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Association of a polymorphism in the gene encoding phosphoenolpyruvate carboxykinase 1 with high-density lipoprotein and triglyceride levels

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Abstract *Aims/hypothesis:* Phosphoenolpyruvate carboxykinase (PCK) is the key enzyme involved in the regulation of gluconeogenesis. The aim of this study was to identify genetic polymorphisms in potential candidate genes for type 2 diabetes by sequencing all exons in the *PCK* genes (*PCK1* and *PCK2*), and examining the association with type 2 diabetes and diabetic phenotypes in a Korean population (775 type 2 diabetic patients and 316 normal control subjects). *Materials and methods:* Twenty-two polymorphisms in *PCK1* and *PCK2* were identified in a Korean population ($n=24$) by direct DNA sequencing. The TaqMan genotyping method was applied for genotyping the remainder of the study population. Associations of *PCK* polymorphisms with the risk of type 2 diabetes and diabetic phenotypes were analysed using logistic and multiple regressions, adjusting for age, sex and BMI. *Results:* Although no significant associations between the genetic polymorphisms in *PCK* genes and the risk of type 2 diabetes were detected, in further haplotype analysis, one of the common haplotypes, *PCK1* ht3, revealed susceptibility to type 2 diabetes ($p=0.006$). One 3' untranslated region (UTR) single nucleotide polymorphism (SNP)

also showed an association with HDL levels among non-diabetic control subjects: individuals homozygous for the major allele (T/T) had the lowest HDL level (1.11 ± 0.32 mmol/l), heterozygotes (T/C) had an intermediate level (1.27 ± 0.37 mmol/l), and those homozygous for the minor allele (C/C) had the highest level (1.39 ± 0.28 mmol/l) ($p=0.000003$). This 3' UTR SNP was also associated with triglyceride levels, with a lower triglyceride level observed among individuals who were homozygous for the minor allele (C/C) than among those who were not. *Conclusions/interpretation:* The strong genetic association of HDL and triglyceride levels with variation/haplotype information identified in this study would be useful for further genetic epidemiological studies of this important gene.

Keywords HDL · Phosphoenolpyruvate carboxykinase · Polymorphism · Triglyceride · Type 2 diabetes mellitus

Abbreviations ESM: electronic supplementary material · HOMA_{IR}: homeostasis model assessment for insulin resistance · ht: haplotype · LD: linkage disequilibrium · PCK: phosphoenolpyruvate carboxykinase · SNP: single nucleotide polymorphism · UTR: untranslated region

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Introduction

Phosphoenolpyruvate carboxykinases (PCKs) mainly function as enzymes that help to regulate gluconeogenesis, and have been implicated as candidate genes for type 2 diabetes mellitus. PCKs are found in the cytosol (encoded by *PCK1*; MIM 261680) and the mitochondria (encoded by *PCK2*; MIM 261650) [1, 2]. The similar gene structures and the amino acid sequence homology of 68–70% between the cytosolic PCK1 and the mitochondrial PCK2 [3] suggest that they are derived from a common ancestor gene. The activity of PCK1 (on chromosome 20q13.31) is controlled by the rate of transcription of its gene, which is regulated by insulin, glucocorticoids, cAMP and diet, to adjust glucose production to meet physiological requirements [4], whereas *PCK2* (on chromosome 14q11.2) seems to be constitutively

active [4]. It has been proposed that PCK1 is best suited to gluconeogenesis from amino acids and that mitochondrial PCK2 best serves gluconeogenesis from lactate [4]. Recent evidence suggests that PCK may primarily function as an integrator of hepatic energy metabolism rather than as a determinant of gluconeogenesis [1, 5, 6].

Previous studies had indicated that the *PCK1* region on chromosome 20q has a genetic linkage to type 2 diabetes. Using affected-sib-pair methods, significant results were obtained in the 20q13 region, in the vicinity of the *PCK1* locus [7]. This was also reported as a candidate region for insulin resistance [8]. It is noteworthy that two other diabetes-susceptibility genes also map to chromosome 20q, both of which are centromeric to the *PCK1* locus, namely *MODY1*, which causes a subtype of MODY, and the gene encoding the agouti signalling protein, non-agouti homologue (mouse) (*ASIP*), which is the human homologue of a murine gene responsible for an obesity-diabetes syndrome in mice [9, 10].

