

Hepatocyte nuclear factor 4 alpha P2 promoter variants associate with insulin resistance

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This study aimed to investigate the associations of hepatocyte nuclear factor 4 (HNF4) alpha single nucleotide polymorphisms (SNPs) and haplotype with insulin resistance and metabolic syndrome parameters. Nine SNPs spanning the HNF4 alpha P2 promoter (rs4810424, rs1884613 and rs1884614) and coding region (rs2144908, rs6031551, rs6031552, rs1885088, rs1028583 and rs3818247) were genotyped in 160 subjects without diabetes or metabolic syndrome. The HNF4 alpha P2 promoter SNPs rs4810424, rs1884613 and rs1884614 were associated with insulin resistance ($p = 0.017$; 0.037 ; 0.024) and body mass index (BMI) ($p = 0.03$; 0.035 ; 0.039). The intron 1D SNP rs2144908 was associated with high-density lipoprotein cholesterol (HDLc) ($p = 0.020$) and the intron 9 SNP rs3818247 showed association with systolic ($p = 0.02$) and diastolic ($p = 0.034$) blood pressure. HNF4 alpha common haplotype CCCGTC associated with higher insulin resistance ($p = 0.022$), fasting blood glucose (FBG) ($p = 0.035$) and lower HDLc ($p = 0.001$). In conclusion, subjects with HNF4 alpha P2 variants and haplotypes have been shown to have a higher insulin resistance and are therefore at a higher risk for developing type 2 diabetes mellitus.

Keywords: insulin resistance, HNF4 alpha, single nucleotide polymorphisms, haplotypes

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INTRODUCTION

Insulin resistance and beta cell dysfunction precede the development of intermediate hyperglycaemia and the subsequent type 2 diabetes mellitus (T2DM). Insulin resistance is a feature of a number of diseases including obesity, glucose intolerance, dyslipidemia and hypertension clustering in the so-called metabolic syndrome (Sesti, 2006) that together are an indicator of risk for type 2 diabetes mellitus. The prevalence of T2DM in school children is on the increase, which is associated with obesity, metabolic syndrome and insulin resistance (Nathan, 2007; Cali & Caprio, 2008; Holst-Schumacher, 2009; Phillips & Phillips, 2009; Amed *et al.*, 2010). The peripheral insulin resistance refers to diminished insulin-mediated uptake of glucose principally by skeletal muscle and adipose tissue, which depends mainly on the control of glucose transporter type 4 (GLUT4) expression and translocation to the plasma membrane (Leclercq *et al.*, 2007). Hepatic insulin resistance refers to insufficient ability of insulin to suppress hepatic glucose production that account for hyperglycemia. Eighty percent of sub-

jects converted to T2DM are insulin resistant (Haffner *et al.*, 2000).

Hepatocyte nuclear factor 4 alpha (HNF4 alpha), mostly expressed in the liver and pancreatic beta cells, is a transcriptional factor essential for normal functioning of hepatocytes and endocrine pancreas (Parviz *et al.*, 2003; Odom *et al.*, 2004). HNF4 alpha controls the expression of several transcription factors (Gonzalez, 2008; Bolotin *et al.*, 2010) and regulates several genes encoding components of insulin secretion and glucose metabolism (Stoffel & Duncan 1997; Wang *et al.*, 2000). Polymorphisms of HNF4 alpha gene may be associated with insulin resistance. The aim of this project was to study the association of HNF4 alpha P2 promoter SNPs and haplotypes with the insulin resistance and metabolic syndrome parameters (waist circumference, body mass index, fasting blood glucose, C-peptide, systolic and diastolic blood pressure, triglyceride, total cholesterol, LDL and HDL cholesterol).

MATERIALS AND METHODS

Ethical approval was obtained from the National University of Malaysia Research and Ethics committee. The subjects were randomly approached through distribution of brochures to office letterboxes around Cheras area. The Hospital and Faculty of Medicine staffs of University Kebangsaan Malaysia were approached. Brochures were also given to participants to distribute to their relatives and friends. In two years, around nine hundred subjects responded, but after several tries, only 262 were included. After biochemical analysis of their blood, 160 of the 262 were found without diabetes or metabolic syndrome

Biochemical analysis. Kits for the measurement of glucose, triglyceride, total cholesterol and HDL cholesterol (reference number 10260, 10724, 10028 and 10018, respectively) were purchased from Human Company (Human GmbH, Wiesbaden, Germany). Human company Elevated control sera (Humatrol P Reference number 13512) was used as quality control for these parameters. C-peptide was measured in an automated quantitative immunoassay analyzer (Immulite, DPC, Los Angeles,

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Abbreviations: HNF4 alpha, hepatocyte nuclear factor 4 alpha; SNP, single nucleotide polymorphism; BMI, body mass index; HDLc, high-density lipoprotein cholesterol, FBG, fasting blood glucose; T2DM, type 2 diabetes mellitus; LDLc, low-density lipoprotein cholesterol; PCR, polymerase chain reaction; DHPLC, denaturing high-performance liquid chromatography; BP, Blood pressure; TG, triglyceride.

