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Polymorphisms in *PNLIP*, encoding pancreatic lipase, and associations with metabolic traits

Abstract Pancreatic lipase (EC 3.1.1.3) is an exocrine secretion that hydrolyzes dietary triglycerides in the small intestine. We developed genomic amplification primers to sequence the 13 exons of *PNLIP*, which encodes pancreatic lipase, in order to screen for possible mutations in cell lines of four children with pancreatic lipase deficiency (OMIM 246600). We found no missense or nonsense mutations in these samples, but we found three silent single-nucleotide polymorphisms (SNPs), namely, 96A/C in exon 3, 486C/T in exon 6, and 1359C/T in exon 13. In 50 normolipidemic Caucasians, the *PNLIP* 96C and 486T alleles had frequencies of 0.083 and 0.150, respectively. The *PNLIP* 1359T allele was absent from Caucasian, Chinese, South Asian, and North American aboriginal samples, but had a frequency of 0.085 in an African sample, suggesting that it is a population-specific variant. In an association analysis of 185 African neonates, the *PNLIP* 1359C/T SNP genotype was significantly associated with concentrations of plasma lipoproteins. These associations were most likely due to linkage disequilibrium with another functional variant at or near *PNLIP*. Thus, we report three new SNPs for the *PNLIP*, which may serve as markers for association analyses and for pharmacogenetic studies of pancreatic lipase inhibitors.

Key words Lipolysis · Lipids · Ethnicity · Complex traits · DNA sequencing · Mutation · Metabolism

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Introduction

Pancreatic lipase (PL; EC 3.1.1.3), a 56-kD protein, is involved in the intestinal hydrolysis of dietary triglyceride to fatty acids. PL deficiency (OMIM 246600) is associated with malabsorption of long-chain triglyceride fatty acids, failure to thrive in infancy and childhood, and absence of PL secretion upon secretin stimulation (Sheldon 1964; Rey et al. 1966). *PNLIP* on chromosome 10q26.1 (Davis et al. 1991) is a 13-exon gene that spans more than 20 kb (Sims et al. 1993) and encodes the 465-amino acid PL mature protein (Lowe et al. 1989). To date, neither *PNLIP* mutations in PL deficiency nor common *PNLIP* single-nucleotide polymorphisms (SNPs) have been reported. We thus sequenced *PNLIP* from the genomic DNA of subjects with PL deficiency and of several normal subjects in order to discover rare mutations and/or common SNPs.

Methods

Study subjects

Cell lines of PL-deficient probands. Lymphoblasts from four PL deficiency patients were purchased from Coriell (Camden, NJ, USA). Subject GM13278 was a 3-year-old Caucasian female with chronic diarrhea, failure to thrive, and complete deficiency of PL activity with secretin stimulation. Subject GM13322 was a 2-year-old Hispanic male with failure to thrive, developmental abnormalities, and complete deficiency of PL activity with secretin stimulation. Subject GM13343 was a 5-year-old Caucasian male with chronic diarrhea, failure to thrive, and complete deficiency of PL activity with secretin stimulation. Subject GM13344 was a 2-year-old Caucasian male with failure to thrive, developmental abnormalities, and complete deficiency of PL activity with secretin stimulation.

Trinidadian African neonates. We assessed 300 consecutive newborn infants from the Mount Hope Women's Hospital

in Trinidad. All newborns were weighed immediately after birth. Cord blood samples were collected from each newborn. Ethnicity was determined as described (Busch et al. 1999), leaving 185 subjects of African ancestry for study. After birth, samples were taken by syringe from the umbilical vein. Ten ml of cord blood was transferred to ethylenediaminetetraacetic acid (EDTA)-containing tubes and was immediately centrifuged at $1700\,g$ at 4°C for 10 min. Plasma was collected and stored for determination of plasma lipoproteins, and buffy coats were saved for extraction of genomic DNA. Each mother gave her informed consent. The protocol was approved by the Ethics Committee of the Faculty of Medical Sciences, University of the West Indies (St. Augustine).