In an effort to identify genetic polymorphisms in potential candidate genes for type 2 diabetes, we sequenced all exons in *PCK1* and *PCK2*, including the -1.5 kb of the 5' flanking region and ± 50 bp exon-intron boundaries, and investigated associations with the risk of type 2 diabetes and diabetic phenotypes in a Korean population.

Subjects and materials

Subjects and measures A total of 775 unrelated patients with type 2 diabetes and 316 unrelated non-diabetic control subjects were recruited. Non-diabetic control subjects were recruited from an unselected population undergoing a routine health check-up at Seoul National University Hospital (Seoul, Korea). Inclusion criteria were as follows: 60 or more years of age, no past history of diagnosis of diabetes, no diabetes in first-degree relatives, fasting plasma glucose level <6.1 mmol/l and HbA_{1c} $<5.8\%$. The diabetic subjects were randomly recruited from patients attending the outpatient clinic of Seoul National University Hospital. Diabetes was diagnosed based on American Diabetes Association criteria [11]. Subjects who tested positive for anti-GAD antibodies were excluded. All subjects enrolled in this study were of Korean ethnicity. The study protocol was approved by the Institutional Review Board of the Clinical Research Institute at Seoul National University Hospital. Informed consent was obtained from all subjects before drawing blood samples. All study subjects were examined in the morning after an overnight fast. Height, weight, waist and hip circumferences and blood pressure were measured. Blood samples were drawn for biochemical measurements (fasting plasma glucose, post-prandial 2-h glucose, fasting plasma insulin, HbA_{1c}, total cholesterol, triglyceride, and HDL cholesterol) and DNA extraction. The insulin resistance index HOMA_{IR} (homeostasis model assessment) was calculated as fasting serum insulin (mU/l) $\times$ fasting plasma glucose (mmol/l) / 22.5 [12]. The clinical characteristics of the subjects are shown in Table 1.

Table 1 Clinical characteristics of type 2 diabetic patients and normal control subjects

Parameter	Type 2 diabetic patients	Normal controls
Sex (M/F)	361/414	146/170
Age (years)	58.9 $\pm$ 10.5	64.8 $\pm$ 4.3
BMI (kg/m ²) ^a	24.4 $\pm$ 2.9	23.7 $\pm$ 3.1
WHR	0.90 $\pm$ 0.06	0.89 $\pm$ 0.06
Systolic blood pressure (mmHg)	134.5 $\pm$ 20.0	132.3 $\pm$ 19.4
Diastolic blood pressure (mmHg)	80.8 $\pm$ 11.9	81.3 $\pm$ 11.5
HOMA _{IR} ^a	4.73 $\pm$ 4.74	1.85 $\pm$ 0.98
Total cholesterol (mmol/l)	5.26 $\pm$ 0.97	5.07 $\pm$ 0.89
Triglycerides (mmol/l) ^a	1.93 $\pm$ 1.32	1.57 $\pm$ 0.81
HDL cholesterol (mmol/l) ^b	1.23 $\pm$ 0.30	1.17 $\pm$ 0.35
Fasting blood glucose (mmol/l) ^a	8.6 $\pm$ 2.6	5.2 $\pm$ 0.7
Fasting serum insulin (pmol/l) ^a	86.4 $\pm$ 81.3	55.8 $\pm$ 27.6
HbA _{1c} (%) ^a	8.08 $\pm$ 1.63	5.22 $\pm$ 0.48

Data are means $\pm$ SD

^a $p < 0.001$

^b $p < 0.01$ for the difference between type 2 diabetes and normal control subjects

Sequencing analysis of the gene encoding human PCK We sequenced all exons, including exon-intron boundaries and the promoter region (~1.5 kb), to discover single nucleotide polymorphisms (SNPs) in 24 Korean DNA samples using the ABI PRISM 3,700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Twenty-two primer sets for the amplification and sequencing analysis were designed based on GenBank sequences (Ref. Seq. of *PCK1* mRNA: NM_133263 and mRNA: NT_026437.11). Information regarding primers is available at Electronic supplementary material (ESM) Table 1. Sequence variants were verified by chromatograms.