USA) using IMMULITE C-peptide kit (catalogue number LKPE1). Insulin resistance was calculated using the Homeostasis Model Assessment (HOMA2) Calculator v2.2. This program uses fasting C-peptide or insulin and blood glucose measurement to calculate insulin resistance.

Genotype analysis. HNF4 alpha SNPs rs4810424, rs1884613, rs1884614, rs2144908, rs6031551, rs6031552, rs1885088, rs1028583 and rs3818247 were selected for genotypic analysis in based on published results from the Finnish and Ashkenazi Jewish studies (Love-Gregory *et al.*, 2004; Silander *et al.*, 2004). All SNPs in this research were amplified by in an Icyler thermocycler (Bio-Rad Laboratories, Inc., Richmond, CA, USA) using a 96 microwell plate. Touchdown PCR was applied for all SNPs except rs1884613 which was amplified and identified by allele specific PCR (AS-PCR). Restriction enzymes (New England Biolabs, USA) were used for identifying the genotypes of rs4810424 (BstXI), rs1884614 (BsgI), rs6031551 (MseI), rs6031552 (CviKI-1), rs1885088 (Sau96) and rs1028583 (PstI). The complete procedures of PCR amplification and genotyping of the SNPs are available from the authors.

Rs2144908 and rs3818247 were genotyped by denaturing high-performance liquid chromatography (DHPLC) (Varian Inc, Palo Alto, CA, USA). A recommendation for running rs2144908 and rs3818247 at 60°C and 58°C, respectively, was obtained when these SNPs PCR amplified sequences were uploaded to the DHPLC melt program (<http://insertion.stanford.edu/melt.html>). These recommended temperatures were confirmed experimentally by running 5 µl of PCR products of these SNPs on the DHPLC at two degrees below and above. Separated peaks of heteroduplex and homoduplex DNA from heterozygous samples of rs2144908 and rs3818247 were detected at 59°C and 57.5°C, respectively, using DHPLC program gradient buffer and time. Known heterozygous, homozygous minor and homozygous major genotypes of rs2144908 and rs3818247 (these samples were sequenced) were included into the runs as quality controls which were run at 59°C and 57.5°C, respectively. Some samples were sequenced by automated 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using terminator cycle sequencing kit v3.1 to confirm the allele specific PCR and DHPLC results.

Statistics. HelixTree 6.0.1 SNP and Variation Suite for Genetic statistics was used to study the deviation of SNPs from the Hardy-Weinberg equilibrium and to study the linkage disequilibrium between SNPs and construct haplotypes and diplotypes of related SNPs. Other statistical analyses were done by SPSS 11.5 program. The missing data were listwise deleted (when any of the variables were missing, the entire observation was omitted from the analysis). The BMI, waist, systolic and diastolic blood pressure, fasting blood glucose, C-peptide, insulin resistance, total cholesterol, triacylglycerol, HDL and LDL cholesterol data were log transformed because they were not normally distributed. These parameters' means and 95% confidence intervals were transformed back and reported, and were called geometric means.

The linearity of correlation of metabolic syndrome parameters: insulin resistance, C-peptide, glucose, BMI, waist, systolic BP, diastolic BP, TG, total cholesterol, HDL cholesterol and LDL cholesterol (dependent variables) were analyzed by bivariate correlations, resulting in three sets of dependent variables. The first set was insulin resistance, C-peptide, fasting blood glucose, BMI, waist, triglyceride, and HDL cholesterol. The second set

was total cholesterol and LDLc and the third set was systolic and diastolic blood pressure. The association of HNF4 alpha SNPs, haplotypes and diplotypes with these three sets of dependent variables was evaluated by multivariate analyses (general linear model) adjusted for age, sex and race as covariates. Wilks' Lambda test was used for more than two groups of independent variables. Hotelling's Trace was used for two groups of independent variable. The difference between means was considered significant if p -value < 0.05.

RESULTS

The demographic and biochemical parameters of the subjects are summarized in Table 1. HelixTree program showed no deviation of all the SNPs included in this study from Hardy-Weinberg equilibrium. Five SNPs showed association with metabolic syndrome parameters: rs4810424, rs1884613, rs1884614, rs2144908 and rs3818247, whereas rs6031551, rs6031552 and rs1028583 showed no association. The homozygous minor and heterozygous genotypes frequencies of rs1885088 were very low in our subjects (0.0 and 0.03, respectively) thus the association of this SNP with metabolic syndrome parameters was not studied.

