

Plasma resistin concentration determined by common variants in the resistin gene and associated with metabolic traits in an aged Japanese population

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Received: 19 May 2009 / Accepted: 5 August 2009 / Published online: 1 September 2009
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Abstract

Aims/hypothesis Resistin is a cytokine derived from adipose tissue and is implicated in obesity-related insulin resistance and type 2 diabetes mellitus. Polymorphisms of the resistin gene (*RETN*) have been shown to affect the

Electronic supplementary material The online version of this article (doi:10.1007/s00125-009-1517-2) contains supplementary material, which is available to authorized users.

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plasma resistin concentration. The aims of this study were to identify polymorphisms of *RETN* that influence plasma resistin concentration and to clarify the relation between plasma resistin level and metabolic disorders in an aged Japanese cohort.

Methods The study participants comprised 3133 individuals recruited to a population-based prospective cohort study (KING study). Plasma resistin concentration, BMI, abdominal circumference, blood pressure, fasting plasma glucose and serum insulin concentrations, HbA_{1c} content and serum lipid profile were measured in all participants. The HOMA index of insulin resistance (HOMA-IR) was also calculated. Eleven polymorphisms of *RETN* were genotyped.

Results A combination of ANOVA and multiple linear regression analysis in screening and large-scale subsets of the study population revealed that plasma resistin concentration was significantly associated with rs34861192 and rs3745368 polymorphisms of *RETN*. Multiple linear regression analysis with adjustment for age and sex also showed that the plasma resistin level was significantly associated with serum concentrations of HDL-cholesterol, triacylglycerol and insulin, as well as with BMI.

Conclusions/interpretation Our results implicate the rs34861192 and rs3745368 polymorphisms of *RETN* as robust and independent determinants of plasma resistin concentration in the study population. In addition, plasma resistin level was associated with dyslipidaemia, serum insulin concentration and obesity.

Trial registration: ClinicalTrials.gov NCT00262691

Funding: This study was supported by Grants-in-Aids for Scientific Research from the Japan Society for the Promotion of Science and the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Keywords Cohort study · Dyslipidaemia · Insulin · Obesity · Polymorphism · Resistin

Abbreviations

FPG	Fasting plasma glucose concentration
HOMA-IR	HOMA index of insulin resistance
HWE	Hardy–Weinberg equilibrium
IRI	Fasting serum immunoreactive insulin concentration
MAF	Minor allele frequency
SNP	Single nucleotide polymorphism
SRE	Sterol regulatory element
SREBP1c	SRE-binding protein 1c

Introduction

Although adipose tissue used to be thought of as an inert energy depot, adipocytes and macrophages in adipose tissue have emerged as a key secretory system [1]. Adipose tissue thus produces and releases various factors that are known as adipocytokines (or adipokines) and include adiponectin, leptin, TNF- α , plasminogen-activator inhibitor 1 and resistin.

Resistin has been implicated as a potential link between obesity and insulin resistance. The circulating concentration of resistin is markedly increased in genetic and diet-induced murine models of obesity. Moreover, the serum resistin concentration was found to be reduced by treatment of mice with rosiglitazone, an insulin-sensitising drug that interacts with peroxisome proliferator-activated receptor γ . These observations thus suggest that resistin might play a role in obesity-related insulin resistance and type 2 diabetes [2].

Macrophages are thought to be the predominant source of circulating resistin in humans [3, 4]. Resistin is also considered to be a biomarker or mediator of metabolic and inflammatory diseases [5–8]. Some studies have demonstrated an association between increased serum resistin concentration and metabolic disorders, including obesity, insulin resistance, type 2 diabetes, hypertension, dyslipidaemia and atherosclerotic cardiovascular disease [9–15]. Moreover, polymorphisms of the human resistin gene (*RETN*) have also been found to be associated with such disorders [16–25]. However, not all studies have reproduced such findings [26–28]. Although polymorphisms in the promoter region of *RETN* have also been shown to influence the circulating level of resistin [25, 29], the genetic and environmental determinants of the circulating resistin concentration remain unclear.