Genotyping with fluorescence polarisation detection For genotyping of polymorphic sites, amplifying primers and probes were designed for TaqMan [13]. Primer Express (Applied Biosystems) was used to design both the PCR primers and the MGB TaqMan probes. One allelic probe was labelled with the FAM dye and the other with the fluorescent VIC dye. PCRs were run in the TaqMan Universal Master mix without UNG (Applied Biosystems), with PCR primer concentrations of 900 nmol/l and TaqMan MGB probe concentrations of 200 nmol/l. Reactions were performed in a 384-well format in a total reaction volume of 5 μ l using 20 ng of genomic DNA. The plates then were placed in a thermal cycler (PE 9700; Applied Biosystems) and heated at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The TaqMan assay plates were transferred to a Prism 7900HT instrument (Applied Biosystems) in which the fluorescence intensity in each well of the plate was read. Fluorescence

data files from each plate were analysed using automated software (SDS 2.1; Applied Biosystems). Information regarding the primers is shown in ESM Table 2.

Statistical analysis χ^2 Tests were used to determine whether the individual variant was in Hardy–Weinberg equilibrium at each locus in the population. Haplotypes and their frequencies were inferred using the algorithm developed by Stephens et al. [14]. Logistic regression analyses were used for calculating p values, controlling for age, sex and BMI as covariates. Multiple regressions, with adjustments for age, sex and BMI, were used for association analyses of metabolic phenotypes. Haplotype associations were analysed using the algorithm developed by Schaid et al. (Haplo.Score and Haplo.GLM) [15], controlling for age, sex and BMI as covariates. The insulin and triglyceride levels were logarithmically transformed to normalise skewed distribution. Only non-diabetic subjects were used for association analyses of metabolic phenotypes, as treatment of type 2 diabetes may have affected the phenotypic values in the diabetic patients.

Results

Fourteen polymorphisms were identified in *PCK1*: five in the 5' flanking region, two in introns, five in exons, and two in 3' untranslated regions (UTRs). Eight polymorphisms were identified in *PCK2*: two in the 5' flanking region, one in an intron, and four in exons (see ESM Table 3). The locations and allele frequencies of identified polymorphic sites are shown in Fig. 1a and b. By pair-wise linkage analysis with 24 Korean DNA samples, which were used for direct sequencing, we found that two sets of SNPs in *PCK1* were in absolute linkage disequilibrium (LD) ($|D'|=1$ and $r^2=1$) (see Fig. 1a).

Among the identified polymorphisms, six SNPs in *PCK1* (+69A>G, +1428C>G, +1573G>C, +3404T>C, +4513T>G and +4824T>C) and four SNPs in *PCK2* (−403C>T, +3894T>C, +5804A>G and +8881C>G) were selected for larger-scale genotyping based on location (SNPs in exons were preferred), LD (only one SNP if there are absolute LDs), frequency (>0.05) and haplotype tagging status. A $>97\%$ genotyping success rate was obtained (on average) by the TaqMan method. Genotyping quality control was performed by duplicate checking of 10% of the samples. No mismatches were detected among duplicates. Six common haplotypes in *PCK1* and four common haplotypes in *PCK2* were constructed in a Korean population (Fig. 1b,d). The minor allele frequencies of genotyped polymorphisms were 0.391 (*PCK1*+69A>G [Leu23Leu]), 0.27 (*PCK1*+1428C>G [Leu184Val]), 0.281 (*PCK1*+1573G>C), 0.356 (*PCK1*+3404C>T), 0.216 (*PCK1*+4513T>G), 0.222 (*PCK1*+4824T>C), 0.487 (*PCK2* −403C>T), 0.202 (*PCK2*+3894T>C [Pro124Pro]), 0.212 (*PCK2*+5804A>G [Lys410Lys]) and 0.364 (*PCK2*+ 8881C>G) in the Korean population ($n=1091$, see ESM Table 3).

The genotype distributions of all loci were in Hardy–Weinberg equilibrium ($p>0.05$, ESM Table 3). Among common haplotypes identified among the Korean population, only those with frequencies >0.05 were used for further analysis (Fig. 1b,d). Haplotypes that were mostly ($>90\%$) tagged by single SNPs were not used for further analysis (ht2 in *PCK1*, ht3 in *PCK2*).

Associations of *PCK* polymorphisms with the risk of type 2 diabetes and diabetic phenotypes were analysed using logistic and multiple regressions, adjusting for age, sex and BMI (775 type 2 diabetic patients and 316 normal control subjects). The clinical characteristics of subjects are shown in Table 1. Our study was designed to detect common variants that might be involved in the risk of type 2 diabetes and relative phenotypes.

