Original Article

New Polymorphism of *ENPP1* (*PC-1*) Is Associated With Increased Risk of Type 2 Diabetes Among Obese Individuals

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The K121Q polymorphism in ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*) is associated with type 2 diabetes and obesity. The possibility of other *ENPP1* polymorphisms influencing these phenotypes has received little attention. Our aim was to examine the associations of tagging single nucleotide polymorphisms (SNPs) and haplotypes of the linkage disequilibrium (LD) block containing K121Q polymorphism with type 2 diabetes in a Polish population, controlling for any effect of obesity. We genotyped 426 type 2 diabetic case and 370 control subjects for seven SNPs in *ENPP1*. In the total group, neither type 2 diabetes nor obesity was significantly associated with any SNP. However, in obese subjects, two SNPs were significantly associated with type 2 diabetes: the Q allele of K121Q (odds ratio 1.6 [95% CI 1.003–2.6]) and T allele of rs997509 (4.7 [1.6–13.9]). In the LD block, four SNPs plus the K121Q polymorphism distinguished six haplotypes, three of which carried the Q allele. Interestingly, the T allele of rs997509 sufficed to distinguish a 121Q-carrying haplotype that was significantly more associated with type 2 diabetes than the other two (4.2 [1.3–13.5]). These other two 121Q-carrying haplotypes were not associated with type 2 diabetes. In conclusion, we found a new SNP, rs997509, in intron 1 that is strongly associated with risk of type 2 diabetes in obese individuals. The molecular mechanisms underlying this association are unknown. *Diabetes* 55:2626–2630, 2006

The ectonucleotide pyrophosphatase/phosphodiesterase 1 gene (*ENPP1* or *PC-1*) is located on the long arm of chromosome 6 (6q23.2) and encodes for a protein that inhibits insulin signaling (1). A polymorphism in exon 4 (K121Q) of the gene has been examined for association with features of insulin resistance, type 2 diabetes, and obesity with inconsistent results. The 121Q allele was associated with insulin resistance in nondiabetic subjects in many (2–8) but not in all (2,3,9,10) populations. Similarly, it was associated with type 2 diabetes in South Asians living in India and in the U.S., as well as in Caucasians living in the U.S. (2,3) and Finland (6) but not Caucasians in Sweden (4) or Denmark (10). Although a recent study (11) found no association of obesity with the 121Q allele, Meyre et al. (12) recently reported that a particular haplotype, defined by 121Q and alleles at two other single nucleotide polymorphisms (SNPs) (a T deletion at IVS20 delT-11 and G allele at A→G+1044TGA/rs7754561), was associated with obesity as well as increased risk of type 2 diabetes. However, the role of obesity is unclear from this study. Does the 121Q allele increase the risk of type 2 diabetes directly or through its effect on obesity?

Several factors may explain the discrepant effects of the 121Q allele on metabolic traits in different populations. Its effect may be modified by environmental factors or functional polymorphisms in other genes (13), either of which may vary across populations. Unknown polymorphisms within *ENPP1* that influence these metabolic traits may be in linkage disequilibrium (LD) with the 121Q allele or may interact with it.

Our aim was to study the association of type 2 diabetes with *ENPP1* in Polish Caucasians by examining the relation of these traits with genomic variation in the block of strong LD that contains the K121Q polymorphism. For this purpose, we genotyped seven markers: the K121Q polymorphism itself, four other SNPs that tag the haplotypes of the LD block containing K121Q, and the two SNPs outside the LD block that were reported by Meyre et al. (12) to define a specific risk haplotype that accounts for the 121Q effect. Furthermore, to distinguish *ENPP1*'s effect on the risk of type 2 diabetes from its effect on obesity, we compared the genotypes of patients with and without type 2 diabetes separately in nonobese and obese individuals.

Case and control subjects were divided according to percent of ideal body weight (IBW) at the median (IBW = 138%) for the combined study groups. Clinical characteristics of patients are summarized according to study group and obesity status in Table 1. Within each obesity stratum, percent IBW was similar in control and case subjects, and age at examination for control subjects was similar to age at diagnosis of diabetes for case subjects. Diabetes duration in case subjects was on average 9.3 ± 7.5 (means ± SD) years, and 52% were being treated with insulin. As expected, a family history of diabetes was frequent in case subjects, and fasting blood glucose was higher in case than control subjects.