Normal controls. Samples from a DNA archive of 50 clinically normal Caucasian subjects were analyzed to determine SNP allele frequencies. For the *PNLIP* 1359C/T SNP, additional samples from a DNA archive — 100 clinically normal subjects, of Chinese, East Indian, North American aboriginal, and African ethnic background — were studied to determine allele and genotype frequencies.

Cell culture

Lymphoblasts were cultured in minimal essential medium (Life Technologies, Rockville, MD, USA) supplemented with 20% heat-inactivated fetal bovine serum at 37°C in an atmosphere of 5% CO_2 and 95% air.

Biochemical studies

Plasma lipoproteins and apolipoproteins from neonatal samples were determined as described (Hegele et al. 1999; Brouwer et al. 2000).

Genomic DNA isolation

Genomic DNA from cultured lymphoblasts was obtained using a commercial method, following the manufacturer's instructions (Puregene, Gentra Systems, Minneapolis, MN, USA). Genomic DNA from neonatal samples had been prepared as described (Busch et al. 1999; Hegele et al. 1999).

Screening *PNLIP* for mutations

In order to amplify *PNLIP* coding regions and intron-exon boundaries from genomic DNA, a primer set was developed, using GeneBank sequences (nos. L11242, L24502, L24522, L24523, L24524, L24525, L24526, L24527, L24528, and L24529). Primer sequences are shown in Table 1. Primers were each designed to anneal at 57°C, which allowed for use of a single amplification apparatus for all reactions. Exons 1 and 2, as well as exons 7 and 8, were amplified by a single primer pair. Amplification conditions were: 94°C for 5 min, followed by 30 cycles comprised of 30 s each at 94°C, 57°C, and 72°C, ending with a single 10-min extension step at 72°C.

Genotyping of *PNLIP* 1359T/C SNP

A rapid method was used to genotype the *PNLIP* 1359T/C SNP within exon 13. Genomic DNA was amplified using primers 5′-TCCTCACTGTCACACTCCAGAT-3′ and 5′-TAACAGCCATTGGAGGTTCTTT-3′. The amplification conditions were: 94°C for 5 min, followed by 30 cycles comprised of 30 s each at 94°C, 57°C, and 72°C, ending with a single 10-min extension step at 72°C. The resulting fragment was 301 base pairs in length. Digestion of the 1359C allele with *Al*wNI (or *Cai*I) produced a single fragment of

Table 1. *PNLIP* genomic DNA amplification primers

Exon(s)	Primer sequence (5′ to 3′)	Amplification product size (bp)
1 and 2	Forward: CTTTCTTTGCATGCCATTTTTC	332
	Reverse: TGAAAAGTGAATCTCTCCCAGC	
3	Forward: GCAGCAGTCATTAAAAAGAGCA	291
	Reverse: GTCTCGATCTCCTGACCTCGT	
4	Forward: TACTTACTGCCCCTCTCCATGT	273
	Reverse: CCAGTGCCTTACCGTTTGTTA	
5	Forward: GGAATTTATCCTAGTCCTCCAGTA	261
	Reverse: CCATGTGTACAGGCTTTTATGC	
6	Forward: TAACGTATCCCTGTTGTTGAGC	203
	Reverse: TTCACTGTTTTTCACCAACCTG	
7 and 8	Forward: AGAACCCGTTCATCCCTTTC	522
	Reverse: TGAGCCTGTACAATTCCAAAGA	
9	Forward: GACTTTGTTCTGTGACCTGCAT	322
	Reverse: TGTATAGGAAAGCACACCAGTCA	
10	Forward: CCTCATTCATCATTGTTTCTTTTC	233
	Reverse: TGCACATTTTCAGTGGACATAA	
11	Forward: GTGTCTTTTATCTCCAAACTGACA	222
	Reverse: AGCGAGACTCCGACTCAAAA	
12	Forward: CAGAAATGCATTGTAAGCTGGT	399
	Reverse: ATCCCCTGTAGGAGGTTTCACT	
13	Forward: TCCTCACTGTCACACTCCAGAT	301
	Reverse: TAACAGCCATTGGAGGTTCTTT	

Table 2. Clinical and biochemical traits in Trinidadian neonates of African descent according to *PNLIP* genotype

