Association of genetic variants in the adiponectin gene with adiponectin level and hypertension in Hong Kong Chinese

Short title: Adiponectin, SNP, and hypertension

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Abstract

Objective: Low plasma adiponectin level can predict the development of hypertension after 5 years in our population. We therefore investigated if single nucleotide polymorphisms (SNPs) in the adiponectin gene influenced plasma adiponectin level and whether they were associated with hypertension.

Design and Methods: We genotyped 14 tagging SNPs in 1616 subjects with persistent normotensive or hypertensive status during a 6.4-year follow-up period in the Hong Kong Cardiovascular Risk Factor Prevalence Study-2 (CRISPS-2). Plasma adiponectin level was measured in 1385 subjects using in-house sandwich enzyme-linked immunosorbent assay.

Results: The minor G allele of the SNP rs266729 was significantly associated with higher odds of hypertension (odds ratio [95% CI]=1.49 [1.13-1.95], P=0.0044) after adjusting for covariates. In stepwise multiple logistic regression, this SNP (P=0.006) was a significant independent factor of hypertension, together with age (P<0.001), body mass index (P<0.001), triglycerides (P=0.021), and insulin resistance index (P<0.001). Among the 14 SNPs, rs266729 (β=-0.067, P=0.0037), -10677C>T (β=0.069, P=0.0027), rs182052 (β=-0.097, P<0.0001), and rs12495941 (β=0.103, P<0.0001) were significantly associated with adiponectin level after adjusting for covariates. No significant sex-interaction was found for the associations of SNPs with hypertension and adiponectin level. Similar results were obtained in haplotype analysis.

Conclusion: In our population, genetic variants in the adiponectin gene influenced plasma adiponectin levels and one of them was associated with hypertension. This study has provided further evidence for a role of adiponectin in the development of hypertension.

Keywords: adiponectin; hypertension; gene; single nucleotide polymorphism

Introduction

Adipose tissue has recently been recognized as an endocrine organ. One of its secretory products is adiponectin, which has been shown to increase insulin sensitivity, via promoting lipid β-oxidation in skeletal muscles and reducing hepatic gluconeogenesis (1, 2). It has direct vasoprotective actions such as stimulation of prostaglandin I_2 synthase and endothelial nitric oxide synthase activity (3). A low circulating level of adiponectin is associated with endothelial dysfunction, diabetes, obesity, inflammation, and coronary artery disease (1, 4).

Hypoadiponectinemia is associated with hypertension in cross-sectional case-control studies (5, 6). We and others previously reported that low adiponectin level is predictive of the development of hypertension in prospective studies (7, 8). Genetic factor accounts for about 40-70% of the variation in adiponectin levels (9). Genetic variants in the gene encoding adiponectin (*ADIPOQ*) have been reported to be associated with adiponectin level in several genome-wide linkage and association studies (9-11). However, inconsistent findings on the association of genetic variants of *ADIPOQ* with adiponectin level (9-13) and hypertension (5, 6, 14) have been reported which could be due to difference in ethnic populations, SNP selection, and study power. To our best knowledge, there was no systematic analysis of SNPs in *ADIPOQ* gene with regard to both adiponectin level and hypertension in Chinese population. As female gender is well-known to be associated with higher adiponectin level (15), it is worthwhile to investigate if there is any interaction between sex and genetic variants on adiponectin level and risk of hypertension.

In this study, we investigated the association of common genetic variants in the *ADIPOQ* gene with adiponectin level and hypertension. We included subjects with persistent normotensive or hypertensive status during a 6.4-year follow-up period in the

population-based Hong Kong Cardiovascular Risk Factor Prevalence Study-2 (CRISPS-2).

Subjects and Methods

Subjects

The Hong Kong Cardiovascular Risk Factor Prevalence Study (CRISPS) was a cohort study of cardiovascular risk factors in the Hong Kong Chinese (16, 17). Of the 2895 subjects who initially participated (CRISPS-1), 1944 enrolled in the follow-up study (CRISPS-2) in 2000-4 after a median interval of 6.4 years. The study protocol was approved by the Ethics Committee of the University of Hong Kong. All subjects gave written consent. In this study, we included all the 285 hypertensive subjects and 1331 normotensive subjects with DNA samples available who were either hypertensive or normotensive at baseline and also at the 6.4-year follow-up assessment. As plasma samples were not available for the majority of subjects at baseline, adiponectin level was measured only in plasma samples at follow-up (n=1385) and thus data at the follow-up were used in all the analyses. There was no significant difference in age, sex, body mass index (BMI), and hypertension prevalence between subjects with and without measurement of adiponectin level (all P>0.05).