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IBW, ideal body weight; LD, linkage disequilibrium; SNP, single nucleotide polymorphism.

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TABLE 1
Clinical characteristics of the study groups according to obesity status*

	Low IBW		High IBW	
	Control subjects	Case subjects	Control subjects	Case subjects
n (men/women)	207 (111/96)	191 (125/66)†	163 (32/131)	235 (78/157)†
IBW (%)	117 ± 14	121 ± 11†	175 ± 33	166 ± 25†
Age at examination (years)	52 ± 15.3	61 ± 9.1†	47 ± 14.5	57 ± 9.7†
Age at diagnosis (cases) (years)		51 ± 9.2		47.7 ± 9.5
Duration of diabetes		9.6 ± 7.1		9.1 ± 7.9
Treatment				
Diet/none		12 (6.3)		13 (5.5)
Oral only		80 (42)		100 (42.6)
Oral/Insulin		99 (52)		122 (52)
Fasting glucose (mmol/l)	4.6 ± 0.75	7.7 ± 3.1†	5 ± 0.68	7.9 ± 3†
HbA_{1c}		7.8 ± 1.6		7.9 ± 1.9
Family history of type 2 diabetes	44 (21)	101 (53)†	43 (26)	111 (47)†

Data are means ± SD or *n* (%), unless otherwise indicated. *Individuals with IBW ≤138% (median) were considered as nonobese and those with IBW >138% were considered obese. *P* values were determined with *t* test for continuous variables and $\chi 2$ for discrete variables. †$P <$ 0.05 for comparison of case and control subjects within each IBW stratum.

Using HapMap data (release no. 16c.1/phase I, June 2005), we defined the haploblock structure of *ENPP1* in the CEPH sample of Utah residents with European ancestry (CEU group). Defining a solid spine of LD as $D' > 0.90$, we identified five haploblocks in the *ENPP1* locus (Fig. 1). The first block is 23 kb long, starts 4.3 kb upstream of the transcription start site, and covers exon 1 and nearly half of first intron. Blocks 2 and 3 are 4 and 2 kb, respectively, and they are located within intron 1. Skipping to the last or fifth block, it is 3 kb long and covers intron 24 and exon 25. The fourth haploblock includes the K121Q polymorphism and eight other SNPs. It covers exons 2–19 and spans 35 kb. The average interval between SNPs in this block is 3.9 kb, with the longest being 8.3 kb. Six haplotypes with frequencies >1% account for 100% of the haplotype diversity in this block. Five SNPs were sufficient to tag these haplotypes (Fig. 1), and these were genotyped in our study groups: rs9375831, rs997509, rs1044498 (K121Q), rs9402349, and rs7769712. We also genotyped the two SNPs (IVS20 delT-11 and A→G+1044TGA/rs7754561) reported by Meyre et al. (12) to define together with the K121Q polymorphism a risk haplotype for obesity and type 2 diabetes. These SNPs are outside of the block of strong LD encompassing the K121Q polymorphism (D' with K121Q was 0.45 for IVS20delT-11 and 0.3 for rs7754561).

Of the total group of 856 individuals, genotypes for all seven polymorphisms were obtained for 796 (94% of case and 92% of control subjects), and all were in Hardy-

