Brief Genetics Report

Role of Allelic Variants Gly972Arg of IRS-1 and Gly1057Asp of IRS-2 in Moderate-to-Severe Insulin Resistance of Women With Polycystic Ovary Syndrome

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To assess the role of insulin receptor, insulin receptor substrate (IRS)-1, and IRS-2 genes in insulin resistance, we explored the genomic DNA in women with polycystic ovary syndrome (PCOS) and a variable degree (mean ± SE) of insulin resistance (homeostasis model assessment index for insulin resistance [$HOMA_{IR}$] 3.2 ± 0.6, n = 53; control subjects 1.56 ± 0.34, n = 102) using direct sequencing. Whereas no novel mutations were found in these genes, gene-dosage effects were found on fasting insulin for the Gly972Arg IRS-1 variant and on 2-h plasma glucose for the Gly1057Asp IRS-2 variant. The Gly972Arg IRS-1 variant was more prevalent in insulin-resistant patients compared with non–insulin-resistant individuals or control subjects (39.3 vs. 4.0 and 16.6%, P < 0.0031, respectively). A multivariate model that included BMI as a variable revealed significant effects of the Gly1057Asp IRS-2 variant on insulin resistance (P < 0.016, odds ratio [OR] 7.2, 95% CI 1.29–43.3). $HOMA_{IR}$ was higher in carriers of both IRS variants than in those with IRS-2 mutations only or those with wild-type variants (6.2 ± 2.3, 2.8 ± 0.5, and 1.8 ± 0.2, respectively; P < 0.01), and it was significantly associated with this genotype (P < 0.0085, OR 1.7, 95% CI 1.09–2.99). We conclude that polymorphic alleles of both IRS-1 and IRS-2, alone or in combination, may have a functional impact on the insulin-resistant component of PCOS. *Diabetes* 50:2164–2168, 2001

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AUC, area under the curve; AUC_{gluc}, AUC for glucose; AUC_{ins}, AUC for insulin; FSH, follicle-stimulating hormone; LH, leutenizing hormone; $HOMA_{IR}$, homeostasis model assessment index for insulin resistance; IGT, impaired glucose tolerance; IR, insulin receptor; IRS, IR substrate; OGTT, oral glucose tolerance test; OR, odds ratio; PCOS, polycystic ovary syndrome; PCR, polymerase chain reaction; UTR, untranslated region.

Severe insulin resistance is a prominent feature of rare genetic syndromes in humans and, albeit in a milder form, of common diseases such as obesity, type 2 diabetes, and polycystic ovary syndrome (PCOS) (1–3). Although clear evidence was provided for involvement of mutations in the insulin receptor (IR) in genetic Type A syndrome and leprechaunism, in complex diseases the role of IR remains elusive, and most of the defects in insulin action are expected at the postreceptor level (2). IR substrate (IRS) genes encoding for key proteins in insulin transduction are attractive candidates, particularly IRS-2, as suggested by recent transgenic animal models (4,5). In humans, the role of IRS genes was suggested by the identification of several more prevalent allelic variants in type 2 diabetes (6–11). PCOS is another good model to study influent genes, because in this complex disease, characterized by chronic anovulation and hyperandrogenism in women, insulin resistance is a major component (3,12). In common forms of PCOS, IR mutations have not been found, and linkage studies excluded IR and IRS-1 as major genes (13,14). To understand genetic determinants of insulin resistance, we investigated the effects of two variants of IRS-1 and IRS-2 in PCOS (n = 53) with values (mean ± SE) for homeostasis model assessment index for insulin resistance ($HOMA_{IR}$) twofold higher than the control population (1.56 ± 0.034, n = 102) (Table 1). For the purpose of this study, the PCOS population was stratified into two groups with normal (group A) and elevated (group B) fasting insulin.