SNPs selection and genotyping

Tagging SNPs were selected in the *ADIPOQ* gene from the HapMap Han Chinese population (Phase II data, release 22). There were 13 tagging SNPs (rs16861194, rs266729, rs182052, rs16861205, rs822396, rs12495941, rs7649121, rs7627128, rs1501299, rs3774262, rs6773957, rs1063539, and rs12629945) which captured all the 21 SNPs from 5kb region upstream to 2kb downstream of the gene (position 188,038,157-188,060,946, GenBank accession number NC_000003, NCBI build 36) with r^2 ≥0.9 and minor allele frequency (MAF) ≥0.05. However, genotyping was not performed for the SNP rs7627128 as it could

not be incorporated into Sequenom multiplex assay. This SNP showed a r^2 of 0.85 with rs7649121 and a r^2 of 0.53 with rs266729. In addition, three SNPs (rs822395, rs2241766, and -10677C>T) identified in our previous study (18), but not in the HapMap project, were also genotyped in this study. Genotyping was performed using the MassARRAY system (Sequenom, San Diego, CA) with the iPLEX^{TM} assay in the Genome Research Centre, University of Hong Kong.

Phenotypes and other variables of interest

Hypertension was defined as blood pressure ≥140/90 mmHg or on anti-hypertensive medication. Pulse pressure was defined as the difference between systolic and diastolic blood pressure. Mean arterial pressure (MAP) was calculated as the sum of diastolic blood pressure and one-third of the pulse pressure. Blood pressure was measured as described previously (16, 17). Circulating total adiponectin level was measured with an in-house sandwich enzyme-linked immunosorbent assay established in our laboratory (intra-assay and inter-assay coefficients of variation of 6.2-8.3% and 5.1-6.4% respectively) (19). Plasma high sensitivity C-reactive protein (hsCRP) was measured as described previously (20, 21). Drinking was defined as consumption of alcoholic drinks at least once a week. Regular exercise was defined as having exercise for ≥30 minutes at least once a week in the past month. Smoking was defined as ever smoking in their lives. Other clinical parameters such as glucose, insulin, and lipid profile were measured as described previously (16, 17).

Statistical analysis

Haploview version 4.1 was used to assess linkage disequilibrium (LD) (22). Analysis of SNPs and haplotypes were performed using program PLINK (version 1.0.6) (23). Variables with skewed distributions were ln-transformed before analysis. Multivariate logistic or

linear regression models were used to estimate the odds ratios (OR) or regression coefficients under the assumption of an additive effect of allele dosage. Correction for multiple testing was performed by the SNP spectral decomposition method (SNPSpD) (24). Under this method, the effective number of independent marker loci (M_{effLi}) was 9 and the experimental-wide significance threshold to keep type 1 error rate at 5% was 0.0057. Correction for testing of multiple phenotypes was not performed as the phenotypes tested were closely related to each other. The *P* values for interaction were estimated by including the multiplicative interaction term in the multivariate regression models in full sample after adjusting for the main effects of all covariates.

In PLINK, haplotypes were inferred using the expectation-maximization algorithm. For haplotype analysis, only haplotypes with frequency >0.05 were tested. An omnibus test was performed to assess the global *P* value of the overall variation at the locus.

Results

Subject characteristics and genotyping

Table 1 shows the characteristics of the 1616 subjects in the sub-cohort who were persistently hypertensive (*n*=285) or normotensive (*n*=1331) during the 6.4-year follow-up period. As expected, hypertensive subjects had worse cardiovascular risk profile and lower adiponectin level ($P<0.001$) than the normotensive subjects.

Among the 15 SNPs genotyped, only the SNP rs7649121 showed significant deviation from Hardy-Weinberg equilibrium ($P<0.0001$ among all subjects or among case- and control-specific subgroups) and was excluded from subsequent analysis. This SNP also showed significant deviation from Hardy-Weinberg equilibrium in the HapMap Han Chinese

population (P=0.0056). The genotyping rates of the remaining 14 SNPs were all ≥99.2%. There were 5 SNP pairs showing high pairwise LD pattern (SNPs 1 & 5, SNPs 6 & 7, SNPs 9 & 11, SNPs 9 & 13 and SNPs 11 & 13; all r^2>0.80).

