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Polymorphisms of Exon 17 of Insulin-Receptor Gene in Pathogenesis of Human Disorders With Insulin Resistance[1]

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Objective To investigate the relationship between polymorphisms of insulin-receptor (INSR) gene and insulin resistance in a population-based study in China. **Methods** Polymerase Chain Reaction (PCR) was used to the amplify Exon 17 of INSR gene and all amplified products were analyzed by direct sequencing. **Results** Six single-nucleotide polymorphisms (SNPs) were found at the following loci: T to TC at the locus of 10699 (Tyr^{984}), G to GC at the locus of 10731 (Glu^{994}), Deletion G at the locus of 10798 (Asp^{1017}), C to T/TC at the locus of 10923 (His^{1058}), C to CA at the locus of 10954 (Leu^{1069}), and T to TA at the locus of 10961 (Phe^{1071}), which might not change the amino acid sequence. The data were in agreement with the test of Hardy-Weinberg balance ($P>0.05$). Among the 345 cases, all clinical indices were higher in males than in females except for HDL cholesterol ($P<0.05$). The proportion of insulin resistance in males (64.4%) was higher than that in females (35.6%, OR=1.83). It implied that the relative risk of developing insulin resistance in males was 1.83 times as high as that in females. The biochemical indices in different loci on Exon 17 showed that the individuals with deletion G on the locus of 10798 had lower TG ($P=0.052$) and higher HDL ($P=0.027$) than those without deletion G on the same site. Homa-Index was lower in those with deletion G than in those without deletion G ($P>0.05$). After sex stratification in analysis，all allele frequencies on the six loci of SNPs of Exon 17 had different distributions between the insulin resistant group and the control group, but $P>0.05$. **Conclusion** SNPs of Exon 17 of INSR gene are unlikely to play a direct role in the pathogenesis of human disorders with insulin resistance.

Key words: Insulin resistance; Insulin receptor gene; Polymorphism

INTRODUCTION

Insulin resistance, involving the process of various metabolic disorders and diseases, is

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a syndrome that severely threatens the health of human beings. It is usually characterized by coexistence of hyperinsulinemia, dislipidemia, obesity, hypertension, and influenced by both environmental and genetic background, the latter was mostly unknown[1]. Therefore, insulin-receptor (INSR) gene has been increasingly selected as a candidate gene to study metabolic disorders and pathogenesis of diseases. At present no data is available in China concerning the population-based relationship between insulin resistance and INSR gene mutation. Previous studies indicated that the consensus sequence for an ATP-binding domain was located between amino acid residues 1003 and 1030 on Exon 17 of the insulin-receptor gene. Activation of receptor tyrosine kinase could trigger multiple biological responses of target cells to insulin[2]. Thus, DNA direct sequencing technique of molecular epidemiology was used to analyze the single-nucleotide sequence for Exon 17 of INSR gene from a specific Chinese cohort to explore the relationship between insulin resistance and polymorphism of INSR gene from a population perspective.

MATERIALS AND METHODS

Subjects

Study subjects were selected from the research cohort of "Intrauterine Growth and Adult Diseases" established in 1995 that included alive singleton born in Beijing from 1948 to 1954. Based on informed consents obtained, epidemiological investigations and physical examinations were conducted for 345 respondents.

Interviews With Special Questionnaire

Interviews included history of occupational exposure, physical activities, smoking, alcohol consumption, previous and current diseases, drug administration two weeks prior to physical examination, as well as main job and income in recent two years, etc.

Physical Examination

Height and weight were measured by one observer. Blood pressure (1 mmHg=0.133 KPa) was measured with an automated recorder (Dinamap 8100, Critikon, Tampa, Florida) after the participants lay down for at least 5 minutes. All the above measurements were carried out three times consecutively, and their mean values and body mass index (BMI=weight/height^2 (kg/m^2)) were calculated for analysis accordingly.

Blood Biochemistry

Fasting plasma glucose (FPG), insulin (FIN), serum total cholesterol (TC), triglyceride (TG), low/high density lipoprotein-cholesterol (LDL-C and HDL-C) were tested after a 12-hour overnight fast. FPG was measured by using GOD-PAP method. TC, HDL-C, and TG were measured by using standard enzymatic methods. LDL-C was calculated by using the Friedewald formula. All these indexes were tested by using the Hitachi-7170A auto-biochemistry analyzer at the Clinical Laboratory of Peking University First Hospital. BA-ELISA was used to test the plasma levels of true insulin at the National Laboratory of Endocrine, Peking Union Medical College Hospital.