	1359C/1359C	1359C/1359T plus 1359T/1359T	Bonferroni *P* value
Number	156	29	
Gestational age (weeks)	38.2 ± 2.4	38.4 ± 2.2	NS
Birth weight (g)	3151 ± 510	3084 ± 458	NS
Total cholesterol (mM)	1.61 ± 0.47	1.43 ± 0.45	<0.05
Triglycerides (mM)	0.45 ± 0.19	0.37 ± 0.12	NS
Apo AI (g/l)	0.80 ± 0.21	0.69 ± 0.24	<0.05
Apo B (g/l)	0.18 ± 0.08	0.15 ± 0.09	<0.05
Lp(a) (g/l)	49.7 ± 58.6	28.0 ± 26.7	<0.05

Apo, Apolipoprotein; Lp, lipoproteins; NS, not significant

301 base pairs, whereas digestion of the 1359T allele produced two fragments, with lengths of 211 and 90 base pairs. These fragments could be easily resolved after electrophoresis in 2% agarose gels.

Statistical analysis

SAS version 6.12 (SAS Institute, Cary, NC, USA) was used for all statistical analyses. We used χ^2 analysis to test deviations of genotype frequencies from those predicted by the Hardy-Weinberg equation and to test differences in allele frequencies between groups. Regression analysis was used to determine sources of variation for plasma lipoprotein traits, and Bonferroni-adjusted pairwise comparisons were used to characterize between-genotype differences in quantitative traits, assuming a dominant model for the *PNLIP* 1359T allele. Log transformation was used to normalize the distribution of quantitative traits regression analysis, but, for clarity, untransformed means ± SDs are shown in Table 2, comparing subjects by genotype. The nominal level of statistical significance for all analyses was $P < 0.05$.

Results

Identification of three *PNLIP* SNPs

Genomic DNA from the cell lines from the four PL-deficient subjects and from five normal Caucasian controls were screened, using the amplification primers shown in Table 1 and the amplification program described above. There were no SNPs found in the PL-deficient subjects that altered the amino acid sequence. Three silent SNPs were found in the PL-deficient subjects, and these are shown in Fig. 1. *PNLIP* 96A/C in exon 3 was found in subject GM13322, who was homozygous for the 96C allele. *PNLIP* 486C/T in exon 6 was found in subject GM13278, who was heterozygous for the 486T allele. *PNLIP* 1359C/T in exon 13 was found in subject GM13322, who was homozygous for the 1359T allele. Homozygosity for 1359T was confirmed, using *Alw*NI genotyping, in subject GM13322.

The *PNLIP* 96A/C and 486C/T SNPs were also found in the Caucasian controls, and direct sequencing of 100 Caucasian chromosomes indicated that the frequencies of the 96C and 486T alleles were 0.083 and 0.150, respectively. However, the *PNLIP* 1359T allele was completely absent from 100 Caucasian chromosomes.

Genotyping for *PNLIP* 1359C/T was then performed in normal subjects from various ethnic groups. The 1359T allele was absent from 200 Chinese, 200 East Indian, and 200 North American aboriginal chromosomes, but had a frequency of 0.085 in 200 African chromosomes. In the African sample, the frequencies were 84.3% for the 1359C/C genotype, 15.1% for the 1359C/T genotype, and 0.6% for the 1359T/T genotype. The observed genotype frequencies did not deviate significantly from those predicted by the Hardy-Weinberg equation. Thus, the *PNLIP* 1359T allele appeared to be an ethnic-specific variant, occurring only in subjects of African descent.

Genetic associations in Trinidadian neonates of African descent

In a sample of 185 African neonates, the *PNLIP* 1359T allele frequency was 0.081, with no difference between males and females, and no deviation of genotype frequencies from those predicted by the Hardy-Weinberg equation (data not shown).