Hypertension

Among the 14 SNPs, the minor allele of SNP 2 was significantly associated with higher odds of hypertension after adjusting for age and sex, and correction for multiple testing (P=0.0044) (Table 2). The association remained significant after further adjusting for BMI, triglycerides, high-density lipoprotein (HDL) cholesterol, homeostasis model assessment of insulin resistance index (HOMA-IR), and 2-h post-oral glucose tolerance test (OGTT) glucose (OR [95% CI] = 1.49 (1.13-1.95), P=0.0044). In a separate analysis, similar results was obtained under the assumption of dominant allelic effect (P=0.0024 in full adjustment model). None of the SNP showed significant sex-interaction (P>0.05). In forward stepwise multiple logistic regression, this SNP was a significant independent factor of hypertension (Table 3).

The minor allele of SNP 2 was associated with higher percentage of taking anti-hypertensive medication (12.2%, 16.1%, and 17.3% for subjects with CC, CG, and GG genotypes respectively, P=0.004) and no significant association was found with other clinical characteristics (data not shown). To examine the relationship between SNP 2 genotype and blood pressure traits, we adjusted the blood pressure values in subjects on anti-hypertensive medication by adding 10 and 5 mmHg to systolic and diastolic blood pressures respectively (25). Using this method, the minor allele of SNP 2 was associated with higher diastolic blood pressure after adjusting for age, sex, BMI, triglycerides, HDL cholesterol, HOMA-IR, and 2-h post-OGTT glucose (β=0.053, P=0.019). The diastolic blood pressures (mean±S.D.)

was 74.4±10.2, 75.2±11.0, and 75.7±10.7 mmHg in subjects with CC, CG, and GG genotypes respectively. There was no significant sex-interaction (*P* for interaction=0.669).

Plasma adiponectin level

Among the 1385 subjects with adiponectin level measured, the level was negatively correlated with BMI, waist circumference, blood pressure, MAP, triglycerides, low-density lipoprotein (LDL) cholesterol, fasting glucose, 2-h post-OGTT glucose, fasting insulin, HOMA-IR and hsCRP, and positively correlated with age and HDL cholesterol (all $P<0.05$ after adjusting for age and sex, where appropriate). The level was significantly higher in women ($P<0.001$) and was not related to drinking, smoking, and regular exercise ($P>0.05$). Among hypertensive subjects, the level was significantly lower in subjects with anti-hypertensive medication treatment than those without (geometric mean [95% CI] = 5.78 [5.34-6.26] and 7.83 [6.35-9.66] mg/l respectively, $P<0.001$ after adjusting for age and sex). In forward stepwise multiple linear regression, female gender, higher age, lower BMI, lower triglycerides, higher HDL cholesterol, lower 2-h post-OGTT glucose, and lower HOMA-IR are independent factors associated with higher adiponectin level, explaining 31.3% variation in adiponectin level (Table 4). Sex-specific analysis showed similar results (data not shown). Therefore, in all subsequent analysis of adiponectin level, all models were adjusted for age, sex, BMI, triglycerides, HDL cholesterol, 2-h post-OGTT glucose, and HOMA-IR.

Several genetic variants were significantly associated with adiponectin level after adjusting for covariates (Figure 1A). The minor alleles of SNPs 2 and 4 were significantly associated with lower level ($\beta=-0.067$, $P=0.0037$ and $\beta=-0.097$, $P<0.0001$ respectively) whereas the minor alleles of SNPs 3 and 8 were significantly associated with higher level ($\beta=0.069$, $P=0.0027$ and $\beta=0.103$, $P<0.0001$ respectively). None of the SNP showed significant

sex-interaction (P>0.05). Figure 1B shows the unadjusted geometric mean (95% CI) of adiponectin level stratified by the genotypes of these four SNPs in men and women.