Diagnosis Criteria

HOMA-IR of the homeostasis model assessment (HOMA)[3,4] was used to assess the

individual insulin resistance, for which HOMA-IR was equal to (FPG mmol/L×FIN pmol/L)/22.5, the larger the value of HOMA-IR is, the greater the individual insulin resistance is, and 75% of HOMA-IR (1.75) was considered as the cut off point, or HOMA-IR≥1.75 as being insulin resistant (case group) and HOMA-IR＜1.75 as insulin non-resistant (control group).

DNA Sequencing

Genomic DNA was prepared by using phenol-chloroform extraction from venous blood leukocytes of the subjects. The regions representing Exon 17 were amplified with standard conditions, as described by Seino *et al*.[5]. The 317 bp fragment was amplified by primers 5′-CCA AGG ATG CTG TGT AGA TAA G (10650-10671 bp) and 5′-TCA GGA AAG CCA GCC CAT GTC (10946-10966 bp)[4]. Each reaction was performed in a volume of 50 μL containing 10 times concentrated buffer, 1.5 mmol/L Mg^{2+}, 0.2 mmol/L dNTPs, 0.1 mmol/L primer and 1 U Taq DNA polymerase. Amplification was conducted. After an initial denaturation at 94℃ for 3 min, the samples were subjected to 25 cycles of amplification: annealing at 55℃ for 1.5 min, extension at 70℃ for 2.5 min, and again denaturation at 94℃ for 1 min. Having compared with markers of standard molecular weight using 0.8% agarose gel electrophoresis, the sequence of amplified DNA was determined by direct sequencing according to Sanger's method on a Megabace 1000 sequence machine (Amersham Biosciences Company) carried out at CASarray Biochip Company. All samples were sequenced from both ends of the amplified fragment to ensure the accuracy of results.

Data Analysis

SPSS10.0 was used for data analysis. Hardy-Weinburg balance test was conducted to evaluate the representativeness of gene frequency. Clinical characteristics were compared between subjects with and without insulin resistance by analyzing variance for continuous variables (expressed as $\bar{x} \pm s$) and Chi-square test for categorical variables. Variables showing skewed distribution were analyzed after log-transformation.

RESULTS

Characteristics of Subjects

Demographic trait showed that there was a significant sex difference of all indices in addition to age. All indices of males were higher than those of females except HDL-C (P<0.05). The clinical data are listed in Table 1. All indices in the insulin resistant group were greater than those in the control group, provided that the value of HDL-C in the insulin-resistant group was lower than that in the control (P<0.05).

The clinical data demonstrated that all indices of males were greater than those of females, showing 64.4% males and 35.6% females in the insulin resistant group (P<0.05, OR=1.83). Obviously the risk of males suffering from insulin resistance was 1.83 times of females (Table 2).

Single-nucleotide Polymorphisms (SNPs) at Different Loci of Exon 17 in INSR Gene

Six polymorphic sites of SNPs were identified at Exon 17 of INSR gene after direct sequencing of the PCR amplified products of 345 cases. A summary of the findings is shown in Table 3, namely, T→TC (hetero-peak TC) at 10699 site, G→GC (hetero-peak GC) at 1

0731 site, G→？ (deletion of G) at 10798 site, C→T/TC (mono-peak T or hetero- peak TC) at 10923 site, C→CA (insert A) at 10954 site, T→TA (hetero-peak TA) at 10961 site.

TABLE 1

Clinical Traits in Subjects of the Insulin Resistant Group and the Control ($\bar{x}\pm s$)

Clinical traits	Male		Female	
	Insulin Resistant	Control	Insulin Resistant	Control
No. subjects	56	128	31	130
Age (year)	48.5±1.2	48.3±1.0	48.3±0.7	48.5±1.1
TG (mmol/L)	2.92±0.63*	1.64±0.35#	2.54±0.52*	1.41±0.38#
HDL-C (mmol/L)	1.08±0.16*	1.19±0.13#	1.22±0.12*	1.48±0.15#
SBP (mmHg)	129.6±14.8*	120.8±11.7#	124.1±13.0*	116.9±14.7#
DBP (mmHg)	82.6±8.9*	75.6±8.8#	72.1±7.9*	68.1±10.3#
BMI (Kg/m^2)	27.3±2.8*	24.0±2.8#	27.2±4.1*	23.4±3.3#

Note. * Significance of difference between insulin resistant group and control group, $P<0.01$. # Significance of difference between males and females, $P<0.01$.