Table 2 Logistic analysis of *PCK* polymorphisms with the risk of type 2 diabetes among patients and normal subjects

Gene	Locus	Location	Amino acid change	Type 2 diabetic patients ($n=775$)	Controls ($n=316$)	p^a
<i>PCK1</i>	+69A>G	Exon 2	Leu23Leu	0.39	0.38	0.79
	+1428C>G	Exon 4	Leu184Val	0.27	0.28	0.37
	+1573G>C	Intron 4	–	0.29	0.26	0.21
	+3404T>C	Intron 8	–	0.35	0.39	0.08
	+4513T>G	3' UTR	–	0.21	0.23	0.14
	+4824T>C	3' UTR	–	0.22	0.23	0.87
	ht1	–	–	0.20	0.18	0.29
	ht3	–	–	0.15	0.11	0.006
	ht4	–	–	0.09	0.08	0.43
	ht5	–	–	0.08	0.09	0.46
<i>PCK2</i>	−403C>T	5'	–	0.49	0.48	0.82
	+3894T>C	Exon 3	Pro124Pro	0.21	0.19	0.21
	+5804A>G	Exon 7	Lys410Lys	0.22	0.20	0.22
	+8881C>G	Intron 9	–	0.36	0.36	0.94
	ht1	–	–	0.32	0.32	0.98
	ht2	–	–	0.25	0.26	0.29
	ht4	–	–	0.19	0.19	0.71

Minor allele frequencies of each site and p values for logistic analyses of the co-dominant model, controlling for age, sex and BMI as covariates, are shown

^a p values of haplotype associations were calculated by the algorithm developed by Schaid et al. (Haplo.Score) [15], controlling for age, sex and BMI as covariates

Table 3 Regression analyses of *PCK* polymorphisms with cholesterol, logarithmically transformed triglycerides (TG) and HDL controlling for age, sex and BMI as covariates among non-diabetic subjects