Haplotype analysis

Haplotypes was constructed using the SNPs 2, 3, 4, and 8 as these SNPs showed significant association with adiponectin level. There were four common haplotypes with frequency >5%, namely, CCGT (36.2%), GCAG (24.1%), CCAG (16.2%), and CCGG (16.1%). Haplotype analysis revealed significant associations with both adiponectin level and hypertension (global P<0.001 and 0.01 respectively after adjusting for covariates). However, the overall haplotype association with adiponectin level became non-significant after controlling for the SNPs 4 (P=0.085) or 8 (P=0.217) alone, but not SNPs 2 (P=0.0007) or 3 (P=0.0007), suggesting that the significant haplotype association was mainly contributed by SNPs 4 and 8. Similarly, the overall haplotype association with hypertension became non-significant after controlling for the SNP 2 (P=0.124) alone, but not SNPs 3 (P=0.011), 4 (P=0.005), or 8 (P=0.004), suggesting that the significant haplotype association was mainly contributed by SNP 2.

Discussion

In this study, we showed that four genetic variants, namely rs266729 (-11377C>G), -10677C>T, rs182052 (-10066G>A), and rs12495941 (-2668G>T) were associated with adiponectin level, with the effect being stronger for rs182052 and rs12495941. Among these SNPs, rs266729, and -10677C>T are located in the gene promoter region whereas rs182052 and rs12495941 are located in the intron 1 region. When compared with other previous studies, there seems to be ethnic difference in the associations of genetic variants with adiponectin level. For example, in the Genetic Epidemiology of Metabolic Syndrome

Study, SNPs rs3774261 (712A>G) and rs6773957 (which were almost in complete LD) were the most strongly associated SNPs in *ADIPOQ* gene in Northern and Western European populations (11). In both the Insulin Resistance and Atherosclerosis Study Family Study and the Framingham Offspring Study (9, 12), the most strongly associated SNP was rs1700539 (-11391G>A), which was not found in our population (18).

Based on the Alibaba2.1 program (26), the SNP rs12495941 is not involved in any putative transcription binding site whereas the presence of the minor allele of the SNP rs182052 results in a loss of a Sp1 binding site and gain of a CCAAT/enhancer binding protein (C/EBP) β binding site. Both binding sites are involved in adipocyte differentiation (27, 28). It has been shown that the first intron of the human *ADIPOQ* gene contains a gene expression enhancer element which responds to C/EBP α, but not C/EBP β (29). It is interesting to investigate whether this enhancer element could also respond to Sp1.

Previous association studies on hypertension produced inconsistent results, likely due to small contribution of individual genes, inadequate power for multiple testing, and confounding by environmental factors. In this study, we included only subjects who were persistently hypertensive or normotensive for the entire 6.4-year follow-up period. This could help to minimize the confounding effect of environmental factors and phenotype misclassification, and hence improve our power. In this way, our study is cross-sectional and may not establish the causal relationship between genetic variants in the *ADIPOQ* gene and hypertension. However, given that we have shown previously in a sub-cohort of the CRISPS study that hypoadiponectinemia is a predictor of hypertension at year 5 (7) and genotypes are randomly assigned at conception, the finding of the SNP rs266729, associated with both adiponectin level and hypertension, may provide further supporting evidence for a

role of adiponectin in the development of hypertension. This SNP showed a difference in MAF of about 4.4% between hypertensive and normotensive subjects, and a difference in diastolic blood pressure of about 1.3 mmHg between subjects with CC and GG genotypes. Such small effect size is consistent with the findings from recent genome-wide association studies (30, 31). Nevertheless, our findings will need to be confirmed by an independent larger prospective cohort with a better power to detect associations with smaller effect size.

We and other previously reported that the SNP rs2241766 (45T>G) was associated with or predict the development of diabetes or hyperglycemic status (18, 32). Although dysglycemia and raised blood pressure co-exist in a large proportion of subjects in our local population (17), it was rs266729, rather than rs2241766, that was associated with hypertension. Interestingly, rs266729 has been reported to be associated with diabetes, severe forms of obesity, carotid intima media thinkness, and other cardiovascular risks (33-37). This SNP was associated with adiponectin level in Caucasians (10, 33, 38). This SNP was also associated with diabetic nephropathy in female type 1 diabetic patients and the presence of the minor G allele was shown to destroy the binding site of transcriptional stimulatory protein, Sp1 (39). In a recent study, the minor allele of this SNP showed altered DNA binding activity, leading to lower basal and inducible promoter activity in mouse 3T3-L1 adipocytes (40).