TABLE 2

Sex Difference in Response to Insulin Resistance

Sex	Insulin Resistant	Control Group	Total	χ^2	P	OR (95% CI)
Males (%)	56 (64.4)	128 (49.6)	184 (53.3)			
Females (%)	31 (35.6)	130 (50.4)	161 (46.7)	5.69	0.018	1.83 (1.11-3.03)
Total (%)	87 (100.0)	258 (100.0)	345 (100.0)			

TABLE 3

Polymorphisms of Exon 17 of INSR Gene Detected by Direct Sequencing of the PCR Amplified Products

Locus	Sequence in Gene Bank: 5′→3′	DNA Codon	Mutation	Possible Amino Acid
10699	T^{10699}AC	984Tyr	TCAC	→ Ser/ His / Tyr
10731	GAG^{10731}	994Glu	GACG	→ Asp/Thr /Glu/Ser
10798	G^{10798}AC	1017Asp	? AC	→ ?
10923	CAC^{10923}	1058His	CAT/CATC	→ His/Ile/Thr
10954	C^{10954}TG	1069Leu	CATG	→ Met/His/Thr /Leu
10961	TT^{10961}C	1071Phe	TTAC	→ Tyr/ Ile/ Tyr/ ?

Biochemcal Alterations of Single-nucleotide Polymorphisms (SNPs) at Different Sites of Exon 17 in INSR Gene

SNPs biochemical indices at different loci of Exon 17 of INSR gene are listed in Table 4. The level of TG in subjects with deletion G at 10798 site was lower than that in people without deletion G at the same site, $P=0.052$. However, the level of HDL-C in people with deletion G was much higher than that in people without deletion G, ($P=0.027$). There was no statistical difference among the different indices at other loci presenting SNPs polymorphic variations.

TABLE 4

Clinical and Biochemical Indices at Different Sites Presenting SNPs Polymorphic Variations of Exon 17 of INSR Gene ($\bar{x}\pm s$)

Locus	Geno-type	N	TG (mmol/L)	HDL-C (mmol/L)	BMI (Kg/m^2)	SBP (mmHg)	DBP (mmHg)	HOMA Index
10699	TT	341	1.79±0.49	1.28±0.16	24.6±3.5	121.1±14.2	73.6±10.7	1.37±0.74
	TC		1.65±0.31	1.34±0.11	25.7±6.1	118.8± 6.2	76.6± 4.4	1.57±0.67
10731	GG	343	1.79±0.49	1.28±0.16	24.6±3.5	121.0±14.2	73.6±10.7	1.37±0.74
	GC	2	1.81±0.02	2.16±0.20	26.5±2.4	127.5± 4.0	81.0± 1.4	1.92±0.61
10798	GG	333	1.82±0.49*	1.28±0.16$^\#$	24.6±3.5	120.9±14.0	73.5±10.7	1.38±0.75
	Del G	12	1.24±0.32	1.51±0.16	25.3±3.3	124.1±17.7	77.3± 8.7	1.31±0.50
10923	CC	121	1.68±0.45	1.28±0.16	24.3±3.5	121.3±14.9	73.7±11.3	1.34±0.72
	TT	64	1.68±0.40	1.32±0.16	24.7±3.2	120.4±15.4	72.6±10.6	1.34±0.74
	CT	160	1.94±0.55	1.27±0.16	24.8±3.6	121.1±13.0	73.9±10.1	1.41±0.76
10954	CC	343	1.79±0.49	1.28±0.16	24.6±3.5	121.1±14.1	73.6±10.7	1.38±0.74
	Ins A	2	1.55±0.40	1.33±0.22	23.7±3.9	107.5± 0.7	66.0±1.89	0.97±0.50
10961	TT	338	1.80±0.49	1.28±0.16	24.6±3.5	121.2±14.2	73.7±10.7	1.38±0.75
	TA	7	1.41±0.35	1.46±0.15	23.4±2.9	112.5±6.2	66.3± 6.2	1.10±0.37
Total		345	1.79±0.49	1.28±0.16	24.6±3.5	121.0±14.1	73.6±10.6	1.37±0.74

Note. $^*P$=0.052; $^\#P$=0.027.

