allele frequencies for the entire sample were used, because they were similar to the frequencies based on one randomly selected co-twin per pair. As a supplement to the sib-pair method, when evidence of linkage to a specific marker was found, intraclass correlation of the appropriate phenotype was calculated within twin pairs stratified into those sharing 2, 1, and 0 allele of IBS. When linkage was present, twins sharing 2 alleles of IBS were expected to have a higher correlation or a lower difference than those sharing 1, which in turn should have a higher correlation than those pairs sharing 0 allele. Because the standard errors of intraclass correlations were difficult to compute, *P* values were reported on the basis of interclass correlations with the same sample size. Similarly the associative action or interaction of the two gene polymorphisms of β 3AR Trp64Arg and PPAR γ Pro12Ala was analyzed, or the squared twin-pair differences in twins sharing 4, 3, and 0-2 alleles of IBS were compared.

Owing to the skewed distributions of insulin and HOMA-IR, the values were naturally logarithmically transformed before statistical analysis. All statistical analyses were done with SPSS11.0.

RESULTS

Gene Polymorphism Distribution

The observed genotype frequencies of Trp64Trp, Trp64Arg, and Arg64Arg were 72.3%, 23.8%, and 3.9%, respectively, while the allele frequencies of Trp, Arg were 84.2% and 15.8%, respectively. The genotype frequencies of Pro12Pro, Pro12Ala, and Ala12Ala were 89.9%, 9.6%, and 0.5%, respectively, while the allele frequencies of Pro, Ala were 94.7% and 5.3%, respectively. The distributions were in accord with Hardy-Weinberg equilibrium. The RFLP and electrophoresis results are shown in Figs. 1 and 2.

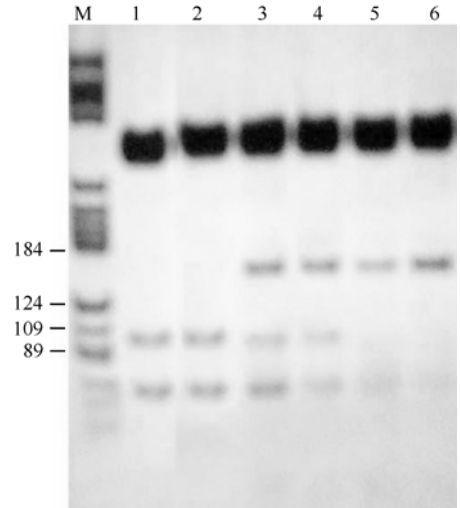


FIG. 1. β 3AR Trp64Arg RFLP polyacrylamide gel electrophoresis. M: DNA marker, pBR322 DNA/HaeIII; Lanes 1, 2: Trp64Trp (99 bp, 62 bp); Lanes 3, 4: Trp64Arg (161 bp, 99 bp, 62 bp); Lanes 5, 6: Arg64Arg (161 bp).

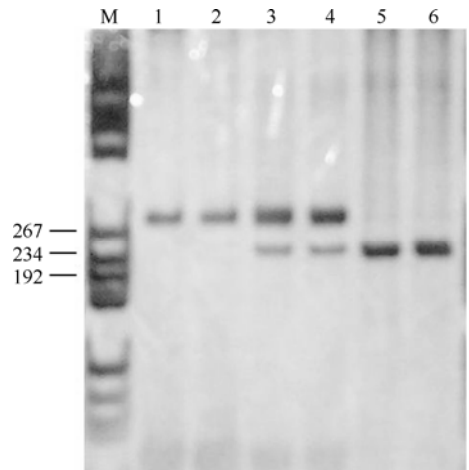


FIG. 2. PPAR γ Pro12Ala RFLP polyacrylamide gel electrophoresis. M: DNA marker, pBR322 DNA/HaeIII; Lanes 1, 2: Pro12Pro (270 bp); Lanes 3, 4: Pro12Ala (270 bp, 227 bp); Lanes 5, 6: Ala12Ala (227 bp).

Association of Gene Polymorphisms with Related Traits

For $\beta 3\text{AR Trp64Arg}$ the numbers of twin pairs sharing 2, 1, or 0 allele of IBS were 78, 23, and 2, respectively. The latter two groups were put together since the number of twin pairs sharing 0 allele was too small. WHR and GLU differences of the twin pairs sharing 0-1 allele of IBS were bigger than those sharing 2 alleles of IBS, but the difference was not significant. As shown in Table 1, the correlations

between WHR, GLU, and INS, HOMA-IR of the twin pairs sharing 2 alleles of IBS were greater than those sharing 0-1 allele of IBS. A significantly positive correlation to HOMA-IR ($r=0.246$, $P=0.026$) was found in twin pairs sharing 2 alleles of IBS, whereas a low correlation ($r=0.048$, $P=0.820$) was observed in the twin pairs sharing 1 allele of IBS (Figs. 3 and 4). It was suggested that $\beta 3\text{AR Trp64Arg}$ polymorphism was associated with these traits or indexes.