Association of P2 promoter SNP rs4810424 with metabolic syndrome parameters

Multivariate analyses of this SNP with the three sets of metabolic syndrome parameters showed a significant Wilks' Lambda test ($P=0.039$) with the first set (insulin resistance, C-peptide, FBG, TG, HDLc, waist and BMI) whereas the second set (total and LDL cholesterol) and the third set (systolic and diastolic blood pressure) were non-significant (Table 2). The subjects having

Table 1. Demography and biochemical parameters of subjects

Parameters	n=160	
Gender	Male %	40.6
	Female %	59.4
races	Malay %	63.7
	Chinese %	26.9
	Indian %	9.4
Age (yrs)		46.8±9.2
Weight (kg)		63.7±10.5
Height (m)		1.60±0.078
BMI(kg/m ²)		24.8±3.56
Waist (cm)		82.2±9.50
Systolic blood pressure (mmHg)		126± 6.6
Diastolic blood pressure (mmHg)		78±9.0
Fasting C-peptide (pmol/l)		800±444
Fasting glucose (mmo/l)		4.9±0.56
Triglyceride (mmol/l)		1.08±0.54
Total cholesterol (mmol/l)		4.7±0.88
HDL cholesterol (mmol/l)		1.29±0.33
LDL cholesterol (mmol/l)		2.89±0.84
Insulin resistance		1.8±1.0

Table 2. Association of rs4810424 with metabolic syndrome parameters

Metabolic syndrome Parameter	Rs4810424					
	CC n(40)	CG n(79)	GG n(35)	Multivariate P-value	GG versus CC P-value	GG versus CG P-value
Insulin resistance	1.87 (1.61–2.17)	1.56 (1.39–2.74)	1.41 (1.17–1.68)	0.039	0.017	0.36
C-peptide	796 (674–939)	679 (604–764)	621 (520–742)		0.046	0.413
Fasting blood glucose	4.95 (4.8–5.1)	4.92 (4.8–5.0)	4.74 (4.59–4.90)		0.055	0.060
Triacylglycerol	1.07 (0.95–1.21)	1.0 (0.91–1.09)	0.99 (0.86–1.12)		0.34	0.89
HDL cholesterol	1.22 (1.15–1.31)	1.26 (1.20–1.30)	1.34 (1.25–1.44)		0.060	0.14
Waist	81.1 (78.5–83.7)	82.3 (80.4–84.2)	79.89 (77.2–82.7)		0.530	0.158
Body mass index	24.8 (23.7–25.9)	24.8 (24.0–25.5)	23.3 (22.2–24.4)		0.066	0.036
Total Cholesterol	4.7 (4.5–5.0)	4.6 (4.3–4.7)	4.5 (4.3–4.7)	0.317	0.22	0.48
LDL cholesterol	2.9 (2.7–3.2)	2.8 (2.6–2.9)	2.6 (2.3–2.8)		0.056	0.18
Systolic blood pressure	123 (119–127)	125 (122–127)	123 (119–127)	0.672	0.91	0.47
Diastolic blood pressure	78 (75–80)	78 (76–80)	76 (74–79)		0.37	0.22

The results are presented as geometric mean and 95% confidence interval of the mean adjusted for age, sex and race as covariate.

homozygous GG variant had significantly lower insulin resistance ($P=0.017$) and C-peptide levels ($P=0.046$) than those with homozygous CC. In addition, the FBG, BMI and LDLc were non-significantly lower in subjects having the homozygous minor GG variant than the homozygous major CC variant ($P=0.055$, 0.06 , 0.056 , respectively), whereas HDLc was non-significantly higher ($P=0.06$). The subjects having heterozygous CG of this SNP showed a significantly higher BMI ($P=0.036$) and non-significantly higher FBG ($P=0.06$) than the subjects carrying homozygous minor GG. The multivariate analysis showed that homozygous minor and heterozygous variants of this SNP were not associated with waist, TG, total cholesterol, systolic and diastolic blood pressure.