The association of the *PNLIP* 1359C/T SNP with metabolic traits, assuming a dominant model for 1359T, was examined in this sample of 185 neonates of African origin, using linear regression analysis, with sex and gestational age included as covariates. The *PNLIP* 1359C/T SNP genotype was a significant source of variation for plasma concentrations of total cholesterol, apolipoprotein (apo) AI, apo B and lipoprotein (Lp) (a). Sex was a significant source of variation for cholesterol and apo AI (data not shown). Gestational age was a significant determinant of birth weight in this sample, as subjects with lower gestational age also had a lower birth weight (data not shown). Pairwise comparisons (shown in Table 2) indicated that subjects with the *PNLIP* 1359T allele had lower plasma total cholesterol, apo AI, apo B, and Lp(a) compared with findings in subjects with the *PNLIP* 1359C/C genotype.
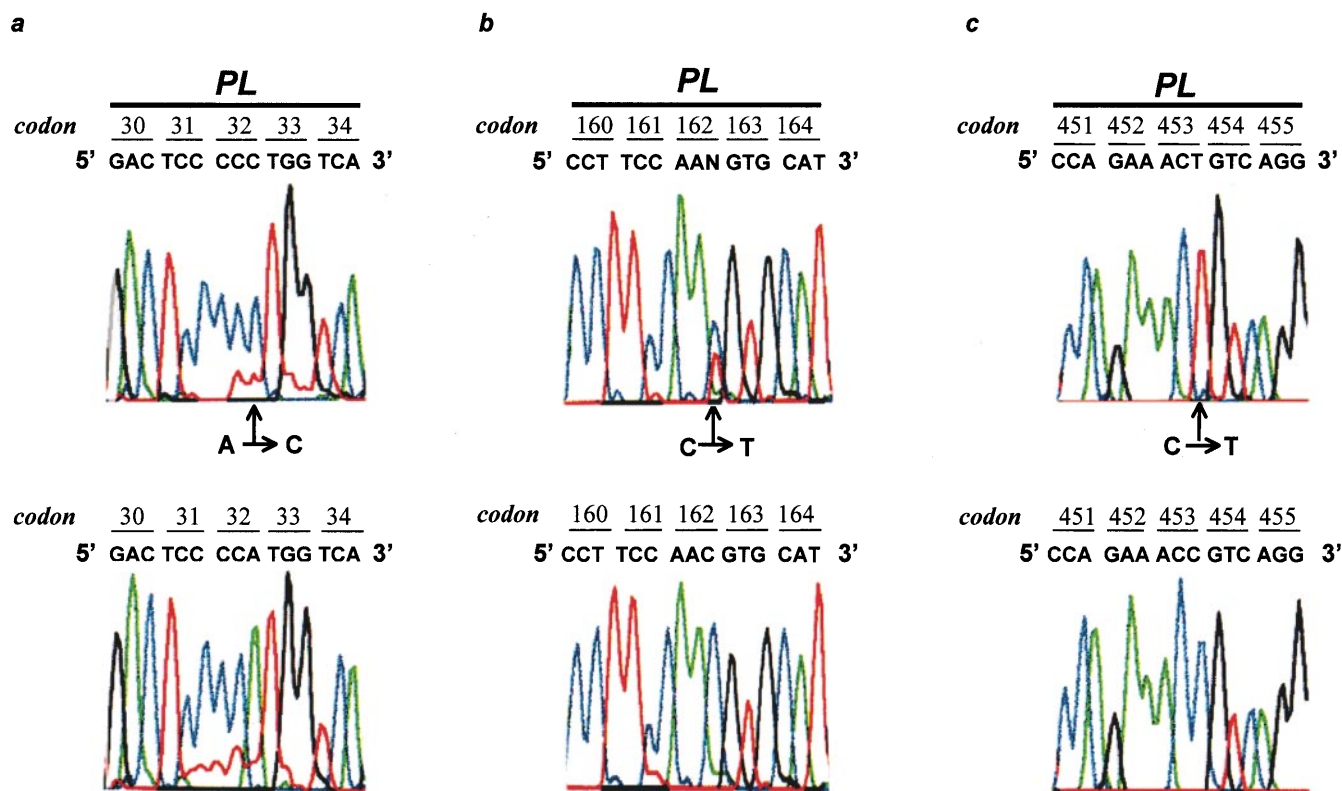
Fig. 1a–c. *PNLIP* single-nucleotide polymorphisms (SNPs). Electrophoretogram tracings of sequences from genomic DNA templates in subjects screened for mutations in *PNLIP*. Mutant sequences are shown in *each upper panel*, and wild-type sequences are shown in *each lower panel*. Codon numbers, orientation, and nucleotide sequences are indicated. Mutant nucleotides are indicated by *arrows*. **a** Subject GM13322 is a homozygote for 96C; **b** subject GM13278 is a heterozygote for 486T; and **c** subject GM13322 is a homozygote for 1359T

