Brief Genetics Report

A Cluster of Three Single Nucleotide Polymorphisms in the 3′-Untranslated Region of Human Glycoprotein PC-1 Gene Stabilizes PC-1 mRNA and Is Associated With Increased PC-1 Protein Content and Insulin Resistance–Related Abnormalities

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Glycoprotein PC-1 inhibits insulin signaling and, when overexpressed, plays a role in human insulin resistance. Mechanisms of PC-1 overexpression are unknown. We have identified a haplotype in the 3′-untranslated region of the PC-1 gene that may modulate PC-1 expression and confer an increased risk for insulin resistance. Individuals from Sicily, Italy, carrying the "P" haplotype (i.e., a cluster of three single nucleotide polymorphisms: G2897A, G2906C, and C2948T) were at higher risk ($P < 0.01$) for insulin resistance and had higher ($P < 0.05$) levels of plasma glucose and insulin during an oral glucose tolerance test and higher levels of cholesterol, HDL cholesterol, and systolic blood pressure. They also had higher ($P < 0.05$–0.01) PC-1 protein content in both skeletal muscle and cultured skin fibroblasts. In CHO cells transfected with either P or wild-type cDNA, specific PC-1 mRNA half-life was increased for those transfected with P ($t/2 = 3.73 \pm 1.0$ vs. 1.57 ± 0.2 h; $P < 0.01$). In a population of different ethnicity (Gargano, East Coast Italy), patients with type 2 diabetes (the most likely clinical outcome of insulin resistance) had a higher P haplotype frequency than healthy control subjects (7.8 vs. 1.5%, $P < 0.01$), thus replicating the association between the P allele and the insulin resistance–related abnormalities observed among Sicilians. In conclusion, we have identified a possible molecular mechanism for PC-1 overexpression that confers an increased risk for insulin resistance–related abnormalities. *Diabetes* 50:1952–1955, 2001

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AF, allele frequency; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; OGTT, oral glucose tolerance test; PCR, polymerase chain reaction; SBP, systolic blood pressure; SNP, single nucleotide polymorphism; SSC, sodium chloride–sodium citrate; SSCP, single-strand conformation polymorphism; UTR, untranslated region.

The molecular mechanisms causing insulin resistance (a condition that plays a major role in type 2 diabetes) are not completely understood (1–3). Tissue overexpression of glycoprotein PC-1, achieved by inhibiting insulin receptor tyrosine-kinase activity and insulin signaling, may induce insulin resistance (4–8). However, mechanisms of PC-1 overexpression are unknown. We identified a haplotype in the 3′-untranslated region (UTR) of the human PC-1 gene that increases mRNA stability and is associated with PC-1 overexpression and insulin resistance. Screening for sequence variants in the PC-1 gene 3′UTR was performed by single-strand conformation polymorphism (SSCP), cloning, and sequencing. Three very close single nucleotide polymorphisms (SNPs) were identified: G2897A (allele frequencies [AFs]: G = 92.5%, A = 7.5%), G2906C (AF: G = 93.2%, C = 6.8%), and C2948T (AF: C = 92.5%, T = 7.5%). These SNPs combined, as indicated by cloning and sequencing polymerase chain reaction (PCR) products from genomic DNA, in only three different haplotypes: haplotype "A" (2897G, 2906G, and 2948C), "P" (2897A, 2906C, and 2948T), and "N" (2897A, 2906G, and 2948T) with frequencies of 92.5, 6.8, and 0.7%, respectively. All alleles were in Hardy-Weinberg equilibrium. The association between A and P haplotypes with insulin resistance was first tested in 205 healthy nondiabetic Caucasians from Sicily. Insulin resistance was defined as the presence of a cluster of abnormalities typical of the syndrome, i.e., values in the higher quartile of the 205 healthy subjects in three or more of the following parameters: glucose and insulin levels during a oral glucose tolerance test (OGTT) and triglyceride, cholesterol–to–HDL cholesterol, and systolic blood pressure (SBP) levels. Insulin resistance was identified in 43 (21%) subjects. Insulin sensitivity was directly measured by euglycemic clamp in 88 of 205 randomly selected individuals. The insulin-mediated glucose disposal values in subjects with the insulin resistance cluster (4.7 ± 0.3 mg · kg^{-1} ·

TABLE 1
Clinical features of healthy Sicilians (n = 205) according to the presence or absence of the "P" allele