Association of P2 promoter SNP rs1884613 with metabolic syndrome parameters

Multivariate analyses of this SNP with the three sets of metabolic syndrome parameters showed a non-significant Wilks' Lambda test with the first set (insulin resistance, C-peptide, FBG, TG, HDLc, waist and BMI), the second set (total and LDL cholesterol) and the third

set ($P=0.131$, 0.347 , 0.282 , respectively) (Table 3). Parameter estimates of the multivariate test showed that subjects carrying homozygous minor variant for this SNP had lower insulin resistant and FBG, and higher HDLc than subjects carrying homozygous major variant ($P=0.037$, 0.042 , 0.038 , respectively). The subjects carrying the heterozygous (CG) variant for this SNP had lower HDLc ($P=0.042$) and higher BMI (0.035) than those with homozygous minor variant. The homozygous major and heterozygous variants of this SNP were not associated with the other metabolic syndrome parameters (C-peptide, TG, waist, total and LDL cholesterol, systolic and diastolic blood pressure).

Association of P2 promoter SNP rs1884614 with metabolic syndrome parameters

The SNP multivariate analyses of the three sets of metabolic syndrome parameters showed a non-significant Wilks' Lambda test with the first set (insulin resistance, C-peptide, FBG, TG, HDLc, waist and BMI), the second set (total and LDL cholesterol) and the third set (systolic and diastolic blood pressure) ($P=0.112$, 0.243 , 0.395 ,

Table 3. Association of Rs1884613 with metabolic syndrome parameters

Metabolic syndrome parameter	Rs1884613					
	CC n(52)	CG n(68)	GG n(34)	Multivariate P-value	GG versus CC P-value	GG versus CG P-value
Insulin resistance	1.79 (1.56–2.04)	1.56 (1.38–1.76)	1.17 (1.17–1.68)	0.131	0.037	0.35
C-peptide	763 (659–882)	685 (603–778)	614 (513–736)		0.068	0.33
Fasting blood glucose	5.0 (4.9–5.1)	4.9 (4.8–5.0)	4.8 (4.6–4.9)		0.042	0.29
Triacylglycerol	1.01 (0.90–1.13)	1.03 (0.93–1.13)	0.98 (0.85–1.11)		0.68	0.55
HDL cholesterol	1.24 (1.16–1.31)	1.24 (1.20–1.32)	1.34 (1.25–1.44)		0.038	0.042
Waist	81.1 (78.5–83.7)	82.3 (80.4–84.2)	79.9 (77.2–82.7)		0.40	0.18
Body mass index	24.8 (23.7–25.9)	24.8 (24.0–25.5)	23.3 (22.2–24.4)		0.12	0.035
Total Cholesterol	4.7 (4.4–4.9)	4.5 (4.4–4.7)	4.5 (4.3–4.8)	0.347	0.49	0.96
LDL cholesterol	2.9 (2.7–3.1)	2.7 (2.5–2.9)	2.6 (2.4–2.9)		0.12	0.60
Systolic blood pressure	123 (119–127)	125 (122–128)	123 (119–128)	0.282	0.86	0.56
Diastolic blood pressure	77 (75–79)	78 (77–80)	76 (73–78)		0.34	0.076

The results are presented as geometric mean and 95% confidence interval of the mean adjusted for age, sex and race as covariate.

Table 4. Association of Rs1884614 with metabolic syndrome parameters

Metabolic syndrome parameter	Rs1884614					
	CC n(46)	CT n(72)	TT n(33)	Multivariate P-value	TT versus CC P-value	TT versus CT P-value
Insulin resistance	1.78 (1.53–2.05)	1.63 (1.44–1.84)	1.35 (1.10–1.62)	0.112	0.024	0.087
C-peptide	761 (650–892)	716 (632–812)	577 (479–695)		0.028	0.059
Fasting blood glucose	4.9 (4.8–5.1)	4.9 (4.8–5.0)	4.8 (4.6–4.9)		0.16	0.43
Triacylglycerol	1.05 (0.93–1.17)	1.01 (0.92–1.12)	1.00 (0.87–1.15)		0.66	0.93
HDL cholesterol	1.24 (1.17–1.32)	1.26 (1.19–1.32)	1.36 (1.27–1.46)		0.057	0.060
Waist	81.9 (79.5–84.4)	81.5 (79.6–83.5)	80.4 (77.6–83.3)		0.43	0.53
Body mass index	24.9 (23.9–25.9)	24.6 (23.8–25.4)	23.3 (22.2–24.4)		0.039	0.061
Total Cholesterol	4.7 (4.5–4.9)	4.6 (4.4–4.8)	4.5 (4.3–4.8)	0.243	0.39	0.75
LDL cholesterol	2.9 (2.7–3.2)	2.7 (2.6–2.9)	2.6 (2.3–2.8)		0.073	0.320
Systolic blood pressure	123 (120–127)	126 (123–129)	121 (116–125)	0.395	0.36	0.059
Diastolic blood pressure	77 (75–79)	78 (76–70)	76 (73–78)		0.32	0.16

The results are presented as geometric mean and 95% confidence interval of the mean adjusted for age, sex and race as covariate.

respectively) (Table 4). However, parameter estimates of multivariate analyses showed that insulin resistance, C-peptide and BMI were significant lower in subjects having homozygous minor TT variant than the homozygous major CC subjects ($P=0.024$, 0.028 , 0.039 , respectively). The subjects having homozygous major or heterozygous variant had non-significantly lower HDLc than those with the homozygous minor variant ($P=0.057$, 0.060 , respectively). The heterozygous and homozygous major variants were not associated with the other metabolic syndrome parameters but the pattern was similar to that for HDLc.