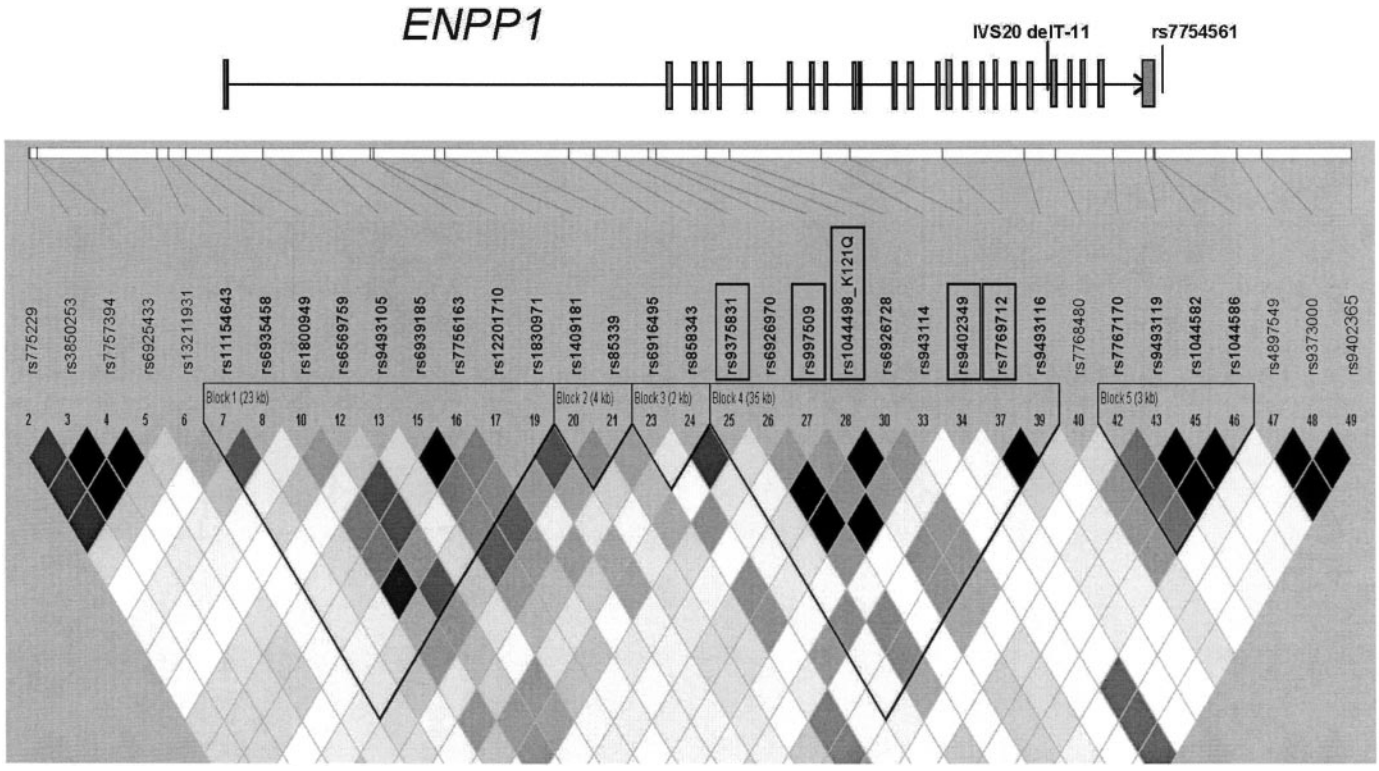

FIG. 1. Modified output of Haploview program showing haplotype blocks ($D' > 0.9$) in the *ENPP1* gene estimated on 90 CEU individuals. Tagging SNPs of the block encompassing K121Q are boxed. Shades of squares represent r^2 values for pairs of SNPs. ■, $r^2 = 1$; ▨, $0 < r^2 < 1$; □, $r^2 = 0$.