	A	P
n	181	24
Age (years)	36.7 ± 0.9	39.75 ± 2.9
Sex (male/female)	97/84	12/12
BMI (kg/m^2)	24.12 ± 0.2	24.37 ± 0.7
OGTT (mmol/l)		
G 0 min	4.78 ± 0.0	4.90 ± 0.1
G 60 min	5.84 ± 0.1	6.66 ± 0.41*
G 120 min	4.7 ± 0.1	5.0 ± 0.2
I 0 min	44.12 ± 1.5	51.11 ± 4.3
I 60 min	321.6 ± 15.0	390.7 ± 47.4*
I 120 min	203.8 ± 11.5	208.8 ± 32.3
Cholesterol (mmol/l)	5.3 ± 0.1	5.4 ± 0.2
HDL cholesterol (mmol/l)	1.4 ± 0.0	1.2 ± 0.1*
Cholesterol/HDL cholesterol	4.1 ± 0.1	4.9 ± 0.4*
Triglycerides (mmol/l)	1.1 ± 0.0	1.3 ± 0.2
SBP (mmHg)	115.0 ± 0.9	120.4 ± 2.8*
DBP (mmHg)	74.1 ± 0.7	75.7 ± 2.1

Data are means ± SE or n. *P < 0.05 vs. A allele carriers. DBP, diastolic blood pressure.

min^{-1}, n = 18) were lower than those in subjects without the insulin resistance cluster (6.37 ± 0.2 mg · kg^{-1} · min^{-1}, n = 70) (P < 0.01) . In these 88 subjects, P frequency was identical to that observed in the entire cohort of 205 individuals (6.8%). In individuals with the insulin resistance cluster (n = 43), P frequency was 14.3 vs. 4.9% (P < 0.01) in those without the insulin resistance cluster (n = 162). P carriers (n = 24, 4 homozygous) were at higher risk (odds ratio 3.23, 95% C.I. 1.46–7.12) for insulin resistance, had higher levels of plasma glucose and insulin during OGTT and higher cholesterol–to–HDL cholesterol and SBP levels (Table 1). Thus, the PC-1 P haplotype is associated with insulin resistance–related metabolic alterations. No linkage disequilibrium was observed between the P and the K121Q PC-1 polymorphism previously reported to be associated with insulin resistance (9–10): P frequency was reported as 7.2 and 6.9% in KK- and Q-carrying individuals, respectively.

In 54 of the 205 subjects studied, we measured skeletal muscle PC-1 content by enzyme-linked immunosorbent assay (ELISA) (8). P frequency (8.8%) in these 54 individuals was similar to that observed in all 205 subjects (6.8%). Muscle PC-1 content had a wide range of distribution (5–69 ng/mg) and, on average, was significantly higher in the 8 P carriers than in the 46 age- (38.5 ± 5.7 vs. 44.0 ± 2.0 years), sex- (6 men and 2 women vs. 30 men and 13 women) and BMI-matched (23.2 ± 0.9 vs. 25.2 ± 0.6 kg/m^2) A carriers (43.6 ± 5.4 vs. 29.8 ± 2.4, P < 0.05 ng/mg protein) (Fig. 1). In addition, no P carriers among subjects with low PC-1 content (i.e., <20 ng/mg, the cutoff value of the lower tertile of the entire cohort) was observed (Fig. 1). Similar data were obtained in cultured skin fibroblasts from three P and six age- (30.7 ± 9.4 vs. 30.0 ± 4.3 years), sex- (two men and one woman vs. four men and two women), and BMI-matched (22.3 ± 1.9 vs. 22.2 ± 0.6 kg/m^2) A carriers (57.0 ± 1.53 vs. 30.8 ± 5.45 ng/mg protein, P = 0.01). Finally, PC-1 gene expression in skeletal muscle (by competitive PCR) (11) was 2.44- to 3.0-fold higher in two P carriers than in two matched A carriers. Thus, the PC-1 P haplotype is also associated with increased tissue PC-1 protein content, an abnormality that may induce insulin resistance (4–8). The 3′UTR may regulate mRNA stability and eventually gene expression and protein content by several mechanisms, including the modulation of mRNA stability by AU-rich elements (9,10). In fact, the three nucleotide changes we identified are localized between two ATTTTA motifs (at 2,834 and 3,212 bp, respectively). To investigate whether the P haplotype may be responsible for changes in PC-1 mRNA stability, CHO cells were transfected with either P or A cDNA. Specific mRNA half-life was increased (P < 0.01 by two-way analysis of variance [ANOVA] test) for P (t1/2 = 3.73 ± 1.0 h, n = 4) versus A (1.57 ± 0.2, n = 4) (Fig. 2); this indicates that the P haplotype stabilizes PC-1 mRNA and suggests that this may, in turn, cause increased PC-1 protein content in P carriers. Compatible with this possibility is the observation that in a subgroup of subjects we studied, skeletal muscle mRNA level was directly correlated to PC-1 protein content (r = 0.64, P < 0.05, n = 10). Finally, to minimize the possibility of a false positive result caused by population stratification among Sicilians, a population of different ethnicity (Gargano, East Coast Italy) was studied. In 130 nondiabetic individuals, P frequency (1.5%) was too low to allow comparison between P (n = 4) and A (n = 126) carriers. However, compared with that observed in these 130 healthy individuals, P frequency was significantly higher (7.8%, P < 0.01) in 135 age- and sex-matched patients with type 2 diabetes (the most likely clinical outcome of insulin resistance) from the same area. As shown in Table 2, higher levels of BMI, blood pressure, and triglycerides and a lower level of HDL cholesterol confirmed the presence of insulin resistance in patients with type 2 diabetes. Therefore, these data replicate the association between the P haplotype and insulin resistance–related abnormalities observed in Sicilians.