Association of intron 1D SNP rs2144908 with metabolic syndrome parameters

The dependent variables of the first set (insulin resistance, C-peptide, FBG, TG, HDLc, waist and BMI) showed a significant Wilks' Lambda test ($P=0.036$) although the parameter estimates of the multivariate analysis showed that only HDLc was significantly higher among subjects having the homozygous minor (AA)

variant than in those carrying the homozygous major one ($P=0.020$) (Table 5). In addition, HDLc was border line higher in the subjects having one copy of this SNP minor variant than those subjects having no minor genotype copies ($P=0.054$). Wilks' Lambda test for the variables of the second set (total and LDL cholesterol) and the third set (systolic and diastolic blood pressure) were non-significant ($P=0.73$, 0.258 , respectively) as well as the parameter estimates showed no association of these variables with rs2144908 genotypes.

Association of intron SNP rs3818247 with metabolic syndrome parameters

The multivariate analyses of the three sets of metabolic syndrome parameters indicated a non-significant Wilks' Lambda test with the first set (insulin resistance, C-peptide, FBG, TG, HDLc, waist and BMI), the second set (total and LDL cholesterol) and the third set (systolic and diastolic blood pressure) (Table 6). The parameter estimates indicated that systolic and diastolic blood pressure were higher among subjects carrying the

Table 5. Association of Rs2144908 with metabolic syndrome parameters

Metabolic syndrome parameter	Rs2144908					
	GG n(54)	AG n(70)	AA n(33)	Multivariate P-value	AA versus GG P-value	AA versus AG P-value
Insulin resistance	1.70 (1.48–1.94)	1.59 (1.40–1.79)	1.52 (1.26–1.81)	0.036	0.33	0.69
C-peptide	728 (630–840)	690 (608–783)	664 (551–799)		0.44	0.73
Fasting blood glucose	4.9 (4.8–5.1)	4.9 (4.8–5.0)	4.8 (4.6–4.9)		0.090	0.21
Triacylglycerol	0.97 (0.85–1.06)	1.04 (0.94–1.14)	1.04 (0.91–1.19)		0.32	0.94
HDL cholesterol	1.23 (1.16–1.30)	1.25 (1.19–1.32)	1.37 (1.27–1.46)		0.020	0.054
Waist	80.7 (78.5–82.9)	82.0 (80.1–84.1)	81.7 (78.8–84.7)		0.58	0.87
Body mass index	24.4 (23.5–25.4)	24.7 (23.9–25.5)	23.8 (22.6–25.0)		0.39	0.22
Total Cholesterol	4.5 (4.3–4.7)	4.6 (4.4–4.8)	4.6 (4.4–4.9)	0.731	0.51	0.78
LDL cholesterol	2.8 (2.6–3.0)	2.8 (2.6–2.9)	2.6 (2.4–2.9)		0.38	0.50
Systolic blood pressure	122 (118–125)	126 (123–129)	123 (119–127)	0.258	0.66	0.24
Diastolic blood pressure	76 (75–78)	79 (77–80)	76 (74–78)		0.75	0.089

The results are presented as geometric mean and 95% confidence interval of the mean adjusted for age, sex and race as covariate.