Insulin Resistance in Relation to SNPs on Different Loci of Exon 17 of INSR Gene in Insulin Resistant and Control Groups

By applying hardy-weinburg balance test to determine the gene frequency of six sites presenting SNP variations, the result showed no significant difference ($P\geqslant 0.05$). In order to analyze the relationship between insulin resistance and SNP polymorphism of Exon 17 of INSR gene, case-control study with sex stratification was conducted to compare the difference of allele distribution on different sites between the insulin resistant and control groups. The result is listed in Table 5.

TABLE 5

Comparison of Allele Frequency on Different Sites Presenting SNP Variations of Exon 17 of INSR Gene in Insulin Resistant and Control Groups

Site	Allele	Male				Female			
		IR (n=56)	Control (n=128)	P	OR	IR (n=31)	Control (n=130)	P	OR
10699	T	111(0.991)	255(0.996)	0.517	0.44	62(1.000)	258(0.992)	1.000	0.81
	C	1(0.009)	1(0.004)			0(0.000)	2(0.008)		
10731	G	111(0.991)	256(1.000)	0.304	0.32	62(1.000)	259(0.996)	1.000	0.81
	C	1(0.009)	0(0.000)			0(0.000)	2(0.004)		
10798	G	11(0.991)	253(0.988)	1.000	1.32	60(0.968)	254(0.977)	0.653	0.71
	Del G	1(0.009)	3(0.012)			2(0.032)	6(0.023)		
10923	C	71(0.634)	147(0.574)	0.301	1.28	30(0.484)	154(0.592)	0.153	0.65
	T	41(0.366)	109(0.426)			32(0.516)	106(0.408)		
10954	C	112(1.000)	255(0.996)	1.000	0.70	62(1.000)	259(0.996)	1.000	0.81
	A	0(0.000)	1(0.004)			0(0.000)	1(0.004)		
10961	T	112(1.000)	251(0.980)	0.328	0.69	62(1.000)	258(0.992)	1.000	0.81
	A	0(0.000)	5(0.020)			0(0.000)	2(0.008)		

Table 5 shows that regardless of sex difference, there was no significant difference in allele frequency on the six sites presenting SNP variations of Exon 17 of INSR gene in

insulin resistant and control groups.

DISCUSSION

The study on 345 respondents revealed that, except for age, all indices in males were greater than those in females (P<0.05), with the exception of HDL-C which was higher in females (P=0.00). In addition, the ratio of insulin resistance of males (64.4%) was greater than that of females (35.6%), with OR=1.83, indicating that the occurrence of insulin-resistance in males was 1.83 times as high as that in females. Therefore, sex was treated as the main confounding factor in our subsequent analysis. Hardy-weinberg balance test showed that this method presented higher effectiveness of gene frequency and the data obtained were valid and reliable for further analysis.

The biochemical analysis of indices in different sites of SNPs of Exon 17 of INSR gene found that the level of TG in people with deletion G on 10798 site was lower than that in people without deletion G on 10798 site. However, the level of HDL-C in people with deletion G on 10798 site was much higher than that in people without deletion G on 10798 site, P=0.027. In the case-control study with sex stratification for analyzing the relationship between insulin-resistance and SNP variations on different sites of Exon 17 of INSR gene, no statistical difference in the allele frequency on the six sites with SNPs (P>0.05) was found in either sex between insulin-resistant and control groups. This might be biased by the insufficient sample size that needs further consideration.

Human insulin-receptor (INSR) is an integral membrane-spanning glycoprotein on the surface of all cells, and a family member of tyrosine kinase receptors. INSR mediates the action of insulin upon target cells. Any defects of INSR in number or functions might decrease the action of insulin- and cause insulin-resistance[6]. Previous studies demonstrated that mutations in INSR gene were detected in many patients with severe or moderate insulin-resistance, and the genetic basis for more common moderate forms of insulin resistance was likely polygenic and heterogeneous[7-9]. It suggested that mutations in INSR gene might be one of the candidate genes contributing to the development of insulin-resistance. Human INSR gene spans a region about 120000 base pairs (bp) on the short arm of chromosome 19 consisting of 22 exons and 21 introns. Mature human INSR is a hetero-tetramer of two α-subunits and two β-subunits. Exons 1-11, which encoded the α-subunit of the receptor, can be depressed over 90000 bp. In contrast, Exons 12-22, which encoded the β-subunit, are located together in a region of 30000 bp. The insulin-binding α-subunit was located outside the cell membrane and could repress the activity of tyrosine kinase. Insulin binding to α-subunit probably induces a conformational change in the receptor that relieves this repression. Membrane-spanning protein tyrosine kinase β-subunit containing 13 tyrosine residues is also named as catalytic subunit. Exons 17-22 are coded for protein tyrosine kinase domains and their activity is required for insulin action[10,11]. Tyrosine residues are auto-phosphorylated by the specific tyrosine kinase on the insulin-binding signal transmitted from α-subunit and caused a series of phosphorylation responses to mediate the biological effect of insulin. Hence, any functional and structural defects in insulin-receptor might impair the biological response to insulin, thus leading to insulin resistance.