To clarify the relation between plasma resistin level and metabolic disorders including obesity, insulin resistance, type 2 diabetes, hypertension and dyslipidaemia, and to

elucidate the determinants of plasma resistin concentration, we have now performed a large-scale, population-based and cross-sectional study in an aged Japanese cohort.

Methods

Study population This study is part of the ongoing KING (KIItaNagoya Genome) study, a community-based prospective observational study of the genetic basis of cardiovascular disease and its risk factors, which has been following 3,975 participants who underwent community-based annual health checkups in Kitanagoya City, Japan, between May 2005 and December 2007. The participants were aged 50 to 80 years at recruitment and are all native to Japan. The participants of the present study comprised 3,133 individuals (1,396 men, 1,737 women) from the original cohort, whose blood samples were available for measurement of plasma resistin concentration. Blood samples for analytic measurements and extraction of DNA were collected from participants after they had fasted overnight. BMI, abdominal circumference, BP, fasting plasma glucose concentration (FPG), fasting serum immunoreactive insulin concentration (IRI), HbA_{1c} content and serum lipid profile were determined for all participants. The HOMA index of insulin resistance (HOMA-IR) was calculated as: FPG (mmol/l) \times IRI (pmol/l)/156.3. Diabetes mellitus, hypertension and dyslipidaemia were defined respectively as (1) an FPG of ≥ 7.0 mmol/l, HbA_{1c} of $\geq 6.5\%$ or treatment with glucose-lowering drugs; (2) systolic BP of ≥ 140 mmHg, diastolic BP of ≥ 90 mmHg or treatment with antihypertensive drugs; and (3) serum total cholesterol concentration of ≥ 5.68 mmol/l, serum HDL-cholesterol concentration of < 1.04 mmol/l, serum triacylglycerol concentration of ≥ 1.69 mmol/l or treatment with antihyperlipidaemic drugs. The prevalence of metabolic syndrome was assessed using the definition proposed by the International Diabetes Federation [30]. The clinical characteristics of all participants are summarised in Table 1. The study was approved by the Ethics Review Board of Nagoya University School of Medicine and all participants provided written informed consent.

Measurement of plasma resistin concentration The plasma concentration of resistin was measured with an ELISA kit for human resistin (LINCO Research, St Charles, MS, USA). In our hands, the lowest level of human resistin detectable by the assay was 0.16 ng/ml and the inter- and intra-assay coefficients of variation were 3.5% and 7.3%, respectively.

Selection of polymorphisms Using public databases, including PubMed, Online Mendelian Inheritance in Man (www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM), accessed 3 April

Table 1 Characteristics of the study participants

Characteristic	Screening study (<i>n</i> =500)	Large-scale study (<i>n</i> =2,633)	Total (<i>n</i> =3,133)	<i>p</i> value
Male, <i>n</i> (%)	249 (49.8)	1147 (43.6)	1396 (44.6)	0.01
Age (years)	62.8±6.7	63.8±6.5	63.6±6.6	1.25×10 ⁻³
Height (cm)	158.3±8.4	157.3±7.8	157.5±7.9	0.74
Weight (kg)	58.0±10.0	56.6±9.5	56.8±9.6	0.18
BMI (kg/m ²)	23.1±3.0	22.8±3.0	22.9±3.0	0.11
Abdominal circumference (cm)	83.7±8.5	83.6±8.3	83.6±8.3	0.81
Systolic BP (mmHg)	134.5±19.7	132.0±19.6	132.4±19.6	0.07
Diastolic BP (mmHg)	77.7±11.9	76.9±11.0	77.0±11.1	0.27
Serum total cholesterol (mmol/l)	5.5±0.8	5.6±0.9	5.6±0.9	0.72
Serum triacylglycerol (mmol/l)	1.3±0.7	1.3±0.8	1.3±0.7	0.24
Serum HDL-cholesterol (mmol/l)	1.6±0.4	1.6±0.4	1.6±0.4	0.76
Serum LDL-cholesterol (mmol/l)	3.3±0.8	3.4±0.8	3.4±0.8	0.82
FPG (mmol/l)	5.4±1.1	5.4±1.2	5.4±1.2	0.79
IRI (pmol/l)	31.6±37.4	38.5±45.9	37.4±44.7	0.05
HOMA-IR	1.2±1.6	1.4±2.1	1.4±2.0	0.09
HbA _{1c} (%)	5.3±0.7	5.4±0.7	5.4±0.7	0.36
Plasma resistin (ng/ml)	11.2±6.6	11.0±7.5	11.0±7.4	0.61
History of disorder				
Hypertension, <i>n</i> (%)	271 (54.2)	1259 (47.8)	1530 (48.8)	0.06
Type 2 diabetes, <i>n</i> (%)	66 (13.2)	298 (11.3)	364 (11.6)	0.18
Dyslipidaemia, <i>n</i> (%)	238 (47.6)	1316 (50.0)	1554 (49.6)	0.73
Metabolic syndrome, <i>n</i> (%)	96 (19.2)	517 (19.6)	613 (19.6)	0.74
Myocardial infarction, <i>n</i> (%)	9 (1.8)	25 (0.9)	34 (1.1)	0.07
Angina pectoris, <i>n</i> (%)	17 (3.4)	71 (2.7)	88 (2.8)	0.22
Cerebral infarction, <i>n</i> (%)	8 (1.6)	46 (1.7)	54 (1.7)	0.95