Table 6. Association of Rs3818247 with metabolic syndrome parameters

Metabolic syndrome parameter	Rs3818247			Multivariate P-value	GG versus TT P-value	GG versus GT P-value
	TT n(54)	GT n(80)	GG n(23)			
Insulin resistance	1.50 (1.29–1.73)	1.65 (1.47–1.85)	1.79 (1.44–2.18)	0.931	0.18	0.51
C-peptide	644 (557–746)	722 (641–813)	766 (612–957)		0.21	0.65
Fasting blood glucose	4.9 (4.8–5.1)	4.9 (4.8–5.0)	4.9 (4.7–5.1)		0.42	0.91
Triacylglycerol	1.04 (0.93–1.15)	1.00 (0.91–1.09)	1.01 (0.85–1.18)		0.81	0.92
HDL cholesterol	1.28 (1.20–1.35)	1.27 (1.21–1.33)	1.24 (1.13–1.35)		0.58	0.62
Waist	80.5 (78.3–82.8)	81.7 (79.8–83.5)	83.3 (79.8–86.9)		0.19	0.42
Body mass index	24.0 (23.1–25.0)	24.6 (23.8–25.4)	24.8 (23.4–26.3)		0.39	0.78
Total Cholesterol	4.5 (4.3–4.7)	4.6 (4.4–4.8)	4.7 (4.4–5.0)	0.504	0.45	0.80
LDL cholesterol	2.6 (2.4–2.8)	2.8 (2.6–3.0)	2.9 (2.6–3.3)		0.15	0.50
Systolic blood pressure	122 (118–125)	124 (121–127)	129 (124–135)	0.200	0.020	0.078
Diastolic blood pressure	76 (74–78)	78 (76–79)	80 (77–83)		0.034	0.17

The results are presented as geometric mean and 95% confidence interval of the mean adjusted for age, sex and race as covariate.

Table 7. Association of most common haplotypes2 and haplotypes1 with metabolic syndrome parameters

Metabolic syndrome Parameter	Most common Haplotypes2			Multivariate P-value	CCCGTC versus GGTATC P-value
	CCCGTC n(72)	GGTATC n(46)			
Insulin resistance	1.78 (1.59–1.98)	1.43 (1.22–1.66)		0.002	0.022
C-peptide	770 (682–870)	630 (541–734)			0.044
Fasting blood glucose	4.9 (4.8–5.0)	4.7 (4.6–4.9)			0.035
Triacylglycerol	1.07 (0.98–1.16)	0.97 (0.87–1.09)			0.19
HDL cholesterol	1.20 (1.14–1.27)	1.37 (1.29–1.46)			0.001
Waist	82.4 (80.4–84.5)	81.4 (78.9–84.0)			0.544
Body mass index	25.0 (24.2–25.9)	23.8 (22.9–24.9)			0.078
Total Cholesterol	4.6 (4.4–4.8)	4.6 (4.4–4.8)		0.114	0.92
LDL cholesterol	2.8 (2.6–3.0)	2.7 (2.5–2.9)			0.31
Systolic blood pressure	124 (121–127)	123 (120–127)		0.730	0.86
Diastolic blood pressure	78 (76–79)	77 (75–79)			0.52

Metabolic syndrome parameter	Most common Haplotypes1			Multivariate P-value	CCCGTC versus GGTATC P-value
	CCCGTC n(39)	GGTATC n(68)			
Insulin resistance	1.71 (1.46–1.97)	1.56 (1.38–1.75)		0.161	0.34
C-peptide	731 (622–860)	681 (602–770)			0.70
Fasting blood glucose	5.0 (4.8–5.1)	4.9 (4.8–5.0)			0.37
Triacylglycerol	0.95 (0.83–1.08)	1.05 (0.95–1.15)			0.22
HDL cholesterol	1.25 (1.17–1.34)	1.28 (1.21–1.34)			0.65
Waist	80.1 (77.7–82.6)	81.3 (79.4–83.2)			0.56
Body mass index	24.6 (23.5–25.6)	24.3 (23.5–25.1)			0.72
Total Cholesterol	4.7 (4.5–4.9)	4.6 (4.4–4.8)		0.178	0.51
LDL cholesterol	2.9 (2.7–3.2)	2.7 (2.5–2.9)			0.15
Systolic blood pressure	124 (120–128)	125 (122–128)		0.872	0.60
Diastolic blood pressure	77 (75–79)	78 (76–79)			0.71

The results are presented as geometric mean and 95% confidence interval of the mean adjusted for age, sex and race as covariate.

Table 8. Association of most common diplotypes with metabolic syndrome parameters

