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Putative association between a new polymorphism in exon 3 (Arg109Cys) of the pancreatic colipase gene and type 2 diabetes mellitus in two independent Caucasian study populations

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The protein encoded by the pancreatic colipase (CLPS) gene is an essential cofactor needed by pancreatic triglyceride lipase (PNLIP) for efficient dietary lipid hydrolysis. Since the inhibition of lipase activity was shown to reduce the incidence of type 2 diabetes mellitus, we tested the hypothesis that genetic variations in the CLPS and PNLIP genes are associated with type 2 diabetes; 47 unrelated subjects were screened for polymorphisms of the CLPS and PNLIP genes. A nested-case control study of 192 incident type 2 diabetes subjects and 384 sex- and age-matched controls taken from the European Prospective Investigation into Cancer and Nutrition Potsdam Cohort (EPIC) was employed for association studies. The Metabolic Intervention Cohort Kiel (MICK) consisting of 716 males was used for verification. A novel putative functional polymorphism (Arg109Cys) was identified in the CLPS gene. The frequencies of the Arg/Cys genotype were 2.6% in EPIC and 2.2% in MICK study subjects. No homozygotes for the Cys/Cys genotype were found in either study population. Logistic regression analysis showed a statistically significant association of the Arg/Cys genotype with an increased risk of type 2 diabetes. The odds ratios estimated by the model were 3.75 (95%CI = 1.13 – 12.49, $p = 0.03$) in EPIC and 4.86 (95%CI = 1.13 – 20.95, $p = 0.03$) in MICK. No comparable associations were found with other traits of the insulin-resistance syndrome (*e.g.*; body mass index, waist to hip ratio). In conclusion, we obtained evidence in two German Caucasian study populations that the variant of the rare CLPS Arg109Cys polymorphism might contribute to increased susceptibility of type 2 diabetes.

Keywords: Pancreatic colipase / Polymorphism / Type 2 diabetes mellitus

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1 Introduction

The pancreatic colipase (CLPS) is an essential protein for efficient dietary lipid hydrolysis, since the pancreatic triglyceride lipase (PNLIP) requires colipase for explicating activity. Various constituents in meals and in bile, particularly bile acids, inhibit the PNLIP. CLPS restores activity to lipase in the presence of inhibitory substances like bile acids. Presumably, colipase functions by anchoring and orienting PNLIP at the oil-water interface. The x-ray structure of the CLPS-PNLIP complex supports this model [1]. Both genes are expressed only in the pancreatic acinar cells [2, 3] and then secreted into the small intestine. The CLPS is secreted as proenzyme, pro-colipase, and then activated by cleavage of a pentapeptide from the N-terminal domain (enterostatin) in the duodenum [4]. The expression of both PNLIP and CLPS is connected to the amount of dietary lipid intake [5, 6].

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Enhanced postprandial triglyceride levels were found in relatives of type 2 diabetics and were associated with a reduced insulin sensitivity, whereas the inhibition of the lipase activity results in a reduced body weight and higher insulin sensitivity and reduced incidence of type 2 diabetes [7 – 9]. In an experimental diabetic animal model, the PNLIP activity correlated with the development of diabetes [10]. Therefore, alterations in the function and/or expression of the CLPS and/or PNLIP could be responsible, in part, for individual variation in lipid metabolism or other risk factors contributing to type 2 diabetes mellitus. So far, only few sequence variations have been described. Screening of cell lines of subjects with clinical deficiency of PNLIP (OMIM 246600) failed to identify any disease-causing mutations [11]. The only population study examining sequence variations of the PNLIP gene showed no association between PNLIP polymorphisms and metabolic parameters of the syndrome [11]. In an effort to identify polymorphisms in the coding and/or regulatory regions of these candidate genes for type 2 diabetes, we sequenced the exons, putative splice sites and promoters of the CLPS and PNLIP genes. Among the identified polymorphisms, the putative functional polymorphism CLPS Arg109Cys was further investigated in two independent population samples to find associations with diabetes type 2 and traits of the insulin-resistance syndrome.