Phenotype	Gene	Locus	Location	Amino acid change	C/C ^a	C/R ^a	R/R ^a	p ^b		
								Co-dominant	Dominant	Recessive
Cholesterol	<i>PCK1</i>	+69A>G	Exon 2	Leu23Leu	113 (5.15±0.90)	150 (5.04±0.86)	42 (5.03±1.05)	0.19	0.14	0.62
		+1428C>G	Exon 4	Leu184Val	144 (5.08±0.90)	124 (5.06±0.94)	20 (5.05±0.63)	0.55	0.55	0.77
		+1573G>C	Intron 4	–	166 (5.13±0.87)	116 (5.01±0.92)	22 (4.86±0.88)	0.24	0.26	0.52
		+3404T>C	Intron 8	–	118 (4.91±0.88)	137 (5.15±0.93)	49 (5.22±1.79)	0.02	0.02	0.20
		+4513T>G	3' UTR	–	178 (4.99±0.89)	109 (5.18±0.95)	15 (5.07±0.66)	0.43	0.26	0.69
		+4824T>C	3' UTR	–	171 (5.08±0.87)	91 (5.10±0.99)	19 (5.06±0.89)	1.00	1.00	1.00
	<i>PCK2</i>	ht1	–	–	200 (5.15±0.93)	95 (4.91±0.78)	8 (5.11±0.94)	0.06	0.03	0.79
		ht3	–	–	241 (5.08±0.89)	57 (5.02±0.94)	5 (5.08±0.68)	0.81	0.72	0.78
		ht4	–	–	256 (5.03±0.85)	46 (5.31±1.07)	1 (4.35)	0.16	0.12	0.53
		ht5	–	–	253 (5.09±0.88)	47 (4.98±0.95)	3 (4.91±0.96)	0.77	0.76	0.96
		ht6	–	–	266 (5.05±0.90)	35 (5.24±0.83)	2 (5.18±0.11)	0.21	0.16	0.87
		–403C>T	5'	–	85 (5.11±0.90)	152 (5.09±0.85)	73 (5.01±0.99)	0.21	0.43	0.21
		+3894T>C	Exon 3	Pro124Pro	211 (5.07±0.86)	85 (5.11±1.00)	15 (4.90±0.800)	0.61	0.91	0.27
		+5804A>G	Exon 7	Lys410Lys	205 (5.07±0.85)	89 (5.10±1.02)	16 (4.94±0.80)	0.70	0.96	0.36
		+8881C>G	Intron 9	–	121 (5.10±0.99)	152 (5.02±0.82)	37 (5.23±0.89)	0.95	0.72	0.51
		ht1	–	–	139 (5.08±0.96)	139 (5.04±0.84)	30 (5.22±0.89)	0.74	0.88	0.64
Log-TG	<i>PCK1</i>	ht2	–	–	169 (5.10±0.89)	115 (5.06±0.93)	24 (5.00±0.87)	0.41	0.50	0.49
		ht4	–	–	202 (5.06±0.89)	92 (5.07±0.92)	14 (5.29±0.93)	0.23	0.41	0.15
		+69A>G	Exon 2	Leu23Leu	113 (0.16±0.20)	150 (0.13±0.20)	42 (0.15±0.19)	0.24	0.12	0.88
		+1428C>G	Exon 4	Leu184Val	144 (0.13±0.20)	124 (0.17±0.19)	20 (0.14±0.19)	0.36	0.19	0.71
		+1573G>C	Intron 4	–	166 (0.15±0.21)	116 (0.14±0.20)	22 (0.12±0.19)	0.67	0.58	0.95
		+3404T>C	Intron 8	–	118 (0.13±0.19)	137 (0.14±0.21)	49 (0.17±0.20)	0.23	0.41	0.24
	<i>PCK2</i>	+4513T>G	3' UTR	–	178 (0.13±0.20)	109 (0.17±0.21)	15 (0.14±0.17)	0.32	0.19	0.76
		+4824T>C	3' UTR	–	171 (0.16±0.19)	91 (0.16±0.20)	19 (0.04±0.16)	0.09	0.41	0.008
		ht1	–	–	200 (0.16±0.20)	95 (0.12±0.20)	8 (0.1±0.18)	0.05	0.04	0.54
		ht3	–	–	241 (0.15±0.20)	57 (0.12±0.21)	5 (0.07±0.27)	0.16	0.16	0.55
		ht4	–	–	256 (0.14±0.20)	46 (0.20±0.20)	1 (–0.02)	0.15	0.11	0.44
		ht5	–	–	253 (0.15±0.20)	47 (0.14±0.18)	3 (0.21±0.15)	0.55	0.67	0.40
		ht6	–	–	266 (0.14±0.20)	35 (0.17±0.21)	2 (0.25±0.27)	0.31	0.32	0.66
		–403C>T	5'	–	85 (0.13±0.20)	152 (0.15±0.20)	73 (0.15±0.21)	0.89	0.68	0.84
		+3894T>C	Exon 3	Pro124Pro	211 (0.14±0.19)	85 (0.14±0.21)	15 (0.17±0.26)	0.87	0.97	0.72
		+5804A>G	Exon 7	Lys410Lys	205 (0.14±0.20)	89 (0.13±0.20)	16 (0.16±0.25)	0.89	0.81	0.87

Table 3 (continued)

Phenotype	Gene	Locus	Location	Amino acid change	C/C ^a	C/R ^a	R/R ^a	p ^b		
								Co-dominant	Dominant	Recessive
HDL	PCK1	+69A>G	Exon 2	Leu23Leu	113 (1.18±0.36)	150 (1.18±0.36)	42 (1.13±0.25)	0.71	1.00	0.46
		+1428C>G	Exon 4	Leu184Val	144 (1.22±0.36)	124 (1.14±0.33)	20 (1.09±0.31)	0.05	0.05	0.33
		+1573G>C	Intron 4	–	166 (1.16±0.33)	116 (1.18±0.37)	22 (1.24±0.31)	0.40	0.42	0.61
		+3404T>C	Intron 8	–	118 (1.17±0.35)	137 (1.17±0.35)	49 (1.19±0.36)	0.87	0.97	0.78
		+4513T>G	3' UTR	–	178 (1.20±0.37)	109 (1.14±0.32)	15 (1.16±0.25)	0.22	0.18	0.80
		+4824T>C	3' UTR	–	171 (1.11±0.32)	91 (1.27±0.37)	19 (1.39±0.28)	0.000003	0.000008	0.006
		ht1	–	–	200 (1.18±0.34)	95 (1.16±0.36)	8 (1.05±0.15)	0.42	0.58	0.30
		ht3	–	–	241 (1.15±0.35)	57 (1.25±0.34)	5 (1.34±0.24)	0.02	0.02	0.39
		ht4	–	–	256 (1.18±0.35)	46 (1.15±0.33)	1 (1.24)	0.80	0.76	0.77
		ht5	–	–	253 (1.18±0.33)	47 (1.14±0.40)	3 (1.17±0.47)	0.36	0.34	0.90
PCK2		ht6	–	–	266 (1.18±0.35)	35 (1.10±0.31)	2 (1.10±0.09)	0.22	0.20	0.84
		–403C>T	5'	–	85 (1.21±0.38)	152 (1.14±0.33)	73 (1.20±0.32)	0.93	0.37	0.27
		+3894T>C	Exon 3	Pro124Pro	211 (1.18±0.34)	85 (1.16±0.38)	15 (1.20±0.30)	0.90	0.75	0.70
		+5804A>G	Exon 7	Lys410Lys	205 (1.17±0.34)	89 (1.17±0.37)	16 (1.24±0.33)	0.72	0.96	0.41
		+8881C>G	Intron 9	–	121 (1.14±0.30)	152 (1.18±0.38)	37 (1.23±0.35)	0.13	0.24	0.18
		ht1	–	–	139 (1.15±0.30)	139 (1.17±0.37)	30 (1.26±0.38)	0.24	0.52	0.13
		ht2	–	–	169 (1.19±0.37)	115 (1.12±0.30)	24 (1.25±0.30)	0.85	0.31	0.15
		ht4	–	–	202 (1.19±0.35)	92 (1.13±0.33)	14 (1.16±0.35)	0.15	0.14	0.55