To eliminate the possibility of existing mutations, the coding regions of IR and IRS-1 genes were entirely sequenced in group B. No novel mutations were revealed in the IR, except for one subject with Ile485Thr. Three silent (Gln276, Asp519, and Ala523) and 25 intronic polymorphisms (not shown) were also detected. Examination of the IRS-1 gene did not reveal any novel mutation, but it did reveal silent polymorphisms: Asp90 (one subject), Gly234 (six subjects), Ala804 (two subjects), and Pro893 (one subject). Variants Ala513Pro and Gly818Arg were rare (1 subject for each), but Gly972Arg was frequent (11 subjects). Because conflicting sequences were reported for IRS-2, the coding region of this gene was sequenced in

TABLE 1
Clinical and laboratory features of patients with PCOS included in the genetic study

Characteristics	PCOS
n	53
Age (years)	26.3 ± 0.8
Diabetic history (%)	39.7
Acanthosis nigricans (%)	30.2
BMI (kg/m^2)	29.9 ± 1.1
LH/FSH	2.1 ± 0.1
Testosterone (nmol/ml)	0.9 ± 0.04
Δ4 Androstenedione (ng/ml)	2.4 ± 0.2
Fasting glucose (mmol/l)	4.2 ± 0.1
AUC_{gluc} (mmol/l · h)	12.8 ± 0.6
2-h glucose (mmol/l)*	6.0 ± 0.3
IGT (%)†	15.5
Insulin (μU/ml)	16.1 ± 2.6
AUC_{ins} (μU/ml · h)	135.4 ± 17.3
2-h insulin (μU/ml)*	77.1 ± 10.4
$HOMA_{IR}$‡	3.2 ± 0.6

Data are means ± SE. *Two-hour values during 75-g OGTT; †IGT was categorized according to American Diabetes Association criteria; ‡$HOMA_{IR}$ index for insulin resistance was calculated as (fasting insulin × fasting glucose)/22.5.

both group A and group B (15). No novel or previously reported mutations (Gly879Ser and Leu647Val) were detected (6,7). Among known polymorphisms, we found Cys816 (17 and 21 subjects) and Gly1057Asp (12 and 22 subjects) in groups A and B, respectively, whereas four novel substitutions were detected at codon 723 (18 and 16 subjects), 829 (13 and 12 subjects), 1031 (1 and 0 subjects), and 1033 (9 and15 subjects). Compared with the reference sequence (6), other nucleotide changes were detected at 16 different sites (Table 2).

Among all of these variations, the variants Gly972Arg of IRS-1 and Gly1057Asp of IRS-2 were more frequent in the PCOS population, and both were in Hardy-Weinberg equilibrium. The allelic frequency (*q*) of the 972Arg variant of IRS-1 was 0.11 (95% CI, 0.06–0.19), not significantly different from 0.083 (0.04–0.13) in control women or from 0.07 (0.051–0.11) in the general population. For 1057Asp of IRS-2, the *q* value was 0.36 (0.027–0.46), which was comparable to that of control women (0.29, 0.023–0.36) or of the general population (0.31, 0.026–0.36). These data suggest that two allelic variants of IRS were not major determinants of PCOS. To further investigate whether they are influent in the insulin-resistant component of PCOS, we analyzed the gene-dosage effect in the population as well as the prevalence rates and the genotype-phenotype correlation in the stratified population as a function of insulin resistance.

When carriers of the Gly972 wild type variant of IRS-1 (G/G, $n = 41$) were compared with heterozygous Gly972Arg (G/C) carriers ($n = 12$), a gene-dosage effect was found on fasting insulin (12.16 ± 1.36 vs. 29.58 ± 9.71 μU/ml, $P < 0.0031$) or $HOMA_{IR}$ (2.37 ± 0.28 vs. 5.94 ± 2.16, $P < 0.0065$, Mann-Whitney *U* test). Similarly, when carriers of the wild-type Gly1057 variant of IRS-2 (G/G, $n = 19$) were compared with heterozygous (G/A) and homozygous (A/A) carriers of Gly1057Asp ($n = 34$), the gene-dosage effect was observed for 2-h glucose (5.16 ± 0.23 vs. 6.54 ± 0.47 mmol/l $P < 0.02$) and 2-h insulin (42.4 ± 6.4 vs. 95.7 ± 14.5 μU/ml, $P < 0.033$, Mann-Whitney *U* test). None of the IRS-2 wild-type (G/G) carriers were glucose-intolerant, but 24% of the Gly1057Asp carriers displayed impaired glucose tolerance (IGT) ($P < 0.032$).