TABLE 1

Intraclass Co-twin Correlation Among Twin Pairs Sharing 1, 2 Alleles of IBS at Different Loci

Variable	$\beta 3\text{AR Trp64Arg}$				PPAR $\gamma 2$ Pro12Ala			
	IBS=1		IBS=2		IBS=1		IBS=2	
	r	P	r	P	r	P	r	P
BMI (Kg/m ²)	0.661	0.000	0.601	0.000	0.238	0.481	0.636	0.000
WHR	0.517	0.006	0.537	0.000	0.694	0.018	0.557	0.000
PBF (%)	0.570	0.002	0.452	0.000	-0.005	0.989	0.500	0.000
GLU (mmol/L)	0.600	0.001	0.672	0.000	0.201	0.554	0.729	0.000
INS (mU/L)	0.024	0.908	0.179	0.108	0.049	0.892	0.092	0.382
HOMA-IR	0.048	0.820	0.246	0.026	0.020	0.956	0.200	0.054

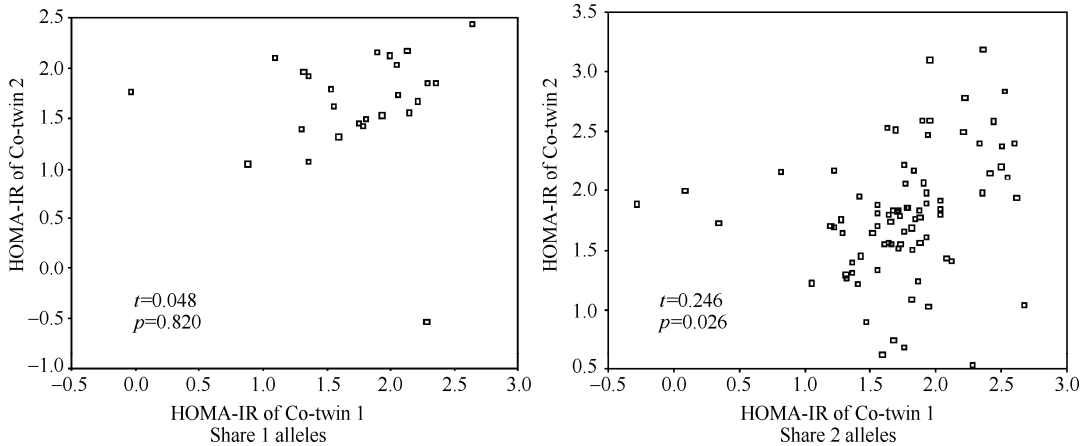


FIG. 3. Intraclass co-twin correlation among twin pairs sharing 1, 2 alleles of IBS at $\beta 3\text{AR Trp64Arg}$ locus.

For PPAR $\gamma 2$ Pro12Ala the number of twin pairs sharing 2, 1, or 0 allele of IBS was 98, 10, and 0, respectively. Intraclass differences of WHR among the twin pairs sharing 1 allele of IBS were greater than those sharing 2 alleles of IBS, but the difference was not significant. Most traits that had an intraclass co-twin correlation of twin pairs sharing 2 alleles of IBS were greater than those sharing 1 allele of IBS, and significant statistic differences were seen in BMI, WHR, PBF, and GLU (Table 1), suggesting that PPAR $\gamma 2$ Pro12Ala polymorphism was associated

with those traits. There were both low intraclass co-twin correlation of insulin and HOMA-IR of twin pairs sharing 1 or 2 alleles of IBS, implying that Pro12Ala had no obvious association with insulin sensitivity.

The separate and combined effects of variations on $\beta 3\text{AR}$ and PPAR $\gamma 2$ loci were also examined. The combined effects of variations were present when twin pairs were assigned to the twin pairs sharing 0-2, 3, and 4 alleles at both loci, which showed less squared twin-pair differences of BMI, WHR, PBF,

INS, and HOMA-IR among twins sharing 4 alleles, with a statistic significance in INS and HOMA-IR, indicating that there might be in some degree combined effects of the two loci on these traits. Similarly, twin pairs assigned to those sharing 2 alleles at $\beta 3AR$, sharing 2 alleles at PPAR $\gamma 2$ and sharing 4 alleles at both loci were compared. The squared twin-pair differences of BMI, INS, and HOMA-IR were less in the latter. The distributions of squared twin-pair differences of HOMA-IR among twins sharing different $\beta 3AR$ and PPAR $\gamma 2$ alleles of IBS are presented in Fig. 4.

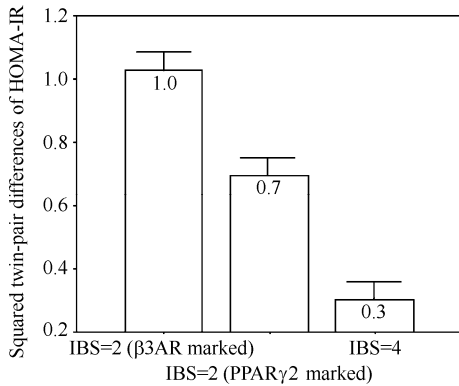


FIG. 4. Squared twin-pair differences of HOMA-IR in twins sharing different alleles of IBS.