2 Materials and methods

2.1 European Investigation into Cancer and Nutrition Cohort

Nested case-control study subjects were taken from the European Prospective Investigation into Cancer and Nutrition Cohort (EPIC) Potsdam study. This population-based, prospective study comprises 27548 people from the area around Potsdam, Germany. Baseline examination was conducted between 1994 and 1998, and included anthropometric and blood pressure (BP) measurements, blood sampling, a self-administered food-frequency questionnaire, and a personal interview on lifestyle habits and medical history [12, 13]. During the first follow-up, on average 2.3 years after recruitment, 192 incident cases of type 2 DM were identified and confirmed by the primary care physician [13]. Cases were matched with two control subjects each by age and sex (n = 384). Gender distribution of the nested case-control study was 59% male and 41% female subjects with a mean age of 55.5 years (35 – 65 years). All study participants had given informed consent and the genotype assessment was agreed to by the local ethics committee. Hypertension was defined as baseline mean systolic reading of greater than 140 mmHg or a mean diastolic reading of greater than 90 mmHg, or known established hypertension requiring medical treatment. Information on drug use was obtained at baseline and comprised details of all medications taken during the previous 4 weeks. Total cholesterol and high-density lipoprotein (HDL) cholesterol (HDL-C) were measured with enzymatic colorimetric methods (Boehringer Mannheim, Mannheim, Germany).

2.2 Metabolic Intervention Cohort Kiel

For this study, 716 male subjects aged 45 – 65 years were recruited from the residents register of the town of Kiel, Germany (Metabolic Intervention Cohort Kiel, MICK). The recruitment period lasted from January 2003 to March 2004. Exclusion criteria were: known diabetes type 1 or 2, diseases with impairment of nutrient digestion or metabolism, intake of lipid-lowering drugs or hormones, operation on the intestine in the past 3 months, hypo- or hyperthyroidism, chronic renal disease, hepatitis, cholestasis, alcoholism or cancer. BP, body weight, height and waist and hip circumference were determined at recruitment by means of standardized procedures. Unknown type 2 diabetes mellitus was diagnosed according to a standardized oral glucose tolerance test [14]. Among this cohort, 182 men were diagnosed with disturbed glucose tolerance: fasting glucose >10 mg/dl determined on two different occasions or postprandial glucose >140 mg/dl; of these 49 had type 2 diabetes mellitus (fasting glucose >126 mg/dl determined on two different occasions or postprandial glucose >200 mg/dl; 523 had normal glucose tolerance and in 11 subjects diagnosis was ambiguous. The latter were excluded from analysis. Another 20 study participants had to be excluded because DNA genotyping failed. Serum and plasma was separated from whole blood by centrifugation and stored at –70°C for later determinations. Serum cholesterol, serum HDL-C and plasma glucose were determined using enzymatic methods with Kone Lab 20i analyzer (Kone, Finland). All samples were measured in duplicate. All study participants had given informed consent and the genotype assessment was approved by the local ethics committee.

2.3 Genetic analyses

DNA was isolated from buffy coat (100 µl) using E.Z.N.A.® Blood DNA MiniKits (Peqlab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's instructions. Sixteen exons including the untranslated (UTR) regions and the flanking splice sites were amplified using intronic primers and the putative promoter regions were analyzed using DNA of 47 unrelated subjects. All PCR and sequencing primers were designed using the OLIGO software and primers were purchased from MWG Biotech AG (Ebersberg, Germany). PCR was then carried out in a volume of 25 µl containing 15 ng DNA, 5 pmol of each primer, 0.05 µMol $MgCl_2$, 0.5 U Taq polymerase, 0.125 mmol of each dNTP, 2.5 µl PCR buffer and sterile

water to a the final volume of 25 µl (Taq polymerase, PCR buffer and $MgCl_2$ were from Invitrogen, Karlsruhe, Germany; dNTPs from Fermentas GmbH, St. Leon-Rot, Germany). PCR conditions were as follows: initial denaturing at 94°C for 5 min, 3 cycles of denaturing at 94°C for 20 s, annealing at varying temperatures for 20 s and extension at 72°C for 30 s, followed by another 3 cycles with the annealing temperature lowered by 2°C, followed by 37 cycles with the initial annealing temperature lowered by 4°C and a final extension step at 72°C for 10 min. Sequencing was performed by terminator cycle sequencing using Big Dye chemistry and ABI 3700 capillary DNA sequencer (Applied Biosystems, Foster City, CA, USA). Genotyping of the EPIC and MICK subjects was performed with the TaqMan system (ABI, Foster City, CA, USA), fluorescence was measured with the ABI Prism 7900 HT sequence detection system. Sequences of PCR primers and TaqMan assay primers and probes are available on request.