We have recently described a PC-1 amino acid variant Gln121 (Q121) that, compared with the more common

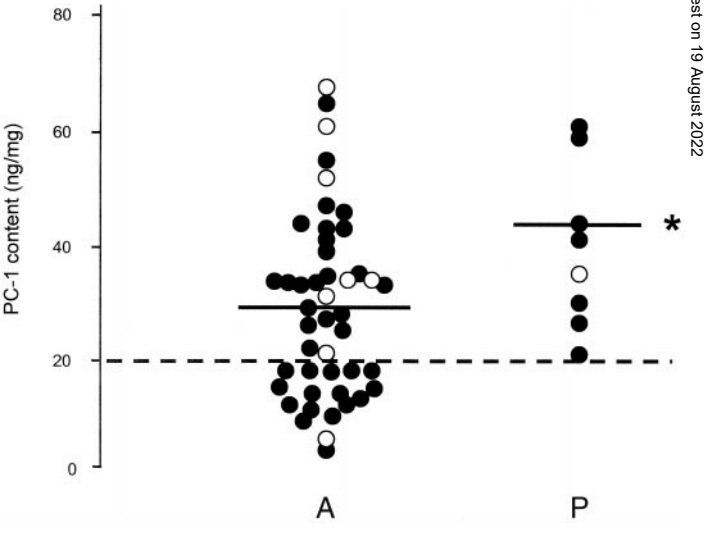

FIG. 1. Individual skeletal muscle (external oblique) PC-1 protein content as measured by ELISA in P and A carriers. ○, subjects carrying the Exon 4 Q121 allele, previously associated with insulin resistance (11–13). ●, Subjects carrying the Exon 4 K121K phenotype. Data were normalized for total protein content. Horizontal bars indicate mean values. *P < 0.05 vs. mean value of A carriers.

TABLE 2
Clinical features of diabetic and healthy individuals from the Gargano area

	Control subjects	Diabetic patients
n	130	135
Age (years)	47.4 ± 0.8	53.2 ± 0.8
Sex (male/female)	56/79	71/64
BMI (kg/m^2)	25.9 ± 0.5	29.8 ± 0.8*
Fasting plasma glucose	5.2 ± 0.1	10.2 ± 0.5*
Cholesterol (mmol/l)	5.5 ± 0.2	5.6 ± 0.2
HDL cholesterol (mmol/l)	1.3 ± 0.0	1.2 ± 0.0*
Triglycerides (mmol/l)	1.3 ± 0.1	1.9 ± 0.1*
SBP (mmHg)	112.5 ± 1.3	142.8 ± 2.6*
DBP (mmHg)	65.4 ± 0.9	82.1 ± 1.1*
P allele frequency (%)	1.5	7.8*

Data are mean ± SE or *n*. *$P < 0.01$ vs. control subjects. DBP, diastolic blood pressure.

variant Lys121 (K121), is a stronger inhibitor of insulin receptor signaling in cultured cells (12) and is associated with in vivo insulin resistance in some (13,14), but not all (15), populations studied. Similar to previous data obtained in the same population (13), among the 205 Sicilians of the present study, those carrying the Q121 allele (n = 57, 3 homozygous) were insulin resistant, as indicated by significantly ($P < 0.05$–0.01) higher levels of both blood glucose and plasma insulin during an OGTT (data not shown). In the population from Gargano, studied to replicate data obtained in Sicilians, Q121 allele frequency was not significantly higher in type 2 diabetic patients than in control subjects (17.5 vs. 14.2%, respectively). These data confirm previous findings (13,14) suggesting that although significantly contributing to insulin resistance, the K121Q PC-1 polymorphism has a minimal, if any, role in type 2 diabetes, a disease that most likely reflects the effect of gene(s) modulating insulin secretion. Finally, in both populations the number of subjects carrying both the Q121 and P haplotypes was too small (i.e., six in Sicily and six [one nondiabetic and five type 2 diabetic subjects] in Gargano) to allow any firm speculation concerning their interaction.