Discussion

We report herein: (1) a novel set of primers that amplified the entire *PNLIP* coding sequence at a single annealing temperature, allowing for effective use of amplification hardware, (2) the use of these amplification primers for genomic DNA sequencing, which has resulted in: (3) the identification of three novel SNPs in *PNLIP*, of which one, namely, 1359C/T, is specific to people of African descent; and (4) novel associations of the *PNLIP* 1359C/T SNP with plasma lipoproteins in neonatal Trinidadians of African descent. There were no *PNLIP* mutations in subjects with PL deficiency.

Two of the novel *PNLIP* SNPs, namely, 96A/C and 486C/T, were shown by direct sequencing of genomic DNA to be relatively common in Caucasians. These SNPs might be useful as markers for association studies. Because these SNPs do not affect naturally occurring restriction sites, other methods, such as mismatch amplification or allele-specific hybridization, will be required to detect them. The development of such methods was beyond the scope of the present report.

The availability of *PNLIP* SNPs may also be helpful for pharamacogenetic studies. Pharmacological inhibition of PL is currently of wide clinical interest. For example, Xenical (orlistat; Hoffmann LaRoche, Nutley, NJ, USA) has been tested in over 17 000 patients in clinical trials and has been prescribed to more than 1 000 000 patients worldwide (Blackburn 1999). There appears be some interindividual variation in response, both with respect to the efficacy and the tolerability of this medication (Hill et al. 1999). Having *PNLIP* markers may be helpful in genetically stratifying people for studies of the sources of interindividual variation in efficacy and tolerability.

The association of *PNLIP* 1359C/T SNP genotype with plasma lipoprotein traits in Trinidadian neonates of African descent, while of some interest, is not likely due to a functional impact of this silent SNP. Rather, this SNP may be in linkage disequilibrium with other variants in *PNLIP*, or another nearby gene on chromosome 10q26.1, which underlie the association. The observations are compatible with suggestions that there may be an early effect of genetic determinants on complex plasma lipoprotein traits (Low 1998). But because there is substantial evolution over time of all plasma lipoproteins when compared with baseline values at birth (van Biervliet et al. 1980), the significance of the observed associations over the long term is unclear. Furthermore, PL is probably not a direct determinant of any neonatal metabolic trait. *PNLIP* mRNA is not expressed in either

human or rat fetal liver (Payne et al. 1994; Yang et al. 2000), and reaches physiological levels only several weeks after birth (Payne et al. 1994). This is in contrast to the PL-related proteins *PNLIPRP1* (OMIM 604422) and *PNLIPRP2* (OMIM 604423), whose function is unknown, but whose expression is evident early in fetal development (Giller et al. 1992; Payne et al. 1994; Yang et al. 2000), indicating that these genes have different temporal expression.

We found no coding sequence or intron-exon boundary SNPs in the cell lines from PL-deficient subjects. There are several possible explanations for this observation. First, because we did not sequence the *PNLIP* promoter region, we cannot rule out the possibility of a disease mutation in that region in some or all PL-deficient subjects. Second, PL requires a 12-kD polypeptide co-factor called pancreatic colipase (OMIM 120105), encoded by the *CLPS* gene. It is possible that the PL-deficient phenotype could result from *CLPS* mutations. Third, there are at least two other *PNLIP*-related genes, called *PNLIPRP1* and *PNLIPRP2* (Giller et al. 1992), whose function is unknown. It is this possible that mutations affecting the function of the *PNLIPRP1* and *PNLIPRP2* gene products might contribute to PL deficiency.

In summary, we have identified genomic DNA amplification primers for *PNLIP*, in addition to common SNPs, which may be useful for association and pharmacogenetic studies. The association of the *PNLIP* 1359C/T SNP with lipoproteins in African neonates probably does not result from a functional change. However, the association is compatible with an effect of genetic determinants on plasma lipoprotein traits, and should be confirmed in larger studies using diverse study samples.

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