As shown in Table 3, when the population was stratified as a function of insulin resistance, the prevalence of the

TABLE 2
Polymorphisms, mutations, and other nucleotide changes in IRS-2 gene compared to reference sequence no. AF073310

Location (codon no.)	Nucleotide no.	Nucleotide change	Amino acid (codon) change
Polymorphisms and mutations			
723 (724)*	2172	C/T[b]	Ser (AGC)/Ser (AGT)
816 (817)	2451	T/C[c]	Cys (TGT)/Cys (TGC)
829 (830)	2490	C/T	Pro (CCC)/Pro (CCT)
1031	3093	G/A	Pro (CCC)/Pro (CCA)
1033	3099	A/G	Pro (CCA)/Pro (CCG)
1057	3170	G > A	Gly (GGC)>Asp (GAC)
Other nucleotide changes			
29	85–87	repeats $(AAC)_n$	$(AAC)_9/(AAC)_8$
40	120	C > G	Asn (AAC) > Lys (AAG)
41	121 and 123	G > C and A > G	Glu (GAA) > Gln (CAG)
42	124	G > A	Glu (GAG) > Lys (AAG)
372	1114 and 1115	C > G and G > C	Arg (CGG) > Ala (GCG)
373	1119	T/G	Ala (GCT)/Ala (GCG)
380	1139 and 1140	C > G and G > C	Ala (GCG) > Gly (GGC)
381	1141, 1142, and 1143	C > G, A > C, and G > C	Gln (CAG) > Ala (GCC)
383	1148	T > C	Leu (CTG) > Pro (CCG)
663	1987	T > C	Phe (TTC) > Leu (CTC)
873	2618 and 2619	G > C	Arg (CGT) > Pro (CCT)
876	2626	C > G	Arg (CGC) > Gly (GGC)
877	2631	G/C	Arg (CGG)/Arg (CGC)
878	2632 and 2634	T > C and A > G	Ser (TCA) > Pro (CCG)
878–879	2634–2635	insert GAG	insert Glu (GAG)
879	2637	T/C	Gly (GGT)/Gly (GGC)

*Number of repeats at codon 29 would change the numbering for codons 723, 816, and 829; †nucleotide T in the codon AGT is the major allele; ‡nucleotide C in the codon TGC is the major allele.

TABLE 3
Prevalence of Gly972Arg (IRS-1) and Gly1057Asp (IRS-2) variants and their combination in total PCOS subjects, groups A and B, and control women

	Total PCOS subjects	Group A	Group B	Control women	P (χ^2)
n	53	25	28	102	
IRS-1 gene					
Gly972Arg (%)	22.6	4.0	39.3	16.6	<0.0031
IRS-2 gene					
Gly1057Asp (%)	64.15	48.0	78.6	54.9	<0.043
Combined mutations (%)	20.75	4.0	35.7	12.6	<0.0093

The P value calculated with the χ^2 test refers to differences between control women and groups A and B. No significant difference was found between total PCOS patients and the control group. Note that the prevalence of Gly1057Asp is not significant if the Bonferroni correction is applied.

Gly972Arg IRS-1 variant in group B was 10-fold higher than in group A ($P < 0.001$) and 2.3-fold higher than in control women ($n = 102$, $P < 0{,}0{,}031$) or in the general population (15.2%, $n = 224$). The Gly1057Asp variant of IRS-2 also appeared more prevalent in group B than in group A, although the difference did not reach statistical significance. Some level of significance (not supported by Bonferroni correction) was reached when prevalence rates in group A and group B were compared with control women (Table 3). Multivariate analysis performed to explain differences between group A and group B indicated a role of BMI ($P < 0.0001$, odds ratio [OR] 1.2, 95% CI, 1.07–1.38) and an additional effect of IRS-2 variant ($P < 0{,}016$, OR 7.2, 95% CI 1.29–43.3). Some effects may also be expected from the IRS-1 variant ($P < 0.01$, OR 12.5, 95% CI 1.2–128.2), but CI values suggested inadequacy of the sample size. In addition to heterogeneity at the gene and phenotype levels, the understanding of IRS effects on insulin resistance seems complicated by the fact that 35.7% of the subjects in group B are carriers of both allelic variants (designated as double-mutated). Introduction in the multivariate model of a genetic variable corresponding to genotype I (carriers of the wild type IRS variants), genotype II (carriers of a mutation on IRS-2 only), and genotype III (carriers of mutations on both IRS-1 and IRS-2) indicated again the influence of the IRS-2 variant ($P < 0.0003$, OR 7.9, 95% CI 1.26–50.6). The effect of the double mutation was difficult to estimate because of the inadequacy of the sample size ($P < 0.0003$, OR 79.2, 95% CI 4.9–1,260).