In conclusion, we have identified a cluster of three SNPs in the 3′UTR of PC-1 gene that affects PC-1 mRNA stability, thus conferring an increased risk for insulin resistance. The present report, taken together with our previous data on the Q 121 variant (12–14), indicates that PC-1 gene changes may modulate whole-body insulin sensitivity by different mechanisms and may therefore play a significant role in insulin resistance.

RESEARCH DESIGN AND METHODS

Subjects. A total of 205 nonobese (BMI <30 kg/m^2) normoglycemic (fasting plasma glucose <116 mg/dl) unrelated Caucasian residents in Sicily were studied. Also, 130 normoglycemic individuals and 135 type 2 diabetic patients (age at onset >40 years, no evidence of ketosis, and BMI >21 kg/m^2) from the Gargano area (East Coast Italy) were analyzed to replicate the association study carried out among Sicilians. Euglycemic-hyperinsulinemic clamp was performed as previously described (13). Informed consent was obtained from participants before entry into the study, which was approved by the local research ethics committee.

PC-1 polymorphism screening. The PC-1 gene is ~50 kb long and is organized in 25 exons (GenBank accession nos. AF110280 –AF110304) on human chromosome 6q22–23. All of the splicing sites, except the intron-2 donor, contain canonical consensus acceptor and donor sequences. A TATA box with a TATA-binding protein recognition site is present at −24. The first 100 bp of the upstream region is very GC-reach rich, a characteristic shared with the first portion of the 5′UTR. We have screened the entire 3′UTR, which is ~786 bp long, from 2,786 to 3,486 bp. Apart from the three SNPs characterizing the P haplotype, another three SNPs were identified at position 3,174 (AF 12.6%), 3,242 (12.7%), and 3,484 (12.5%). Although all three of these SNPs were in linkage disequilibrium with the P haplotype (AF = 6.4, 6.3, and 6.6% in A carriers and 47, 48, and 48% in P carriers, for SNP at 3,174, 3,242, and 3,484 bp, respectively), none was associated with variables related to insulin resistance independent of the P haplotype. Exon 4 K121Q polymorphism has been screened by *Ava*II restriction enzyme as previously described (13). All other exons were analyzed in 40 individuals, and negative data were obtained (13). To screen the whole PC-1 3′UTR sequence, two amplification reactions were performed. Primers 3′UTR FW (5′GCCAACCTTTAGCCAAGAAGAC-3′) and 3′UTR RV (5′CCAATAGTAAGAAAGATTCAAATATC-3′) were used to obtain a 5′–386 bp PCR product, and primers 3′UTRII FW (5′TAGCTGTGAACATTGTCTGG3′) and 3′UTRII RV (5′ATAGGGCCACATTTGAAGCC-3′) were used to amplify a partially overlapping 482-bp additional fragment. PCR was performed in standard reaction conditions (13), with an initial denaturation at 94°C for 2 min, followed by 37 cycles each composed of 40 s at 94°C, 40 s at 62°C, and 30 s at 72°C. A final extension of 5 min at 72°C was added. All samples were screened by SSCP performed as previously described (13) on 8% PAGE (19:1 Acrylamide-Bisacrylamide) containing 10% glycerol in Tris boric acid EDTA for 18 h at 10 W and at room temperature. Gels were then silver-stained. This condition was chosen after performing several SSCP analyses aimed at optimizing electrophoretic pattern resolution. Each sample carrying an altered electrophoretic pattern was further characterized by automatic sequencing after cloning (at least five clones) in PCR-TOPO vector (Invitrogen, Carlsbad, CA). To minimize the possibility of false negative results, 20 samples bearing the most frequent pattern were also automatically sequenced, and none of them had sequence variations.

PC-1 content. PC-1 content in external oblique muscle specimens and cultured skin fibroblasts was measured as previously described (8).

PC-1 gene expression. RNA extraction, reverse transcription, and competitive PCR were performed as previously described (11).