^aC/C, C/R and R/R represent individuals homozygous for the common allele, heterozygotes and subjects homozygous for the rare allele, respectively. Haplotypes and their frequencies were inferred using the algorithm developed by Stephens et al. [14]. Genotype and haplotype distributions (mean±SD of cholesterol [mmol/l], log-transformed TG [mmol/l] and HDL [mmol/l]), and p values (co-dominant, dominant and recessive models for regression analyses) are shown

^bp values of haplotype associations were calculated using the algorithm developed by Schaid et al. (Haplo.GLM) [15], controlling for age, sex and BMI as covariates

Fig. 1 Gene maps and haplotypes of *PCK* genes (*PCK1* and *PCK2*). Coding exons are marked by black blocks, and 5' and 3' UTRs by white blocks. The first base of the translational start site is denoted as nucleotide +1. Asterisks indicate polymorphisms genotyped in a larger population ($n=1,087$). The frequencies of polymorphisms not subjected to larger scale genotyping were based on sequence data ($n=24$). **a** Polymorphisms identified in *PCK1* on chromosome 20q13.31 (Ref. Genome Seq. NT_011362.9). **b** Haplotypes of *PCK1*. Haplotypes with frequencies >0.05 are shown. Others* contain rare

haplotypes: GCGTTC, ACGCTT, AGCTTT, ACGCTC, GG GTGT, GCCTTT, AGGTTT, AGGCGT, GGGTTT, GCGTGC, AC GCGC, GCGCGT, GCGCTT, ACCTGT, GCCTTC, ACGTTT, GCGCGC, GGGCTT, ACCTGC, GGGCGT, GCCTGT, AGCCTT, ACCCTC, ACCCTT, GCGCTC, AGGCGC and ACG TTC. **c** Poly morphisms identified in *PCK2* on chromosome 14q11.2 (Ref. Genome Seq. NT_026437.11). **d** Haplotypes of *PCK2*. Haplotypes with frequencies >0.02 are shown. Others** contain rare haplotypes: TCGG, CCGC, CCAG and CTGC

Only non-diabetic subjects were used for association analyses of metabolic phenotypes, including cholesterol, log-transformed triglyceride, HDL, log-transformed insulin and BMI, because treatment for type 2 diabetes may affect these values.

Although no significant associations between the genetic polymorphisms in *PCK* genes and the risk of type 2 diabetes were detected, in further haplotype analysis one of the common haplotypes, *PCK1* ht3, revealed susceptibility to type 2 diabetes ($p=0.006$, Table 2).