TABLE 2
Risk of diabetes according to *ENPP1* genotypes and obesity

SNP	Obese (IBW >138%)	Subjects	Genotypes [*n* (%)] 11	12	22	OR (95% CI) for diabetes in carriers of minor allele	OR (95% CI) for diabetes in carriers of minor allele after adjustment for relevant covariates	Breslow-Day test for interaction (*P* value)
rs9375831			AA	AT	TT			
	No	Case	12 (6.2)	79 (41.4)	100 (52.4)	1.5 (1.03–2.3)	2 (1.3–3.2)	0.017
		Control	11 (5.3)	66 (31.9)	130 (62.8)			
	Yes	Case	9 (3.8)	84 (35.8)	142 (60.4)	0.77 (0.5–1.1)	0.71 (0.46–1.1)	
		Control	13 (8.0)	62 (38.0)	88 (54)			
rs997509			TT	CT	CC			
	No	Case	0	9 (4.7)	182 (95.3)	0.68 (0.3–1.6)	0.48 (0.7–1.3)	0.0039
		Control	0	14 (6.8)	193 (93.2)			
	Yes	Case	0	25 (10.6)	210 (89.4)	4.7 (1.6–13.9)	5.1 (1.7–15.7)	
		Control	0	4 (2.4)	159 (97.6)			
pc1_KQ			QQ	QK	KK			
rs1044498	No	Case	1 (0.5)	32 (16.8)	158 (82.7)	0.6 (0.37–0.99)	0.58 (0.33–1)	0.0047
		Control	5 (2.4)	48 (23.2)	154 (74.4)			
	Yes	Case	6 (2.6)	59 (25.1)	170 (72.3)	1.6 (1.003–2.6)	1.7 (1.004–2.8)	
		Control	2 (1.2)	29 (17.8)	132 (81.0)			
rs9402349			CC	CA	AA			
	No	Case	2 (1.1)	40 (20.9)	149 (78.0)	0.96 (0.6–1.5)	1 (0.6–1.7)	0.6
		Control	6 (2.9)	41 (19.8)	160 (77.3)			
	Yes	Case	2 (0.9)	44 (18.7)	189 (80.4)	0.8 (0.5–1.3)	0.9 (0.5–1.4)	
		Control	4 (2.45)	34 (20.86)	125 (76.69)			
rs7769712			CC	CA	AA			
	No	Case	1 (0.5)	23 (12.0)	167 (87.5)	0.79 (0.4–1.4)	0.86 (0.45–1.7)	0.2
		Control	0	32 (15.46)	175 (84.54)			
	Yes	Case	4 (1.7)	40 (17.0)	191 (81.3)	1.3 (0.8–2.3)	1.4 (0.8–2.6)	
		Control	1 (0.6)	23 (14.1)	139 (85.3)			
IVS20delT			DD	DI	II			
	No	Case	8 (4.2)	51 (26.7)	132 (69.1)	0.79 (0.5–1.2)	0.9 (0.6–1.5)	0.78
		Control	6 (2.9)	69 (33.3)	132 (63.8)			
	Yes	Case	8 (3.4)	77 (32.8)	150 (63.8)	0.85 (0.6–1.3)	0.9 (0.6–1.4)	
		Control	11 (6.8)	54 (33.1)	98 (60.1)			
rs7754561			GG	GA	AA			
	No	Case	12 (6.3)	50 (26.2)	129 (67.5)	1.4 (0.9–2.1)	1.3 (0.8–2.1)	0.1
		Control	9 (4.35)	75 (36.23)	123 (59.42)			
	Yes	Case	12 (5.1)	77 (32.8)	146 (62.1)	0.86 (0.6–1.3)	0.9 (0.6–1.4)	
		Control	7 (4.3)	49 (30.1)	107 (65.6)			

In the obese group (IBW >138%), there were 163 control and 235 type 2 diabetic case subjects. In the nonobese group (IBW ≤138%), there were 207 control and 191 type 2 diabetic case subjects. ORs and *P* values are for comparisons of frequency of carriers of the minor allele in type 2 diabetic case and control subjects within IBW strata. Adjusted analysis was performed with logistic regression within strata of IBW with the following covariates: sex, family history of type 2 diabetes, age, and IBW.

Weinberg equilibrium (*P* values = 0.3–1.0). In the total group, neither type 2 diabetes nor obesity was significantly associated with the genotypes of any of the polymorphisms, but when the group was stratified by the median IBW, one SNP was associated with type 2 diabetes in the stratum below the median and two were associated with type 2 diabetes in the stratum above the median (Table 2).

In nonobese individuals (IBW ≤138%), the risk of type 2 diabetes for carriers of the minor allele A of SNP rs9375831 was higher than that for noncarriers (odds ratio [OR] 1.5) but not significant after adjustment for multiple comparisons). In obese individuals (IBW >138%), the direction of the difference for this SNP was reversed but the association was not significant (OR 0.77 [95% CI 0.5–1.1]). However, the difference between these ORs was statistically significant ($P < 0.02$, Breslow Day test). In obese individuals, the minor alleles of rs997509 and rs1044498 (K121Q) were significantly associated with type 2 diabetes. For SNP rs997509, carriers of the minor allele T were more frequent in type 2 diabetic case than control subjects (10.6 and 2.4%, respectively; 4.7 [1.6–13.9]). Similarly for SNP rs1044498 (K121Q), carriers of the 121Q allele were more frequent in type 2 diabetic case than control subjects (27.7 and 19%, respectively; 1.6 [1.003–2.6]). Among nonobese individuals, the associations were reversed. Carriers of the T allele of rs997509 were less frequent in type 2 diabetic case than control subjects (4.7 and 6.8%, respectively; 0.7 [0.3–1.6]), as were carriers of