It is known that there are nine conservative residues on the region of tyrosine kinase domain that play key roles in stimulating the activity of tyrosine together with neighboring homogeneous conservative sequences[10]. Three of the nine resideus are located on Exon 17, namely Tyr965, Tyr972, and ATP binding site on 1003-1030 site. Suzuki[12] found that mutation of codon 970 in Exon 17 was present in a regulatory domain of the insulin-receptor

gene, acting in a cis-dominant fashion to reduce the levels of insulin receptor mRNA. Met-985 insulin-receptor variant was associated directly with the development of hyperglycemia and insulin resistance[13]. Therefore, Exon 17 of INSR gene was selected as the candidate gene of insulin resistance. The result of DNA direct sequencing showed that the six sites presenting SNP variations were all located near or just on these three conservative sequences. For example, Phe1071 on the 10798 site was located in ATP-binding site between the 1003-1030 sites, which might play a certain role in tyrosine activity. Analysis of the relationship between allele frequency and insulin resistance suggested that compared with the control group, the frequency of deletion G on 10798 site was higher in insulin-resistant females, but the difference was not statistically significant. It may be due to the small sample size (8 subjects with 4 males). There were polymorphisms on sites 10954 and 10961 in our study. Except for HDL, the biochemical indexes of subjects with SNP variation were lower than normal. Allele frequency analysis implied that all the subjects with SNP variation belonged to the control group. It seems that SNP variation on these two sites could decrease the sensitivity of insulin-resistance. Nevertheless further study is merited. Sites 10699 (Tyr984) and 10923 (His1058) were two common silent polymorphism sites of Exon 17, mainly presenting heterogeneous genotype[7,14]. A T to TC mutation was detected on 10699 site (Tyr984) which might not cause the variation of tyrosine coding for it. Two samples were detected C→T/CT mutation on 10923 site (His1058) without variation of histidine. The result was consistent with Kim's study in Japanese[7]. Moreover, it was found that the distribution of three polymorphisms of 10923 site was relatively equal in the study population with *P* value approaching 1, in both the distribution of clinical and biochemical indices and allele frequency analysis. It suggested that the polymorphic variation of the 10923 site (His1058) be a common genetic polymorphism in Chinese, and its significance in the onset of insulin-resistance requires further evidences. Pang in her study in Chinese showed that mutation in 1058 codon (His1058) of INSR gene might be associated with insulin-resistance in hypertensive patients and subjects with positive hypertensive history[15].

It is worth considering that although the six sites presenting polymorphism were found to have a single nucleotide substitution at different codons in Exon 17 of INSR gene, there might be no change in the amino acid sequence. It is inferred that these substitutions might not directly participate in insulin-resistance development, and disequilibrium might exist in these sites and candidate genes of insulin resistant individuals. The genes that play key roles in insulin resistance development, might be located in other exons, introns, regulatory regions, or other related genes. The deletion G on 10798 site coding for Phe1071 might possibly play an important role in changing the amino acid codon, because the ATP binding-site between 1003-1030 sites of Exon 17 is located in this region, which is a key factor to fix phosphorus during auto-phosphorylation responses. The structure defect of this codon might cause failure to undergo auto-phosphorylation[11], i.e. having a direct impact on the process of γ-autophosphorylation of Mg^{2+}-ATP and causing extreme insulin-resistance.

In a word, it is a complicated problem to study the relationship between insulin resistance and mutation of INSR gene. The identification of specific gene variants that influence insulin-resistance and other traits of insulin resistance syndromes will have profound influences on our understanding of the molecular and pathophysiologic basis of these disorders, from which new and more effectively preventive and therapeutic interventions will be possible[9]. In this study, limited by many factors, only Exon 17 of INSR gene has been directly sequenced to describe the relationship and possible molecular mechanism between insulin-resistance and the six SNP sites on Exon 17 of INSR gene in specific population in China. It is hopeful that more evidences need to be obtained based on a larger sample study.

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