Metabolic syndrome parameter	Most common diplotypes					
	CCCGTC, CCCGTC (Diplotype No 1) n(21)	GGTATC, CCCGTC (Diplotype No 2) n(36)	GGTATC, GGTATC (Diplotype No 3) n(24)	Multivariate P-value	Diplotype No1 versus Diplotype No 3 P-value	Diplotype No 2 versus Diplotype No 3 P-value
Insulin resistance	1.89 (1.54–2.27)	1.74 (1.49–2.02)	1.38 (1.12–1.68)	0.064	0.030	0.071
C-peptide	810 (652–1007)	766 (649–904)	607 (495–744)		0.058	0.083
Fasting blood glucose	5.0 (4.8–5.2)	5.0 (4.8–5.1)	4.8 (4.6–4.9)		0.11	0.11
Triacylglycerol	1.05 (0.88–1.23)	1.10 (0.97–1.24)	1.03 (0.88–1.20)		0.91	0.54
HDL cholesterol	1.21 (1.10–1.33)	1.19 (1.10–1.28)	1.37 (1.25–1.49)		0.065	0.018
Waist	79.9 (76.5–83.4)	82.6 (79.9–85.4)	80.4 (77.2–83.7)		0.83	0.31
Body mass index	24.6 (23.2–26.0)	24.9 (23.9–26.0)	23.34 (22.1–24.7)		0.20	0.070
Total Cholesterol	4.7 (4.4–5.0)	4.6 (4.3–4.8)	4.7 (4.4–5.0)	0.402	0.99	0.63
LDL cholesterol	2.9 (2.6–3.3)	2.8 (2.5–3.0)	2.7 (2.4–3.0)		0.33	0.65
Systolic blood pressure	121 (116–127)	126 (122–130)	122 (117–127)	0.446	0.85	0.19
Diastolic blood pressure	76 (73–79)	79 (76–81)	76 (73–79)		0.84	0.16

The results are presented as geometric mean and 95% confidence interval of the mean adjusted for age, sex and race as covariate.

homozygous minor (GG) variant than the subjects carrying the homozygous major (TT) (0.02, 0.034, respectively).

Associations of common haplotypes with metabolic syndrome parameters

Six-SNP haplotype and diplotype blocks were identified with significant LD. This block is constructed from rs24 (rs4810424), rs13 (rs1884613), rs14 (rs1884614), rs08 (rs2144908), rs51 (rs6031551) and rs52 (rs6031552) (Fig. 1). Wilks' Lambda test of multivariate analysis showed that the most common haplotypes2 were associated with the first set of metabolic syndrome parameters (insulin resistance, C-peptide, FBG, TG, HDLc, waist and BMI) ($P=0.002$). The parameter estimates of this multivariate analysis indicated that subjects carrying the haplotype2, GGTATC, had higher HDLc and lower insulin resistance, C-peptide and FBG than subjects carrying the haplotype2, CCCGTC ($P=0.001, 0.022, 0.044, 0.035$) (Table 7). Wilks' Lambda tests and parameter estimates of multivariate analyses of the second set (total and LDL cholesterol) and the third set (systolic and diastolic blood pressure) of metabolic syndrome param-

eters were not associated with haplotypes2. The most common haplotypes1 (CCCGTC and GGTATC) were not associated with the three sets of metabolic syndrome parameters, however, these haplotypes1 showed a similar pattern to the most common haplotypes2.

Association of the common diplotypes with metabolic syndrome parameters

The association of the most common diplotypes with the first set of metabolic syndrome parameters (insulin resistance, C-peptide, FBG, TG, HDLc, waist and BMI) was border line (Wilks' Lambda test p -value=0.064) (Table 8). The parameter estimates of these metabolic syndrome parameters indicated that subjects carrying the GGTATC,GGTATC diplotype were less insulin resistant than the subjects carrying the CCCGTC,CCCGTC or GGTATC,CCCGTC diplotype ($P=0.030, 0.071$, respectively). In addition, HDLc was higher in subjects having the GGTATC,GGTATC diplotype than in subjects having the CCCGTC,GGTATC or CCCGTC,CCCGTC diplotype ($P=0.018, 0.065$, respectively). Wilks' Lambda tests and the parameter estimates of total and LDL cholesterol, systolic and diastolic blood pressure showed that these parameters were not associated with the above most common diplotypes.

DISCUSSION

The results showed that the homozygous major variants for the HNF4 alpha P2 promoter SNPs (rs4810424, rs1884613 and rs1884613) associated with insulin resistance, C-peptide and BMI whereas the intron 1D SNP rs2144908 associated with HDL cholesterol. In addition, the haplotypes2 (CCCGTC) comprising the homozygous major variants of rs4810424, rs1884613, rs1884614, and rs2144908 showed an additional significant association with insulin resistance, C-peptide, fasting blood glucose and HDL cholesterol. The individuals carrying homozygous major variants for these SNPs might be at a higher risk of developing metabolic syndrome (prediabetic stage) and hence T2DM. The risk for developing T2DM will be increased in the subjects carrying the risk haplotype (CCCGTC).