2.4 Statistical analyses

Allele and genotype frequencies were determined by gene counting. The study populations were tested as a whole and cases/controls individually for the distribution of genotypes according to the Hardy-Weinberg-equilibrium with a χ^2 (one degree of freedom) test. Statistics were computed with SAS software 9.1 (SAS Institute, Cary, NC, USA). In both study groups, some data on genotyping, biochemical, BP and anthropometric measurements were missing and, therefore, analysis of covariance (ANOVA) was performed in a final EPIC study sample of 184 cases and 380 controls and in 680 subjects from MICK. HDL-C and total cholesterol (TC) were logarithmically transformed prior to analysis. Mean values were adjusted for covariates as indicated in the legends. Odds ratios (OR) and corresponding 95% confidence intervals (CI) were determined by conditional logistic regression analysis in the EPIC study. In MICK, unconditional logistic regression analyses were employed. Multivariate logistic regression analysis was conducted adjusting for age, sex, body mass index (kg/m^2) (BMI), waist-hip-ratio (WHR) and the presence of hypertension.

3 Results

By sequencing all exons, putative splice sites and the promoter regions of the PNLIP and CLPS genes, the locations and allele frequencies of polymorphic sites were identified (Tab. 1). None of the single nucleotide polymorphisms (SNPs) found in the PNLIP gene seems to implicate functional consequences. Two PNLIP SNPs were located in intron 11, and one synonymous SNP was found in exon 5. In the CLPS gene, two insertion/deletion polymorphisms in the promoter region, one SNP in the 5′-UTR and one SNP in exon 3 (Arg109Cys) were identified. Since the latter polymorphism may have functional consequences, we choose this novel coding polymorphism for association studies. We analyzed the other SNPs using TRANSFAC® Professional (http://www.biobase.de/pages/products/transfac.html) and PupaSNPs (http://pupasnp.bioinfo.ochoa.fib.es). With this *in silico* search, these non-coding SNPs were not located within intron boundaries or putative binding sites of the promoter/5′-UTR. Therefore, these SNPs seem to be not functional. Haplotype data for the PNLIP and CLPS gene are not as yet available in public databases (hapmap consortium, perlegen). Based on our own re-sequencing data, the large differences in allele frequencies did not allow haplotype calculations.

Table 1. Polymorphisms in the PNLIP and CLPS genes[a)]

Region	Position	Sequence	aa change	Minor allele frequency
PNLIP				
Exon 5 (rs2915748)	37061791	ccaaC/Tgtgc	none	0.090 T
Intron 11	37075666	ggtgC/Tgccc	none	0.079 T
Intron 11	37075701	atagA/Gttgt	none	0.083 G
CLPS				
Promoter	26563445-6	gaga-/GAcct	none	0.284 GAins
Promoter (rs10653763)	26562584-5	acatAT/-aaa	none	0.386 ATdel
5′UTR (rs3748050)	26562195	actcA/Gccat	none	0.256 G
Exon3	**26560064**	**tggaC/Tgctc**	**Arg109Cys**	**0.064 T**

a) The base positions for the PNLIP gene are given according to the human chromosome 10 genomic contig (NT_030059) and base positions for the CLPS gene according to the human chromosome 6 genomic contig (NT_007592).

The CLPS Arg109Cys polymorphism was investigated in 192 incident diabetic patients and 384 age- and sex-matched controls from the EPIC. In addition, the polymorphism was reinvestigated in MICK subjects. Although this cohort was primarily recruited for intervention studies, 182 subjects with impaired glucose tolerance, of these 49 had type 2 diabetes, were diagnosed by oral glucose tolerance test at study entry. The frequencies of the Arg/Cys genotype were 2.6% in EPIC and 2.2% in MICK. No homozygotes for the Cys/Cys genotype were found in either study population. The genotype distributions in study populations were in compliance with the Hardy-Weinberg equilibrium.

Logistic regression analysis showed that the rare genotype was significantly associated with increased risk of type 2 diabetes (Tabs. 2, 3). The OR estimated by the model with adjustment for sex, age and BMI were 3.75 (95% CI = 1.13–12.49, $p = 0.03$) in EPIC and 4.86 (95% CI = 1.13–20.95, $p = 0.03$) in MICK study subjects. The OR estimates

Table 2. Association of CLPS Arg109Cys with type 2 diabetes in EPIC

	Cases (%)	Controls (%)	OR[a] (95% CI)	*p*	OR[b] (95% CI)	*p*	OR[c] (95% CI)	*p*
Arg/Arg	184 (95.8)	377 (98.2)	1.00		1.00		1.00	
Arg/Cys	8 (4.2)	7 (1.8)	2.29 (0.83 – 6.30)	0.1	3.75 (1.13 – 12.49)	**0.03**	4.44 (1.12 – 17.66)	**0.04**

a) Matched on age and sex.
b) Matched on age and sex, adjusted for BMI.
c) Matched on age and sex, adjusted for BMI, WHR and presence of hypertension.