DISCUSSION

In our study, the observed $\beta 3AR$ Trp64Arg allele frequencies of Trp and Arg were 84.2% and 15.8%, respectively, while the PPAR $\gamma 2$ Pro12Ala allele frequencies of Pro and Ala were 94.7% and 5.3%, respectively. Our findings concerning these frequencies are consistent with those found in the Chinese and Japanese populations^[10, 20-21]. 64Arg variant frequency is lower than that in Pima Indian^[12] and higher than that in Caucasian of North Europe and America^[11]. 12Ala variant frequency is lower than that in Caucasian^[22].

Our study applied sib-pair analysis by an alternative way to illustrate the linkage of a gene controlling the quantitative trait and genetic markers by comparing the correlations among the twin pairs sharing 2, 1, or 0 allele of IBS, thus avoiding estimation of IBD status. HOMA-IR was used as a marker of insulin sensitivity. Twins sharing 2 $\beta 3AR$ Trp64Arg alleles of IBS had a higher correlation than those sharing 1 allele of IBS, whereas twins sharing 2 PPAR $\gamma 2$ Pro12Ala alleles of IBS had not a significant higher correlation than those sharing 1 allele of IBS. At the same time, the differences in twin-pairs sharing 2

alleles at $\beta 3AR$ and 2 alleles at PPAR $\gamma 2$ were greater than in those sharing 4 alleles at both loci, indicating that the two gene loci are associated with insulin sensitivity, especially the former. At the same time, the HOMA-IR of the twins with mutant alleles was greater than that of their sib-pairs for 25 twin pairs with different $\beta 3AR$ genotype and 10 twin pairs with different PPAR $\gamma 2$ genotype, but the difference had no significance. Such a phenomenon might be partially resulted from the small sample size. The exactitude of HOMA-IR standing for insulin sensitivity should also be considered. Another possible cause is the fact that mutations at the two gene loci might not be the main genetic factor for insulin sensitivity.

Efforts have been made to identify the variants susceptible to insulin resistance. A large number of studies^[14, 23-27] have been published showing either an association between insulin resistance and type 2 diabetes or between obesity and the Trp64Arg polymorphisms in the beta-3-adrenoceptor gene or lack of association in either cases. It is hard to find a clear reason for these discrepant findings. Our literature review also suggests that some of the studies comparing homozygous with heterozygous subjects for the Arg allele have identified a much stronger association of homozygous subjects for Arg at codon 64 with obesity than that of Trp64Arg carriers. This may be the best evidence supporting that the Trp64Arg variant is associated with obesity. It is well known that obesity exists in many forms, i.e. upper- versus low-obesity, uncomplicated versus complicated obesity, early onset versus late-onset obesity, and the same with insulin resistance. Few studies published so far have defined the type of subjects with insulin resistance and only a few studies are population-based. Therefore, more studies are needed to investigate the different forms of obesity and insulin resistance consequently, including population-based cohorts and other genetic traits in order to clarify the association between insulin resistance and Trp64Arg polymorphisms in the beta-3-adrenoceptor gene.

Different Pro12Ala polymorphism of the PPAR $\gamma 2$ has also been reported. The ongoing debate now is focusing on whether the Pro12Ala is indeed a variant that predisposes to IR and which amino acid allele carries an increased risk of IR. Lindi *et al.*^[28] reported that the Pro allele is susceptible to insulin resistance and T2DM. Two other case-control studies showed that the Ala allele is associated with an increased susceptibility to T2DM in some populations^[29-30]. Malecki^[31] considered that it can be speculated that alleles of the Pro12Ala polymorphism might influence glucose homeostasis by affecting insulin sensitivity and secretion. While carriers of the Pro allele are characterized by increased resistance to

insulin compared to the Ala carriers, there is evidence that the latter might have decreased insulin secreting capacity. Thus, the phenotypic differences between the T2DM populations examined for the association might, at least partially, be responsible for the discrepancy in reported results. Further studies are necessary to prove this hypothesis.

Few studies^[32-33] have investigated the interaction with other putative candidate genes for insulin resistance. We attempted to provide evidence that polymorphism Pro12Ala of the PPAR γ 2 and Trp64Arg of the β 3AR gene may have a synergistic effect on insulin resistance. A similar result has been reported^[32] for obesity in a case-control study including children and adolescents.

In conclusion, the frequencies of the β 3AR Trp64Arg and PPAR γ 2 Pro12Ala polymorphisms in Chinese twin population are similar to those in other Asia populations. The 64Arg and 12Ala variants may be associated with insulin resistance and obesity. These two gene loci may have a synergistic effect on insulin resistance. However, a larger sample and a larger promotion of obesity and different forms of insulin resistance are needed to confirm our preliminary findings.

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