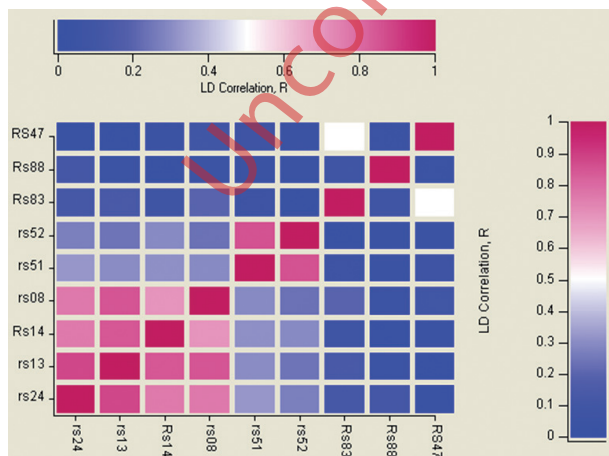


Figure 1. Linkage disequilibrium correlation between SNPs rs24, rs4810424; rs13, rs1884613; rs14, rs1884614; rs08, rs2144908; rs51, rs6031551; rs52, rs6031552.

Andrulionyte *et al.* (2006) found that female carriers of the C allele of rs4810424 had a 1.7-fold elevated risk for the conversion to diabetes compared to woman with the common genotype. Recently, a large cohort study (17831 individuals from Sweden and Finland) has reported that homozygous CC of rs4810424 predicts further T2DM in individuals from Botnia Study in Sweden (Holmkvist *et al.*, 2008). Hansen *et al.* (2005) found that homozygous minor (TT) individuals for rs1884614 had non-significantly lower insulin resistance, C-peptide and BMI than homozygous major CC individuals. In contrast, Holmkvist *et al.* (2008) reported that the rs1884614 homozygous minor TT genotype predicted further T2DM in the Botnia Study (Sweden) in T2DM relative subjects (Holmkvist *et al.*, 2008). Wanic *et al.* (2006) and Holmkvist *et al.* (2008) found that the intron 1D SNP rs2144908 did not associate with diabetic parameters (insulin resistance, C-Peptide and glucose). In contrast, Silander *et al.* (2004) reported that the homozygous AA of this SNP was associated with low BMI, acute insulin response to glucose and glucose disposition index, high fasting insulin, and high differences between 2-hour and fasting glucose and insulin during oral glucose tolerance test (OGTT).

The discrepancies in the association study of HNF4 alpha SNPs and metabolic parameters such as glucose, insulin, insulin resistance and BMI may be due to the characteristics of the subjects. The Hansen *et al.* (2005) subjects were normal glucose tolerant whereas Silander *et al.* (2004) and Holmkvist *et al.* (2008) subjects were unaffected offspring of T2DM and family related T2DM, respectively. On the other hand, the mean BMI of normal subjects in the Wanic *et al.* (2006) study was high (30 kg/m²), which markedly affected the metabolic syndrome parameters. Low frequencies of HNF4 alpha SNPs homozygous minor genotypes in some populations resulted in too few subjects within the subgroups, for example 14 subjects with homozygous AA in the Silander *et al.* (2004) study that resulted in low power of statistical analysis.

The HNF4 alpha P2 promoter risk variants showed higher fasting glucose, which could be due to increased hepatic cell glucose production. This suggestion was supported by the Holmkvist *et al.* (2008) finding that HNF4 P2 promoter variants were associated with increased hepatic glucose production. This increased blood glucose was compensated by increased pancreatic beta cell insulin secretion (C-peptide was high in the HNF4 alpha P2 risk variants). However, the hepatic response to that secreted insulin might be slightly impaired, resulting in insulin resistance in the individuals carrying the HNF4 P2 promoter risk variants. The beta cell responsiveness to the increased blood glucose would be reduced in the long term until insulin secretion decreased more than 50%, resulting in the diagnosis of T2DM. This suggestion was confirmed by the Lehman *et al.* (2007) finding that the age of T2DM onset was decreased in HNF4 alpha P2 promoter risk variants.

The non-coding variants of HNF4 alpha may exert effects on gene expression (Bonnycastle *et al.*, 2006). It was found that HNF4 alpha was associated with a large number of promoters in the liver, an important organ for glucose production, and pancreatic islet cells responsible for insulin secretion (Odom *et al.*, 2004). HNF4 alpha plays an important role in regulating glucose metabolism, hepatic gluconeogenesis (Stoffel and Duncan, 1997; Puigserver *et al.*, 2003; Rhee *et al.*, 2003), and lipoprotein and cholesterol metabolism (Rhee *et al.*, 2006).

In conclusion, HNF4 alpha P2 promoter SNPs were associated with insulin resistance, C-peptide and BMI whereas the intron 1D SNP rs2144908 associated with HDL cholesterol. The haplotype comprising the P2 promoter and intronic 1D SNPs confirmed these associations. Individuals carrying the risk genotypes for the HNF4 alpha P2 promoter SNPs might be at a higher risk of developing T2DM and the risk may be further aggravated with the haplotype that contains these risk genotype SNPs.

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