TABLE 3
Haplotype distribution in obese subjects (IBW >138%) according to diabetes status (163 control and 235 type 2 diabetic case subjects)

Haplotype	rs9375831	rs997509	K121Q	rs9402349	rs7769712	Score	Control proportion	Case proportion	Empirical *P* value
1	A	C	K	C	A	−1.19	0.125	0.096	0.23
2	A	C	K	A	A	−1.14	0.138	0.114	0.25
3	T	C	K	A	A	−0.17	0.630	0.622	0.87
4	T	C	Q	A	A	−0.65	0.016	0.010	0.51
5	T	C	Q	A	C	0.85	0.071	0.087	0.4
6	T	T	Q	A	A	2.89	0.011	0.046	0.004

Global statistics score = 14.5, df = 6, $P = 0.024$.

the 121Q allele (17.3 and 25.6%, respectively; 0.6 [0.37–0.99]). The differences between nonobese and obese individuals and the ORs for type 2 diabetes for carriers of the minor alleles of each SNP were significantly different ($P < 0.004$ and $P < 0.005$, respectively, Breslow-Day test).

In logistic analyses, adjustment for covariates such as sex, age, family history of diabetes, and percent of IBW did not change the unadjusted ORs (Table 2). The frequency distribution of the six haplotypes of the haploblock encompassing the K121Q polymorphism was significantly different in the obese stratum (Table 3, $P = 0.024$ for global statistic). Three haplotypes carried the K allele and three carried the Q allele of the K121Q polymorphism. Only one of the Q allele haplotypes, TTQAA, was more frequent in case than control subjects (4.6 and 1.1%, respectively; $P = 0.004$). Differences between case and control subjects for the other five haplotypes were not significant.

The T allele of the rs997509 SNP suffices to identify the risk haplotype T*T*QAA. In fact, the distribution of the risk haplotype reflected the distribution of the T allele among obese type 2 diabetic case and control subjects (5.3 vs. 1.2%, $P = 0.002$). Moreover, confining the comparison just to obese individuals carrying the 121Q allele, those who were also carriers of the T allele of rs977509 were significantly more associated with type 2 diabetes than those carrying the C allele (OR 4.2 [95% CI 1.3–13.5]). When carriers of the T allele of rs997509 SNP were excluded from the analysis, the frequency of the remaining 121Q carriers was not different in type 2 diabetic case and control subjects (1.2 [0.7–2.8]).

We also examined the haplotype described by Meyre et al. (12) for association with type 2 diabetes. The haplotype (QdelTG) formed by the 121Q allele and two other SNPs of *ENPP1* (IVS20 delT-11 and rs7754561) was more frequent in obese type 2 diabetic case than obese control subjects, but the difference was not statistically significant (7.6 and 4.5%, respectively; $P = 0.07$). In nonobese individuals, the frequency of this haplotype in type 2 diabetic case and control subjects was almost identical (4.4 and 4.9%, respectively; $P = 0.5$).

DISCUSSION

We examined the association of type 2 diabetes and obesity with DNA sequence differences in the 35-kb LD block that includes the K121Q polymorphism of *ENPP1*, conditional on obesity status. Situating the study in the Caucasian population in southeastern Poland reduced the risk of false-positive associations due to population stratification. Also selecting control subjects from among the spouses of case subjects minimized confounding due to environmental or lifestyle factors.

In this study, two *ENPP1* polymorphisms were associated with type 2 diabetes in the presence of obesity. Specifically, in addition to the 121Q allele, our study found a new SNP, rs997509, that is a marker of the functional polymorphism within *ENPP1* that contributes to the development of type 2 diabetes among obese individuals. The minor T allele of this SNP distinguishes one of the three 121Q-carrying haplotypes, which is strongly associated with type 2 diabetes among obese subjects and accounts for the association seen between the 121Q and type 2 diabetes among obese individuals. Association of the T allele of rs997509 with type 2 diabetes has not been previously reported.