Another way to understand the effect of both variants on insulin resistance is to analyze metabolic profiles in three distinct genotypes (groups I, II, and III). Patients in group II were more insulin-resistant and glucose-intolerant than patients in group I, whereas group III was associated with a higher degree of insulin resistance (Table 4). However, the significance of these data should be considered with caution, because they are not supported by the Bonferroni correction. The logistic regression (plurimodal model) revealed no significance for age, diabetic history, BMI, androgens, and leutenizing hormone (LH)–to–follicle stimulating hormone (FSH) ratio, but $HOMA_{IR}$ appeared to be significantly associated with genotype III ($P < 0.0085$, OR 1.7, 95% CI 1.09–2.99).

Whereas several other major genes are actively investigated as candidates for the endocrine syndrome of PCO (14), these data provide evidence for the involvement of Gly972Arg and Gly1057Asp variants in the insulin-resistant component of PCOS. Although the variant Gly972Arg of IRS-1 has been extensively studied in the literature, the effects of the Gly1057Asp variant of IRS-2 are poorly understood. In our patients, Gly1057Asp was associated with high fasting insulin, in contrast to type 2 diabetes, where the same variant was associated with reduced fasting insulin and protective effect (7,11). Moreover, we found this variant twofold more prevalent in both PCOS

TABLE 4
Phenotypic features of patients with PCOS as a function of genotypes

	Group I nonmutated	Group II mutated on IRS-2	Group III double mutated	P
Genotype (designation)	($n = 18$)*	($n = 23$)	($n = 11$)	
IRS-1 (codon 972)	G/G	G/G	G/C	
IRS-2 (codon 1057)	G/G	G/A or A/A	G/A or A/A	
Phenotypic feature				
Age (years)	26.5 ± 1.4	27.4 ± 1.3	23.3 ± 1.7	NS
Diabetic history (%)	44.4	34.8	36.4	NS
Acanthosis nigricans (%)	33.3	26.1	27.3	NS
BMI (kg/m^2)	30.7 ± 2.1	28.7 ± 1.7	30.9 ± 2.1	NS
Fasting insulin (μU/ml)	10.1 ± 1.1	13.7 ± 2.2	30.7 ± 10.5	<0.01
2-h insulin (μU/ml)	43.9 ± 6.7	96.7 ± 20.4	94.2 ± 20.3	NS
AUC_{ins} (μU/ml × h)†	97.6 ± 21.7	138.8 ± 23.7	182.5 ± 50.8	NS
$HOMA_{IR}$‡	1.8 ± 0.2	2.8 ± 0.5	6.2 ± 2.3	<0.01
Fasting glucose (mmol/l)	4.2 ± 0.1	4.3 ± 0.2	4.2 ± 0.1	NS
2-h glucose (mmol/l)*	5.0 ± 0.2	7.0 ± 0.7	5.8 ± 0.3	<0.04
AUC_{gluc} (mmol/l × h)†	11.2 ± 0.5	14.3 ± 1.2	12.3 ± 0.5	NS
IGT (%)§	0.0	37.8	10.0	<0.03

Data are means ± SE and nominal variables of prevalence rate (%). Three groups were defined as a function of the mutations in IRS-1 and IRS-2. Statistical significance between groups I, II, and III was performed with Kruskal-Wallis or χ^2 tests and was considered significant for $P < 0.05$ (without Bonferroni correction). *One patient possessing uniquely the variant Gly972Arg was excluded; †AUC_{ins} and AUC_{gluc} values obtained during OGTT; ‡when insulin resistance is considered of nominal variable (0, 1) as a function of cutoff values used for initial stratification criteria of groups A and B, Kruskal-Wallis test gave the value $P < 0.002$; §values for OGTT were available for $n = 16$, $n = 19$, and $n = 10$ for groups I, II, and III, respectively.