Quantitative data are means±SD

Differences in characteristics between the screening and large-scale studies were evaluated by multiple linear or logistic regression analysis with adjustment for age and sex

2008) and dbSNP (www.ncbi.nlm.nih.gov/SNP, accessed 15 April 2008), we selected 11 previously identified polymorphisms of *RETN* that have a minor allele frequency (MAF) of >1% in Japanese individuals and are uniformly distributed over the entire region of the gene (Electronic supplementary material [ESM] Table 1). The average distance between the polymorphisms was 177 bp. Polymorphisms are referred to by refSNP IDs obtained from the dbSNP website.

Genotyping of polymorphisms Venous blood was collected from each participant into tubes containing EDTA and genomic DNA was isolated with a kit (Qiagen, Chatsworth, CA, USA). Genotyping of the ten single nucleotide polymorphisms (SNPs) was performed with a fluorescence- or colorimetry-based, allele-specific, DNA primer-probe assay system (Toyobo Gene Analysis, Tsuruga, Japan) as described previously [31] (ESM Table 1). The number of ATG repeats in the 3' untranslated region of *RETN* was determined by fragment analysis. Genomic DNA (20 ng) was subjected to

PCR in a final volume of 25 µl with a forward primer labelled at the 5' end with 6-carboxy-fluorescein and with a reverse primer (ESM Table 1). A 1 µl portion of the reaction mixture was then mixed with 9 µl of formamide and 0.2 µl of ROX-500 size markers (Applied Biosystems, Foster City, CA, USA), heated at 94°C for 2 min and fractionated by capillary electrophoresis with an ABI 3100 system (Applied Biosystems). The genotyping success rate was >99% for all polymorphisms (average, 99.9%). To confirm the accuracy of genotyping by the allele-specific DNA primer-probe assay, we randomly selected 100 DNA samples and subjected them to PCR followed by restriction fragment length polymorphism analysis or direct DNA sequencing. In each instance, the genotype determined by the allele-specific DNA primer-probe assay system was identical to that determined by the confirmatory methods. In addition, we genotyped all samples with a positive control (90 samples with a positive control at a time) and confirmed that the positive control was genotyped correctly.

Statistical analysis History of disorders and other clinical variables were compared between the screening and large-scale study populations using multiple logistic regression analysis and multiple linear regression analysis, respectively, adjusted for age and sex. Allele frequencies were estimated by the gene-counting method and the χ^2 test used to identify departures from Hardy–Weinberg equilibrium (HWE). The final p values were determined by multiplying raw p values by the number of genotyped polymorphisms for Bonferroni correction; a final p value of <0.05 was considered significant for departure from HWE after Bonferroni correction. Haplotype analysis using Haploview 4.0 software (www.broad.mit.edu/mpg/haploview/index.php, accessed 17 April 2008) was performed to detect linkage disequilibrium.