A recent study has demonstrated the significant genetic correlation of plasma adiponectin with HDL cholesterol and fasting insulin (41), in keeping with the well-known close association of hypoadiponectinemia with insulin resistance (1), an established risk factor for hypertension. Hypoadiponectinemia may also cause hypertension through several potential mechanisms such as insulin resistance, sympathetic activation, increased circulating fatty acid

levels via reduced fatty acid oxidation, impaired endothelium-dependent vasodilation, and vascular inflammation (7). Although hypertension was associated with lower adiponectin level (5-7), there were only few studies on the association of genetic variants with hypertension, which were all in Asians. One study in Taiwan only genotyped the SNP rs2241766 (45T>G) and reported no significant association with hypertension (6). Another Chinese study only genotyped the SNPs rs2241766 (45T>G) and rs1501299 (276G>T), and reported no significant association with hypertension (14). In a Japanese study which examined two SNPs in men subjects, a rare genetic variant, I164T (with MAF of 1.5% in hypertensive subjects and 0.2% in normotensive subjects), has been shown to be associated with lower adiponectin level and higher odds of hypertension (5). Although rare mutation in key regulatory pathway can often give rise to extreme phenotypes, it is unlikely to be the cause of common multifactorial diseases like hypertension in the general population (42).

In conclusion, several genetic variants in the *ADIPOQ* gene were associated with adiponectin level in this Chinese population and among these variants, the SNP rs266729 was associated with hypertension among subjects who were persistently hypertensive or normotensive during a 6.4-year follow-up period. No significant sex-interaction was found between genetic variants and adiponectin level or hypertension. Taken together with previous reports on hypoadiponectinemia in the prediction of hypertension (7, 8), our study has provided further evidence for a role of adiponectin in the development of hypertension.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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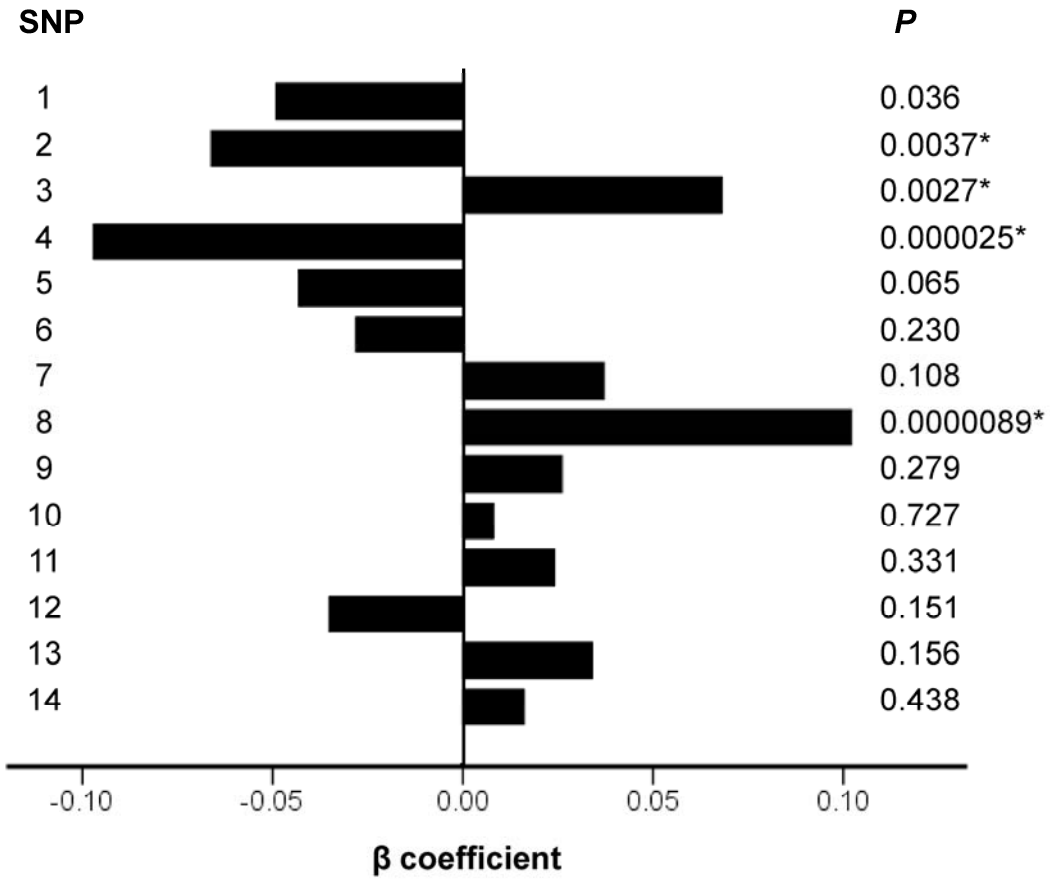
(a)