and the general population, which may be explained by ethnic variations or different efficiencies in the detection of this mutation (6,7). The effect of this variant on IRS-2 remains unknown, but its location near Tyr1032 and Tyr1061 and the replacement Gly/Asp may predict alterations in IRS-2 function. Finally, these findings, together with the recent integrative role of IRS-2 in transgenic mice (16,17), warrant further investigation on the possible role of IRS genes in the pathogenesis of insulin resistance.

RESEARCH DESIGN AND METHODS

Caucasian women of European extraction were recruited in accordance with the Helsinki Declaration (as revised in 1983) after informal consent. Diagnosis of PCOS was based on prolonged oligomenorrhea and/or amenorrhea and two of the following criteria: *1*) hyperandrogenism (androstenedione and/or testosterone), *2*) increased LH-to-FSH ratio (>2), and *3*) criteria for PCOS in a transvaginal ultrasound scan (3,18). Patients with Cushing's syndrome, nonclassical adrenal 21-hydroxylase deficiency, hyperprolactinemia, androgen-secreting neoplasms, Type A syndrome of severe insulin resistance (fasting insulin >100 μU/ml), and type 2 diabetes (fasting blood glucose >7 mmol/l) were excluded. Before DNA sampling, patients were maintained on a free diet containing 300 g carbohydrates daily and were submitted to a standard (75 g glucose) oral glucose tolerance test (OGTT). No upper limit was considered in the recruitment of obese (BMI >27 kg/m^2) patients, whereas positive family history of diabetes was considered when at least first- or second-degree relatives had type 2 diabetes.

A population (n = 154) was registered over a period of 2 years, and 53 case subjects were consecutively included for the genetic study, based on the absence of medication before DNA sampling. They were recruited as group A (with normal fasting insulin) and group B (with high fasting insulin). The cutoff value was established at 10.8 μU/ml insulin, which represented the mean + 2 SD of insulin in 111 normal individuals (7.8 ± 1.7 μU/ml, mean ± SD). This value was close to the median (11 μU/ml insulin) of the PCOS population and corresponds to a stratification of hyperinsulinemic patients (group B) >50th percentile of fasting insulin (13).

Significant differences were detected between group A and group B concerning the prevalence of acanthosis nigricans (12.0 vs. 46.4%, $P < 0.006$), and values (mean ± SE) for BMI (25.9 ± 1.4 vs. 33.4 ± 1.4, $P < 0.001$), the area under the curve (AUC) for glucose (AUC_{gluc}; 11.1 ± 0.5 vs. 14.1 ± 0.9, $P < 0.003$), 2-h glucose (5.2 ± 0.3 vs. 6.4 ± 0.5, $P < 0.01$), the AUC for insulin (AUC_{ins}; 76.0 ± 11.9 vs. 179.9 ± 24.8, $P < 0.0008$) and 2-h insulin (41.0 ± 0.1 vs. 103.7 ± 15.2, $P < 0.002$, Mann-Whitney U test), respectively. Patients in group B had a higher prevalence of obesity (78.5 vs. 40.0%) and IGT (25 vs. 4.8%) than group A, respectively. Their $HOMA_{IR}$ (4.8 ± 1.0) was 3.4-fold higher than in group A (1.4 ± 0.1) and twofold higher than the value of 2.17 ± 1.5 found in a consecutive series (n = 51) of obese women without PCOS, matched for BMI and age (J.F. Brun, unpublished results). Differences in the degree of insulin resistance between group A and group B were further confirmed by intravenous glucose tolerance test in a limited number of patients (n = 7 in group A and n = 6 in group B). The index for in vivo insulin sensitivity was almost twofold lower in group B than in group A (3.9 ± 1.5 vs. 7.4 ± 2.3 10^{-3} pmol/l · min^{-1} insulin). Because two Type A patients with lean phenotype, acanthosis nigricans, and confirmed mutations in the IR were excluded, the remaining patients with hyperadrogenism, insulin resistance, and acanthosis nigricans in group B were designated as HAIRAN syndrome (19).