Plasma resistin concentration was compared among genotypes by one-way ANOVA and Scheffe's multiple comparison test. Multiple linear regression analysis with a stepwise forward selection procedure was performed to identify polymorphisms that independently influence plasma resistin level; the dependent variable was plasma resistin concentration; independent variables included the genotypes of each polymorphism. The proportion of the variance of plasma resistin concentration (R^2) explained by polymorphisms was assessed on the basis of results of one-way ANOVA and multiple linear regression analysis: $R^2 = (\text{explained sum of squares by ANOVA or regression model}) / (\text{total sum of squares of plasma resistin level})$. The significance level for inclusion in and exclusion from the model was $p=0.05$.

We also performed simple linear regression analysis to evaluate the relation between plasma resistin concentration and physical or clinical variables including age, sex, BMI, abdominal circumference, FPG, HbA_{1c}, IRI, HOMA-IR, systolic or diastolic BP, serum concentrations of total cholesterol, HDL-cholesterol or triacylglycerol, and history (0=no history, 1=positive history) of type 2 diabetes, hypertension, dyslipidaemia, metabolic syndrome, myocardial infarction, angina pectoris or cerebral infarction. To clarify the isolated relations of physical or clinical variables to plasma resistin level, we performed multiple linear regression analysis with adjustment for age and sex; the dependent variable was plasma resistin concentration, the independent variables included age, sex (male=1, female=0) and each physical or clinical variable. We performed similar analyses with adjustment for age, sex and BMI. The relation between each physical or clinical trait and each polymorphism was assessed by multiple linear regression analysis or multiple logistic regression analysis with adjustment for age and sex, or for age, sex and BMI.

We also applied a triangulation approach to determine whether associations between polymorphisms of *RETN* and clinical variables might be mediated by plasma resistin

[32]. The effect sizes of plasma resistin concentration for clinical variables were estimated by multiple linear regression analysis with clinical variables as dependent variables and independent variables including age, sex and plasma resistin level. The effect size per minor allele of *RETN* polymorphisms for plasma resistin level were estimated by multiple linear regression analysis with plasma resistin level as the dependent variable and independent variables including age, sex and the genotype of each polymorphism. The effect size per minor allele of *RETN* polymorphisms for clinical variables was estimated by multiple linear regression analysis with clinical variables as dependent variables and independent variables including age, sex and the genotype of each polymorphism.

Statistical analyses were performed with SPSS 14.0J (SPSS, Chicago, IL, USA). The Bonferroni correction was applied to analysis of the relation between plasma resistin level and history of disorders (raw p value $\times 7$ for multiple linear regression analysis), other clinical variables (raw p value $\times 12$ or 11 for multiple linear regression analysis) or *RETN* polymorphisms (raw p value $\times 11$ for ANOVA in screening and large-scale study populations). It was also applied to analysis of the relation between *RETN* polymorphisms and history of disorders (raw p value $\times 7$ for multiple logistic regression analysis) or other clinical variables (raw p value $\times 12$ or 11 for multiple linear regression analysis). A corrected p value of <0.05 was considered statistically significant. Power was calculated on the basis of the observed effect and sample size with the use of a general linear model for ANOVA, with $\alpha=0.0045$ (0.05/11, with 11 being the number of polymorphisms genotyped in the study).

Study design We initially evaluated the relation between plasma resistin concentration and physical or clinical variables in all participants. We then performed a screening study with the 11 polymorphisms of *RETN* and 500 participants randomly selected from the total study population. One-way ANOVA and multiple linear regression analysis with a stepwise forward selection procedure identified three polymorphisms as being independently related to plasma resistin concentration. These three polymorphisms, as well as the rs1862513 (–420C→G) polymorphism, were then examined for their association with plasma resistin level in a large-scale study with the remaining 2633 participants. Finally, we also assessed the relation between selected polymorphisms and metabolic traits.