Table 3. Association of CLPS Arg109Cys with type 2 diabetes in MICK

	Cases (%)	Controls (%)[a]	OR[b] (95% CI)	*p*	OR[c] (95% CI)	*p*	OR[d] (95% CI)	*p*
Arg/Arg	45 (93.7)	500 (98.4)	1.00		1.00		1.00	
Arg/Cys	3 (6.3)	8 (1.6)	4.10 (1.05 – 16.04)	**0.04**	4.86 (1.13 – 20.95)	**0.03**	5.66 (1.18 – 27.09)	**0.03**

a) Controls were sampled from subjects without disturbed glucose tolerance.
b) Adjusted for age.
c) Adjusted for age and BMI.
d) Adjusted for age, BMI, WHR and presence of hypertension.

Table 4. Association of CLPS Arg109Cys with disturbed glucose tolerance in MICK

	Cases (%)	Controls (%)	OR[a] (95% CI)	*p*	OR[b] (95% CI)	*p*	OR[c] (95% CI)	*p*
Arg/Arg	171 (96.6)	500 (98.4)	1.00		1.00		1.00	
Arg/Cys	6 (3.4)	8 (1.6)	2.13 (0.73 – 6.23)	0.2	2.51 (0.81 – 7.7)	0.1	2.42 (0.77 – 7.62)	0.1

a) Adjusted by age.
b) Adjusted by age and BMI.
c) Adjusted by age, BMI, WHR and presence of hypertension.

did not considerably change when we adjusted for BMI, WHR and the presence of hypertension. In MICK, the rare Arg/Cys genotype was more frequent in subjects with impaired (3.4%) than in subjects with normal (1.6%) glucose tolerance. However, ORs were not statistically significant, independent of the model used (Tab. 4). Mean BMI, WHR, waist circumference, blood pressure, HDL-C and TC were analyzed for their association with CLPS Arg109Cys. As shown in Tabs. 5 and 6, no associations between polymorphism and traits of the insulin-resistance syndrome were found. Only in EPIC were individuals with the Arg/Cys genotype shown to have significantly (p = 0.03) lower baseline plasma HDL levels than subjects with the Ala/Ala genotype after adjustment for age and gender.

Table 5. Anthropometric and metabolic variables according to CLPS Arg109Cys genotype in EPIC subjects

	Arg/Arg	Arg/Cys	*p*
Subjects (*n*)	550	14	
Age (years)[a]	55.6 (55.0, 56.2)	55.0 (51.5, 58.5)	0.8
Body mass index (kg/m^2)[a]	28.0 (27.6, 28.4)	27.8 (25.5, 30.1)	0.9[b]
Waist hip ratio[a]	0.907 (0.901, 0.912)	0.909 (0.875, 0.943)	0.9[b]
Waist circumference (cm)[a]	93.6 (92.6, 94.5)	94.0 (87.8, 100.2)	0.9[b]
Systolic BP (mmHg)[a]	136.6 (135.0, 138.2)[c]	132.5 (123.1, 142.0)	0.4[d]
Diastolic BP (mmHg)[a]	86.7 (85.8, 87.6)[c]	86.0 (80.6, 91.5)	0.8[d]
HDL-C (mg/dl)[e]	38.1 (37.4, 38.8)	33.5 (29.9, 37.5)	**0.03**[d]
TC (mg/dl)[e]	185.6 (182.6, 188.6)	177.1 (160.1, 196.1)	0.4[d]

a) Data expressed as arithmetic means (95% CI).
b) Refers to testing equality of adjusted means, adjusted for age and sex.
c) Calculated mean (95% CI) from 500 individuals, since blood pressure measurements were not obtained from 50 subjects. Data were not stratified by diabetes type 2 and/or disturbed glucose tolerance.
d) Refers to testing equality of adjusted means, adjusted for age, sex and BMI.
e) Data expressed as geometric means (95% CI).