Our finding regarding the effect of T allele of rs997509 challenges the previous understanding of the contribution of genetics variation of *ENPP1* to type 2 diabetes. The K121Q polymorphism has been the obvious candidate for being the functional variant that underlies the association of the *ENPP1* locus with metabolic traits. At least in people of European origin, it is the only frequent variant within the gene that results in an amino acid change (8,14,15). In functional studies, the 121Q allele variant binds more strongly to the insulin receptor and inhibits its protein kinase activity more effectively than the K variant (1). In our study, only one of three haplotypes carrying the 121Q allele was associated with type 2 diabetes, and this haplotype is uniquely identified by the T allele of the rs997509 SNP. It is unknown whether the subgroup of 121Q alleles carrying the T allele of rs997509 accounts for the functional characteristics attributed to 121Q or the two polymorphisms have different effects.

One possibility is that the T allele of rs997509 is functional and fully responsible for the effect of the risk haplotype, TTQAA, while the 121Q variant is just a silent marker of it. The rs997509 SNP is located in the 3′ end of intron 1 in a region that may contain a regulatory element, as suggested by the Five Way Regulatory track of the University of California Santa Cruz genome browser (16). However, another polymorphism, rs9493114, which is in complete LD with rs997509 ($r^2 = 1$), is located in intron 8. At this point, it is not clear how many other sequence differences are present and possibly functional on the haplotype tagged by the T allele of rs997509. This will require extensive sequencing of the haploblock encompassing the K121Q polymorphism. The function of these two known SNPs, and possibly additional DNA sequence differences identified through deep sequencing, should be studied further at the population level as well as in vitro.

Among the limitations of this study is the possibility that some of the findings are due to population stratification. However, the risk of this was minimized by organizing the study in a relatively homogeneous population within Poland. A second limitation is the multiple comparison

problem resulting from the number of SNPs genotyped and the stratified analysis. Indeed, after a conservative Bonferroni adjustment for 14 multiple comparisons (seven SNPs and two obesity strata), most of the single marker associations became not significant. However the strongest association (rs997509, uncorrected $P = 0.002$) was still significant after a Bonferroni adjustment.

RESEARCH DESIGN AND METHODS

Study participants were Caucasian residents of southeastern Poland. Type 2 diabetic case subjects were recruited from among patients attending the Clinic of Metabolic Diseases at the Medical College of the Jagiellonian University in Krakow, Poland. Type 2 diabetes was diagnosed according to World Health Orgainization definition (17), as previously described in detail (18,19). Only patients with type 2 diabetes diagnosed after age 35 years who required no insulin therapy for at least 2 years after diagnosis were recruited. The control group consisted of individuals with normal fasting glucose, mainly the spouses of type 2 diabetic patients. During the period 2002–2004, 856 individuals, not known to be related, were enrolled into this study: 453 type 2 diabetic patients and 403 nondiabetic control subjects. Subjects underwent a standardized physical examination, providing a medical history and a blood sample for biochemical measurements and DNA extraction. Study protocols were in accordance with the Helsinki Declaration, and they were approved by the ethical committee of the Medical College of the Jagiellonian University in Krakow. All participants signed the informed consent forms at entry into the study.

For estimation of the haploblocks within the *ENPP1* locus, HapMap data (release no. 16c.1/phase I, June 2005) were used. Since the K121Q polymorphism was not genotyped for that release, we genotyped this polymorphism in the HapMap CEU sample.

SNPs rs997509 and K121Q were genotyped by restriction fragment–length polymorphism using enzymes BsrB I and *Ava*II, respectively. Other SNPs were genotyped by the AcycloPrime-FP SNP Detection System Wallace Victor (2) Multilabel Plate Reader (Perkin Elmer). A total of 10% of the samples were regenotyped for quality control, and there was 100% concordance of genotypes. Data were analyzed with SAS software (SAS version 8.02). χ^2 and Student's t test (two sided) were used for comparison of categorical and continuous variables, respectively. Analysis stratified by median of IBW (138%) was performed. ORs were estimated and interactions were tested using Breslow-Day test. Logistic regression was used to adjust for differences between case and control subjects, within strata of IBW in the distributions of sex, age, family history of diabetes, and IBW.

HaploView software (20) was used to calculate Hardy-Weinberg equilibrium and LD, identify haplotype blocks, and estimate the frequency of the haplotypes. Haploblocks were defined as solid spines of LD ($D' > 0.9$). To identify haplotype-tagging SNPs, *tagSNP* software was used (21). K121Q was forced as htSNP and other SNPs were selected by software. Difference in haplotype distributions between study groups was tested with R package haplo.score (22).

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