(b)

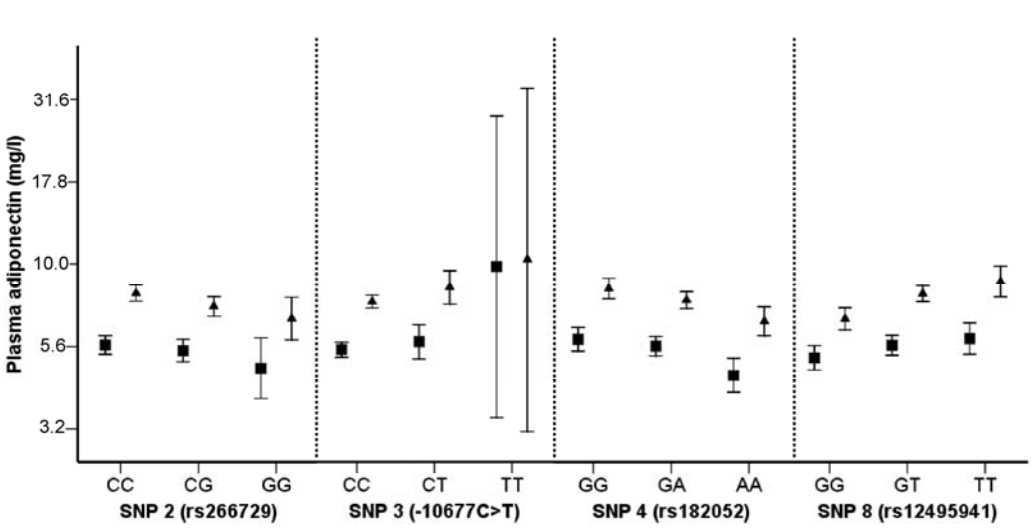


Figure 1. Associations of SNPs with adiponectin level. (A) The bar shows the magnitude of β coefficients in multiple linear regression analysis of adiponectin level (ln-transformed)

after adjusting for age, sex, BMI, triglycerides (ln-transformed), HDL cholesterol, 2-h post-OGTT glucose (ln-transformed), and HOMA-IR (ln-transformed). *P values that can pass multiple testing correction (P<0.0057). (B) Unadjusted geometric mean of plasma adiponectin level stratified by different genotypes of the four SNPs that showed significant association with plasma adiponectin. The error bar shows the 95% CI of the geometric mean. Men and women are represented by a square and a triangle respectively.

Table 1 Clinical characteristics of the 1616 subjects at the follow-up.

	Normotensive (*n*=1331)	**Hypertensive (*n*=285)**
Age, years	48.8±10.5	63.2±9.9‡
Women, %	56.0	50.9
BMI, kg/m^2	23.5±3.3	26.5±3.9‡
Waist circumference, cm	78.0±9.4	85.9±10.0‡
Systolic blood pressure, mmHg§	113.9±11.3	153.9±14.3‡
Diastolic blood pressure, mmHg§	72.1±8.2	86.3±12.7‡
Pulse pressure, mmHg§	41.8±9.0	67.6±15.8‡
MAP, mmHg§	86.0±8.3	108.9±11.0‡
Triglycerides, mmol/l	1.09 (1.07-1.12)	1.49 (1.41-1.58)‡
HDL cholesterol, mmol/l	1.37±0.37	1.24±0.34‡
LDL cholesterol, mmol/l	3.24±0.80	3.54±0.90*
Fasting glucose, mmol/l	5.14 (5.09-5.18)	5.90 (5.74-6.05)‡
2-h post-OGTT glucose, mmol/l	6.55 (6.43-6.67)	8.38 (7.99-8.78)‡
Fasting insulin, mIU/l	6.91 (6.72-7.10)	10.10 (9.41-10.84)‡
HOMA-IR	1.58 (1.53-1.63)	2.65 (2.44-2.87)‡
hsCRP, mg/l	0.61 (0.57-0.65)	1.18 (1.05-1.34)‡
Adiponectin, mg/l‖		
Men	5.63 (5.34-5.94)	5.26 (4.71-5.87)‡
Women	7.91 (7.56-8.28)	7.11 (6.44-7.86)‡
Smoking, %	29.8	31.6
Drinking, %	9.8	9.2
Regular exercise, %	29.2	44.3
Anti-hypertensive medication, %	-	79.3

Data are expressed as mean±S.D. or geometric mean (95% CI) unless otherwise stated.