Results

Relation of plasma resistin concentration to physical or clinical variables We initially assessed the relation of plasma resistin level to various physical and metabolic

variables in all study participants ($n=3133$). Simple linear regression analysis revealed that age, sex, abdominal circumference, BMI, systolic BP, diastolic BP, serum triacylglycerol and HDL-cholesterol concentrations, IRI and HOMA-IR were significantly related to plasma resistin concentration. To clarify the independent relations of physical or metabolic variables to plasma resistin level, we performed multiple linear regression analysis with adjustment for age and sex (model 1) or for age, sex and BMI (model 2). BMI, serum concentrations of triacylglycerol and HDL-cholesterol, and IRI were significantly associated with plasma resistin level independently of age and sex; the relation between plasma resistin and serum concentrations of triacylglycerol and HDL-cholesterol, and IRI remained significant after adjustment for age, sex and BMI (Table 2). We also evaluated the relation between

plasma resistin concentration and prevalence of hypertension, type 2 diabetes mellitus, dyslipidaemia, metabolic syndrome, myocardial infarction, angina pectoris and cerebral infarction; evaluation was by multiple linear regression analysis with adjustment for age and sex (model 1) or for age, sex and BMI (model 2). However, no significant association was detected (Table 2).

Screening study We first assessed the relation between plasma resistin concentration and the 11 genotyped polymorphisms in the screening population ($n=500$) by one-way ANOVA. The genotype distributions of the 11 polymorphisms were in HWE in this population, with nine polymorphisms accounting for 90% of alleles with a MAF of >0.01 and an r^2 of ≥ 0.8 as revealed by the Tagger function in Haploview. The plasma resistin concentration

Table 2 Relation between plasma resistin concentration and physical or clinical variables

Variables	Model 1			Model 2		
	Effect size \pm SE	<i>p</i> value	R^2	Effect size \pm SE	<i>p</i> value	R^2
Independent variable						
Sex	1.466 \pm 0.264	2.88×10^{-8}	0.010			
Age (years)	0.112 \pm 0.020	2.42×10^{-8}	0.010			
Clinical variables						
BMI (kg/m ²)	0.128 \pm 0.044	0.04	0.003			
Abd. circumference (cm)	0.042 \pm 0.016	0.10	0.002	0.008 \pm 0.032	1.00	<0.001
Systolic BP (mmHg)	0.009 \pm 0.007	1.00	<0.001	0.005 \pm 0.007	1.00	<0.001
Diastolic BP (mmHg)	0.019 \pm 0.012	1.00	<0.001	0.012 \pm 0.012	1.00	<0.001
Total cholesterol (mmol/l) ^a	-0.051 \pm 0.152	1.00	<0.001	-0.074 \pm 0.152	1.00	<0.001
Triacylglycerol (mmol/l) ^a	0.850 \pm 0.175	1.58×10^{-5}	0.007	0.776 \pm 0.180	1.81×10^{-4}	0.006
HDL-cholesterol (mmol/l) ^a	-1.757 \pm 0.318	4.12×10^{-7}	0.009	-1.625 \pm 0.331	1.09×10^{-5}	0.006
LDL-cholesterol (mmol/l) ^a	0.057 \pm 0.161	1.00	<0.001	0.006 \pm 0.162	1.00	<0.001
FPG (mmol/l)	-0.041 \pm 0.113	1.00	<0.001	-0.089 \pm 0.115	1.00	<0.001
IRI (pmol/l)	0.011 \pm 0.003	1.78×10^{-3}	0.005	0.009 \pm 0.003	0.02	0.003
HOMA-IR	0.184 \pm 0.064	0.05	0.003	0.146 \pm 0.066	0.32	0.001
HbA _{1c} (%)	0.150 \pm 0.181	1.00	<0.001	0.078 \pm 0.183	1.00	<0.001
History of disorder						
Hypertension	-0.013 \pm 0.27	1.00	<0.001	-0.205 \pm 0.277	1.00	<0.001
Type 2 diabetes	0.672 \pm 0.41	0.71	<0.001	0.525 \pm 0.413	1.00	<0.001
Dyslipidaemia	-0.170 \pm 0.268	1.00	<0.001	-0.203 \pm 0.268	1.00	<0.001
Metabolic syndrome	0.873 \pm 0.332	0.06	0.002	0.543 \pm 0.374	1.00	<0.001
Myocardial infarction	1.287 \pm 1.266	1.00	<0.001	1.223 \pm 1.264	1.00	<0.001
Angina pectoris	0.081 \pm 0.795	1.00	<0.001	0.024 \pm 0.794	1.00	<0.001
Cerebral infarction	0.026 \pm 1.009	1.00	<0.001	0.035 \pm 1.008	1.00	<0.001