No reliable associations were detected with fasting blood sugar, BMI, insulin, $HOMA_{IR}$ and WHR (see ESM Table 4). However, several SNPs and haplotypes have significant correlations with various metabolic traits (cholesterol: *PCK1*+3404T>C and *PCK1* ht1; log-transformed triglyceride: *PCK1*+4824T>C and *PCK1* ht1; HDL: +1428C>G, +4824T>C and *PCK1* ht3) (Table 3). Among them, one 3' UTR SNP, +4824T>C, showed a gene-dose-dependent association with HDL levels among non-diabetic control subjects: individuals who were homozygous for the major allele (T/T) had the lowest HDL level (1.11 ± 0.32 mmol/l), heterozygotes (T/C) had an intermediate level (1.27 ± 0.37 mmol/l), and individuals homozygous for the minor allele (C/C) had the highest level (1.39 ± 0.28 mmol/l)

($p=0.000003$, Table 3). In addition, this 3' UTR SNP was also associated with triglyceride levels; a lower triglyceride level was found among individuals who were homozygous for the minor allele (C/C) than those who were not.

In additional analysis, 3' UTR SNP and *PCK1* ht3 also showed significant association ($p=0.002$ and 0.0002 , respectively) with BMI among diabetic subjects, but these significant associations were unreliable because the treatment for type 2 diabetes might have affected the phenotypic values in diabetics (see ESM Table 5).

Discussion

In a previous report [16], one promoter SNP ($-232C>G$; $-534C>G$ in this study), which is in absolute LD ($|D'|=1$ and $r^2=1$) with three nearby SNPs ($-1,399C>T$, $-1,269C>T$, $-534C>G$, +69A>G, Fig. 1a), was reported to be associated with the risk of type 2 diabetes in both Oji-Cree and Caucasian populations. However, no significant association was revealed in the Korean population. *PCK1* ht3 only showed significant association with the risk of type 2 diabetes. Although it is hard to decipher the discrepancies between the previous study and the present study, a dif-

ferent genetic background and environmental factors or their interaction among different ethnic groups could be a plausible explanation.

In the present study, the significance of the association ($p=0.000003$ in the co-dominant model) between HDL and this 3' UTR SNP (*PCK1*+4824T>C) was retained even after the strictest correction for multiple tests (170 tests: 17 comparisons in case-control, 17 comparisons in five different diabetic phenotypes [cholesterol, triglyceride, HDL, insulin and BMI] with three alternative analysing models [co-dominant, dominant and recessive models]).

Although the mechanisms involved in the different HDL and triglyceride levels associated with the alternative genotypes in the 3' UTR are not currently understood, there are several possible explanations. The crucial role of the non-coding portion of genomes is now widely acknowledged. In particular, mRNA UTRs are involved in many post-transcriptional regulatory pathways that control mRNA localisation, stability and translation efficiency. Initiation of protein synthesis could be influenced by sequence elements in both 5' and 3' UTRs. The post-transcriptional events play an important yet incompletely understood role in regulatory gene expression and cellular behaviour; many of the identified *cis*-acting elements for translational regulation occur within the 3' UTR [17].

Recent data suggest that *PCK1* is involved in glyceroneogenesis as well as gluconeogenesis [1, 2]. Glyceroneogenesis in both liver and adipose tissue is the *de novo* synthesis of glycerol 3-phosphate from gluconeogenic precursors. Each glycerol 3-phosphate is used to esterify fatty acyl-CoA to form triglyceride. It has been shown that mice lacking hepatic *PCK1* develop fatty livers and have elevated plasma fatty acids and triglyceride levels following a fast [1, 5, 6]. It has also been reported that removal of *PCK1* results in an increase in the mitochondrial redox state and dramatic inhibition of the TCA cycle, which results in reduced oxidation of fats via beta oxidation, reduced production of ketones and subsequent hepatic steatosis [18]. These results suggest that mutations in *PCK1* may affect glyceroneogenesis and/or cataplerosis, which would affect the storage and release of fatty acids. Further biological and/or functional evidence would be needed to clarify the mechanism(s) involved in different blood HDL and triglyceride levels mediated by alternative *PCK1* polymorphisms.

In summary, 22 polymorphisms in *PCK1* and *PCK2* were identified in a Korean population ($n=24$) by direct DNA sequencing. Associations of *PCK* polymorphisms with the risk of type 2 diabetes and diabetic phenotypes were analysed using logistic and multiple regression analyses, with adjustment for age, sex and BMI. Although no significant associations between the genetic polymorphisms in *PCK* genes and the risk of type 2 diabetes were detected, it was revealed that *PCK1* ht3 is significantly associated with the risk of type 2 diabetes ($p=0.006$). One 3' UTR SNP, +4824T>C, was strongly associated with HDL and triglyceride levels among non-diabetic control subjects ($p=0.000003$).