Table 6. Anthropometric and metabolic variables according to CLPS Arg109Cys genotype in MICK subjects

	Arg/Arg	Arg/Cys	*p*
Subjects (*n*)	666	14	
Disturbed glucose tolerance (%)	25.2	42.9	0.2[a]
Age (years)[b]	59.0 (58.6, 59.4)	60.4 (57.5, 63.2)	0.4
Body mass index (kg/m^2)[b]	27.4 (27.1, 27.7)	27.0 (24.8, 29.1)	0.7[c]
Waist hip ratio[b]	0.990 (0.984, 0.995)	0.993 (0.956, 1.030)	0.9[c]
Waist circumference (cm)[b]	100.1 (99.1, 101.0)	98.6 (92.1, 105.0)	0.6[c]
Systolic BP (mmHg)[b]	129.5 (128.3, 130.8)	127.9 (119.3, 136.5)	0.7[d]
Diastolic BP (mmHg)[b]	80.5 (79.8, 81.3)	81.9 (76.7, 87.2)	0.6[d]
HDL-C (mg/dl)[e]	51.4 (50.5, 52.4)	51.1 (44.7, 58.3)	0.9[d]
TC (mg/dl)[e]	222.7 (219.5, 225.9)	226.1 (204.7, 249.7)	0.8[d]

a) Fisher's exact test (two-sided).
b) Data expressed as arithmetic means (95% CI).
c) Refers to testing equality of adjusted means, adjusted for age.
d) Refers to testing equality of adjusted means, adjusted for age and BMI. Data were not stratified by diabetes type 2 and/or disturbed glucose tolerance.
e) Data expressed as geometric means (95% CI).

4 Discussion

PNLIP and CLPS are essential components for duodenal hydrolysis of dietary triglycerides. The genes encoding PNLIP and CLPS have been proposed [11, 15] as potential candidate genes for type 2 diabetes due to possible roles in dietary lipid hydrolysis. However, only one association study between polymorphic sites of the PNLIP and metabolic parameters of the syndrome has been published so far [11]. The authors could not detect any significant associations in Caucasians. We screened the splice sites as well as the coding and promoter regions of the CLPS and PNLIP genes for polymorphisms. Only a small number of polymorphic sites were found. This is in agreement with earlier studies [11, 15] and reflects the annotated CLPS/PNLIP SNPs in public databases

(dbSNP, HGBase). However, we have identified a novel non-synonymous SNP in codon 109 of the CLPS gene, which results in an Arg to Cys substitution. Since CLPS contains ten Cys residues, which form five disulfide bridges, an additional Cys residue at position 109 may alter the bridge formation and therefore the stability, conformation and/or function of the protein. These assumptions should be tested *in-vitro* by comparing wild-type and mutant proteins in future studies.

In the two German Caucasian study populations (EPIC and MICK), we found consistently more carriers of the Cys allele among subjects with type 2 diabetes and an association of the Arg/Cys genotype with increased risk of type 2 diabetes after adjustment for confounding effects. A similar, but statistically non-significant, association was found with impaired glucose metabolium in MICK. Concordant evidence in both study populations for associations between Arg/Cys genotype and common traits of the insulin-resistance syndrome were not found. Thus, we could only demonstrate an association with type 2 diabetes. Assuming an influence of the newly discovered CLPS SNP on triglyceride digestion, it could be speculated, that Arg109Cys is linked to type 2 diabetes via postprandial serum triglyceride levels and lipoprotein pathways. Of course, this hypothesis must be proven proofed in future studies. We found significantly lower baseline levels of HDL in subjects with the Arg/Cys genotype in comparisons to homozygote Arg carriers. Reduced HDL-C could be a result of prolonged lipidemia [16].

At present, little is known about the nature of genetic variation underlying complex diseases such as type 2 diabetes. One hypothesis proposes that the genetic factors underlying common diseases will be alleles that are themselves quite common in the population [17, 18]. The Trp64Arg polymorphism of the β3-adrenergic receptor gene and its association with BMI and type 2 diabetes across different study populations [19] is an example supporting the "common disease, common variant" hypothesis. The opposite hypothesis proposes that rare variants are more likely to be involved in complex disorders [20, 21]. For example, several rare mutations in the melanocortin-4 receptor gene predispose to obesity [22]. Our study on the CLPS Arg109Cys SNP seems to be also a good example for the "common disease, rare variant" hypothesis since our findings are based on two separate study populations. Nevertheless, it should be noted that, due to the low frequency of the minor allele, both our studies may lack sufficient power and, therefore, ORs are considerably overestimated. Therefore, the potential contribution of the Arg/Cys genotype to the development of type 2 diabetes has to be reassessed in larger population studies.

In summary, we have identified a putative functional, non-synonymous SNP in codon 109 of the CLPS gene. There was consistent evidence in two German Caucasian study populations that the Arg/Cys variant of the CLPS-Arg109Cys polymorphism might contribute to increased susceptibility of type 2 diabetes.

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