Functional studies on mRNA stability

Mutagenesis. All of the experiments were carried out with the plasmid pRK7-PC1 (4) with a 5′ deletion of 1,374 bp in order to distinguish its expression from that of endogenous native PC-1. Mutagenesis of the 3′UTR of human PC-1 was performed using the QuickChange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The following oligonucleotide primers were used complementary to the antisense and sense strand of pRK7-PC1, respectively (the introduced base changes are underlined): 5′CCAGAGTTAGAACAGAGCCCTCCGTGATGCGGACATCTCAGGGAAACTTGCGTACTCAGCACAGTAG-

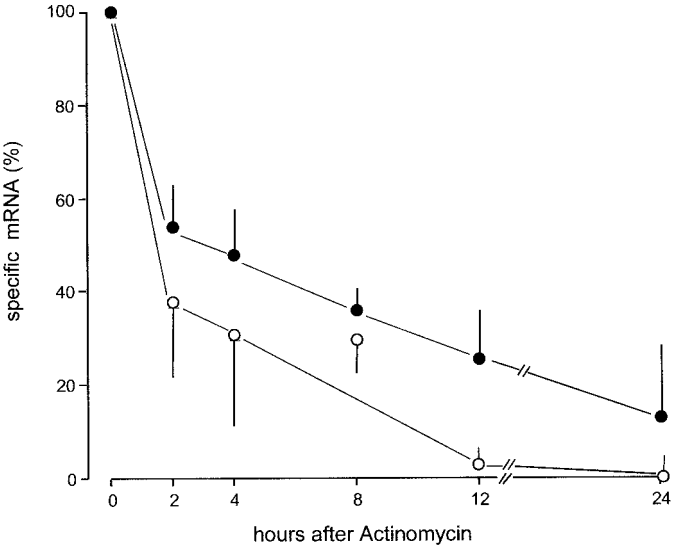

FIG. 2. Specific mRNA content (by Northern blot after transcription inhibition by 5 μg/ml Actinomycin-D) in CHO cells transfected with either P (●) or A (○) cDNA. Data (means ± SE) were normalized for the expression of endogenous G3PDH gene and expressed as percentage of basal values (i.e., in the absence of Actinomycin-D treatment). The two curves are significantly different ($P < 0.01$), as tested by two-way ANOVA.

TGGAGAGTGTTCC-3′ and 5′GGAACACTCTCCACTACTGTGCTGAGTACGC-AAGTTTCCCTGAGATGTCCGCATCACGGAGGGCTCTGTTCTAACTCTGG-3′.

To identify the G2897A and G2906C variations, DNA from different clones were first amplified by PCR, digested with *Nla*IV and *Hph*I, and then sequenced. The C2948T variation was verified by sequencing several clones containing both the G2897A and G2906C variations.

Cell transfection. CHO cells were transfected with either pRK7PC-1UTR-WT or pRK7PC-1UTR-P plasmids using Lipofectamine Plus Reagent Kit (Life Technologies, San Giuliano Milanese, Italy) according to manufacturer's instructions. After 24 h of transfection, cells were rinsed in phosphate-buffered saline at 4°C and treated with 5 μg/ml actinomycin-D (Sigma Chemical, St. Louis, MO), and then samples were collected at different time points.

Northern blot analysis. Total cellular RNA was prepared using Trizol Ls reagent (Life Technologies). RNA (10–15 μg) was fractionated in 1% agarose gel containing 2.2 mol/l formaldehyde and transferred to Hybond-N nylon membrane (Amersham, Arlington Heights, IL). Filters were washed at 65°C for 15 min with 0.1× sodium chloride–sodium citrate (SSC) and 0.5% SDS and prehybridized for 4 h at 42°C in 50% formamide, 5× SSC, 5× Denhardt's, 50 mmol/l sodium phosphate buffer, 0.1% SDS, and 500 μg/ml salmon sperm DNA. Probes, PCR fragments encompassing parts of the exon 24 and 3′UTR of the human PC-1 cDNA clone, and PCR-amplified fragments of the G3PDH gene were ^{32}P-labeled using a random-primed reaction kit (Boehringer, Mannheim, Germany). Hybridization was carried out overnight at 42°C in 50% formamide, 20× SSC, 1× Denhardt's, 20 mmol/l sodium phosphate buffer, 20% dextran sulfate, 0.1% SDS, and 100 μg/ml salmon sperm DNA containing the denatured probe. Filters were then washed twice for 15 min at room temperature with 2× SSC and 0.1% SDS, once at 65°C for 30 min with 0.1× SSC and 0.1% SDS, and then exposed to Kodak X-Omat films. Membranes were then stripped for 1 h at 85°C with 20 mmol/l Tris and 0.1% SDS and reprobed with labeled G3PDH. Scanning densitometry was carried out by Snap Scan 1,212 Agfa.

Statistical analysis. Values are given as means ± SE. Mean values were compared by Student's *t* test or Mann Whitney *U* test, as appropriate. Allele frequencies were compared by χ^2 test. Two-way ANOVA was used to compare the time-course of mRNA half-life.

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