Although the statistical power to detect significant association of this study (775 type 2 diabetic patients and 316 normal control subjects) might be low for rare variants, our study was designed to detect common variants that might be involved in the risk of type 2 diabetes. In addition, the differences in quantitative traits (which are dependent on sample sizes [genotype distributions], means of traits and SDs among populations) among 316 normal controls might not be detected, especially for rare allele (e.g. unequal sample sizes for populations).

Although our results showed several significant associations of polymorphisms with various metabolic traits (cholesterol, log-transformed triglyceride and HDL), replication of our finding in an independent dataset and/or functional validation of polymorphisms should be performed in the future. The strong association of HDL and triglyceride levels with the *PCK1* polymorphism and the variation/haplotype information identified in this study would be useful for genetic epidemiological studies of other metabolic diseases.

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References

1. Beale EG, Hammer RE, Antoine B, Forest C (2004) Dysregulated glyceroneogenesis: *PCK1* as a candidate diabetes and obesity gene. *Trends Endocrinol Metab* 15:129–135
2. Nordlie RC, Lardy HA (1963) Mammalian liver phosphoenolpyruvate carboxykinase activities. *J Biol Chem* 238:2259–2263
3. Modarelli S, Christ B, Bratke J, Zahn S, Heise T, Jungermann K (1996) Molecular cloning, sequencing and expression of the cDNA of the mitochondrial form of phosphoenolpyruvate carboxykinase from human liver. *Biochem J* 315:807–814
4. Watford M, Hod Y, Chiao YB, Utter MF, Hanson RW (1981) The unique role of the kidney in gluconeogenesis in the chicken. The significance of a cytosolic form of phosphoenolpyruvate carboxykinase. *J Biol Chem* 256:10023–10027
5. Beale EG, Forest C, Hammer RE (2003) Regulation of cytosolic phosphoenolpyruvate carboxykinase gene expression in adipocytes. *Biochimie* 85:1207–1211
6. She P, Shiota M, Shelton KD, Chalkley R, Postic C, Magnuson MA (2000) Phosphoenolpyruvate carboxykinase is necessary for the integration of hepatic energy metabolism. *Mol Cell Biol* 20:6508–6517
7. Zouali H, Hani EH, Philippi A et al (1997) A susceptibility locus for early-onset non-insulin dependent (type 2) diabetes mellitus maps to chromosome 20q, proximal to the phosphoenolpyruvate carboxykinase gene. *Hum Mol Genet* 6:1401–1408
8. Hani el H, Zouali H, Philippi A et al (1996) Indication for genetic linkage of the phosphoenolpyruvate carboxykinase (*PCK1*) gene region on chromosome 20q to non-insulin-dependent diabetes mellitus. *Diabetes Metab* 22:451–454
9. Bell GI, Xiang KS, Newman MV et al (1991) Gene for non-insulin-dependent diabetes mellitus (maturity-onset diabetes of the young subtype) is linked to DNA polymorphism on human chromosome 20q. *Proc Natl Acad Sci U S A* 88:1484–1488
10. Wilson BD, Ollmann MM, Kang L, Stoffel M, Bell GI, Barsh GS (1995) Structure and function of ASP, the human homolog of the mouse agouti gene. *Hum Mol Genet* 4:223–230

11. Committee E (1997) Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 20:1183–1197
12. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC (1985) Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28:412–419
13. Livak KJ (1999) Allelic discrimination using fluorogenic probes and the 5' nuclease assay. *Genet Anal* 14:143–149
14. Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet* 68:978–989
15. Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA (2002) Score tests for association between traits and haplotypes when linkage phase is ambiguous. *Am J Hum Genet* 70:425–434
16. Cao H, van der Veer E, Ban MR et al (2004) Promoter polymorphism in PCK1 (phosphoenolpyruvate carboxykinase gene) associated with type 2 diabetes mellitus. *J Clin Endocrinol Metab* 89:898–903
17. Conklin D, Jonassen I, Aasland R, Taylor WR (2002) Association of nucleotide patterns with gene function classes: application to human 3' untranslated sequences. *Bioinformatics* 18:182–189
18. Burgess SC, Hausler N, Merritt M et al (2004) Impaired tri-carboxylic acid cycle activity in mouse livers lacking cytosolic phosphoenolpyruvate carboxykinase. *J Biol Chem* 279: 48941–48949