*P<0.05, †P<0.01, and ‡P<0.001 for normotensive versus hypertensive subjects after adjusting for age and sex, where appropriate.

§Subjects on anti-hypertensive medication were excluded (*n*=226).

‖Data were available in 1145 normotensive (514 men and 631 women) and 240 hypertensive (120 men and 120 women) subjects.

Table 2 Associations of SNPs with hypertension.

No.	SNP	Alleles (Major: minor)	MAF		OR (95% CI)	*P*
			NT	HT		
1	rs16861194	A:G	0.168	0.147	0.82 (0.62-1.10)	0.186
2	rs266729	C:G	0.239	0.283	1.40 (1.11-1.77)	0.0044†
3	-10677C>T*	C:T	0.064	0.070	1.10 (0.73-1.66)	0.660
4	rs182052	G:A	0.407	0.428	1.17 (0.94-1.44)	0.162
5	rs16861205	G:A	0.161	0.144	0.83 (0.62-1.11)	0.200
6	rs822395	A:C	0.151	0.151	0.85 (0.63-1.16)	0.300
7	rs822396	A:G	0.133	0.125	0.78 (0.56-1.08)	0.140
8	rs12495941	G:T	0.419	0.412	1.01 (0.82-1.25)	0.903
9	rs2241766	T:G	0.298	0.288	0.99 (0.79-1.24)	0.933
10	rs1501299	G:T	0.260	0.275	1.06 (0.85-1.33)	0.613
11	rs3774262	G:A	0.301	0.290	1.00 (0.80-1.25)	0.989
12	rs6773957	A:G	0.441	0.439	0.97 (0.79-1.20)	0.778
13	rs1063539	G:C	0.296	0.291	1.02 (0.81-1.28)	0.875
14	rs12629945	G:A	0.184	0.173	0.99 (0.75-1.30)	0.933

HT, hypertension; NT, normotension.

All OR and *P* values were adjusted for age and sex.

*No rs number is assigned for this SNP.

†*P* values that can pass multiple testing correction ($P<0.0057$).

Table 3 Stepwise multiple logistic regression for hypertension in the sub-cohort of 1616 subjects.

Parameter	**OR (95% CI)**	***P***
Age, years	1.14 (1.12-1.16)	<0.001
BMI, kg/m^2	1.19 (1.12-1.25)	<0.001
Triglycerides, mmol/l (ln-transformed)	1.56 (1.07-2.28)	0.021
HOMA-IR (ln-transformed)	2.14 (1.51-3.04)	<0.001
SNP 2 (rs266729)	1.46 (1.11-1.92)	0.006
Nagelkerke r^2	0.444	

Age, sex, BMI, triglycerides (ln-transformed), HDL cholesterol, 2-h post-OGTT glucose (ln-transformed), HOMA-IR (ln-transformed), hsCRP (ln-transformed), and SNP 2 were allowed to enter into the stepwise model.

Table 4 Stepwise multiple linear regression for adiponectin level (ln-transformed).

Parameter	**β**	***P***
Age, y	0.213	<0.001
Female gender	0.154	<0.001
BMI, kg/m^2	-0.091	0.001
Triglycerides, mmol/l (ln-transformed)	-0.098	<0.001
HDL cholesterol, mmol/l	0.234	<0.001
2-h post-OGTT glucose, mmol/l (ln-transformed)	-0.098	<0.001
HOMA-IR (ln-transformed)	-0.140	<0.001
R^2	0.313	

Age, sex, BMI, systolic blood pressure, MAP, triglycerides (ln-transformed), HDL cholesterol, 2-h post-OGTT glucose (ln-transformed), HOMA-IR (ln-transformed), and hsCRP (ln-transformed) were allowed to enter into the stepwise model.