Allelic frequencies or prevalence rates of IRS variants were determined in a control population (n = 224) randomly selected from 1,000 individuals representative of the general population and registered by the Nîmes Obstetricians and Hematologist Association (NOHA) in the Languedoc-Roussillon Region in southern France. The control population had a female-to-male ratio of 1.2, age (mean ± SE) 40.8 ± 0.6 years, and BMI 24.3 ± 1.7 and was recruited based on: *1*) the absence of medication for at least 3 months before DNA sampling, *2*) no personal history of diabetes (fasting blood glucose <7 mmol/l, mean 4.7 ± 0.5 mmol/l, n = 224), *3*) no cardiovascular complications (diastolic blood pressure <90 mmHg at rest, mean cholesterol 4.2 ± 0.8, and triglycerides 1.1 ± 0.4 mmol/l), and *4*) no family history (parents) of hypertension or type 1 or type 2 diabetes. Allelic frequencies in the PCOS were compared with this large population of 224 individuals and were also compared with the subpopulation of women (n = 102) who were fertile (at least one child) and who had no episodes of hypertension, intrauterine death, or growth retardation during pregnancy and no personal history of PCOS (i.e., an absence of menstrual dysfunction).

Genotyping. Genomic DNA was obtained from whole blood using a Nucleon BACC 3 DNA isolation kit (Amersham, Buckingham, U.K.). Candidate genes (IR and IRS-1) were investigated by direct sequencing of the entire coding regions under conditions previously described (20). For the IRS-2 gene, fragments were amplified by polymerase chain reaction (PCR) with 22 oligonucleotide pairs. Two pairs of primers corresponded to 751 bp covering 638 bp at 5′ untranslated region (UTR) and the first 112 bp of exon 1 (GenBank AF 073310). We used 19 pairs of primers to amplify fragments from 239 to 371 bp corresponding to the large exon 1 (Genome Data Base accession no. AB000732) and exon 1–intron boundary (AF074850). Another pair of primers was used to amplify a fragment of 268 bp that covered the intron–exon 2 boundary and 3′ UTR (AF 074851). PCR products were purified by Centricon 100 (Amicon; Millipore, Bedford, MA) and then sequenced from both ends on an ABI 373A DNA sequencer using the AmpliTaq FS ABI Prism Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Roissy, France), as previously described (19). High quality chromatograms were obtained in each case and visually inspected, and litigious sequences were repeated by sequencing from both ends. Preliminary experiments indicated an efficiency <85% in the screening of the Gly1057Asp IRS-2 mutation with *Ban*I (261-bp fragment); therefore, all subjects were genotyped by direct sequencing for the IRS-2 variant.

Assays. Plasma glucose was measured with a Beckman analyzer, and hormones were measured by radioimmunoassay. Plasma insulin was measured with a P2796 kit (DiaSorin, Antony, France), which displayed 28% cross-reactivity with proinsulin. FSH and LH were measured with Kryptor kits (CisBio International, Gif-sur-Yvette, France), whereas androstenedione and total testosterone were assayed with kits from Immunotech (Tassigny, Marseille, France).

Data and statistical analysis. AUC_{gluc} and AUC_{ins} were obtained from OGTT values (30, 60, 90, and 120 min) by the trapezoidal method on Microsoft Excel. Numerical variables were expressed as the mean ± SE and analyzed with nonparametrical tests (Mann-Whitney U or Krukal-Wallis), whereas nominal variables were analyzed by χ^2 test. Significant values were considered at $P < 0.05$ (StatView 5.0; Abacus Concepts, Berkley, CA). For the logistic regression analysis, one model was tested to define genetic factors (IRS alleles) compared with other confounding variables in the phenotype that would explain insulin resistance in groups A and B. All variables with $P < 0.2$ were included in the model. A second model (plurimodal) was tested to compare three distinct genotypes (I, II, and III, as indicated in Table 4). One patient with a mutation only on IRS-1 was excluded. A unique variable was introduced for insulin resistance (fasting insulin or $HOMA_{IR}$), and another was introduced for glucose intolerance (2-h glucose or IGT).

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