^a From serum

The *p* and R^2 values for age and sex were obtained by simple linear regression analysis. Those for other variables in models 1 and 2 were obtained by multiple linear regression analysis with adjustment either for age and sex or for age, sex and BMI, respectively. The *p* values shown have been corrected by the Bonferroni method by multiplying the raw *p* values by seven for history of disorders or by 12 or 11 for other clinical variables

Effect size is the partial regression coefficient

Abd, abdominal

was found to be significantly related to 7 of the 11 polymorphisms (Table 3), with rs34861192 and rs3219175 among those showing the lowest p values (6.14×10^{-60}) and highest coefficients of determination ($R^2=0.428$). Haplotype analysis with the screening study population ($n=500$) revealed linkage disequilibrium among polymorphisms including rs34861192, rs1862513 and rs3219175, with

rs34861192 and rs3219175 being in perfect linkage disequilibrium (Fig. 1). Multiple linear regression analysis with a stepwise forward selection procedure revealed that rs34861192, rs3745368 and rs3833230 polymorphisms were significantly and independently associated with plasma resistin concentration (Table 4). In three tests, these three polymorphisms captured four of ten (40%) alleles at

Table 3 Comparison of plasma resistin concentration among genotypes for *RETN* polymorphisms by one-way ANOVA in the screening study

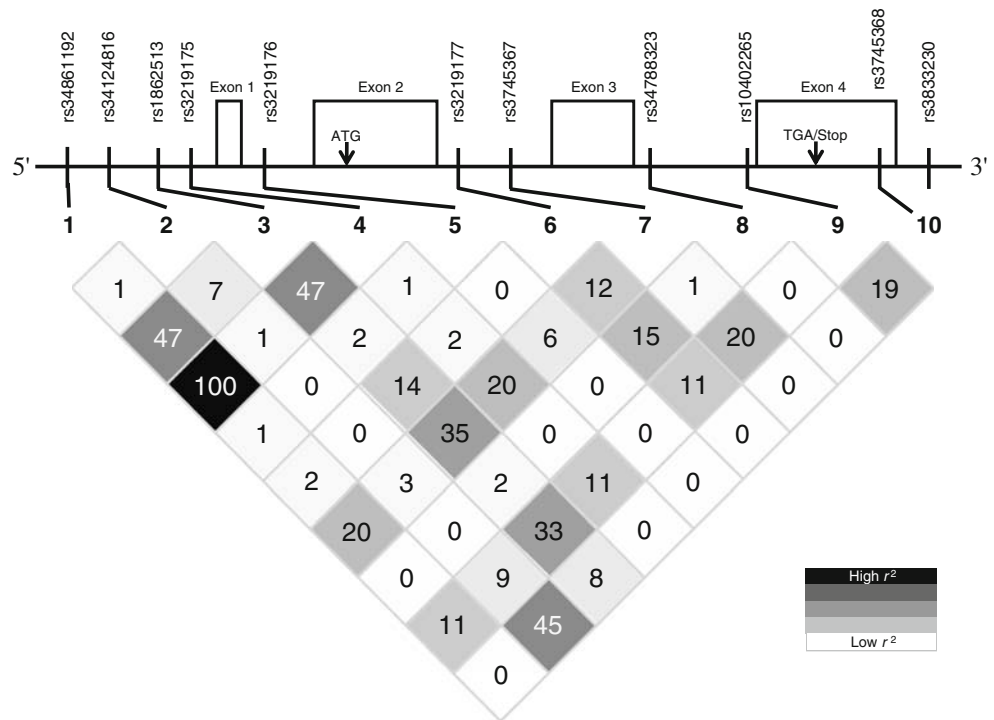
Polymorphism	MAF	Genotype	Participants (n)	Plasma resistin (ng/ml)	R^2	p value (ANOVA)	p value (HWE)	Power
rs34861192	0.220	GG	306	8.0±0.2	0.428	6.14×10^{-60}	1.00	1.000
		AG	168	15.1±0.5				
		AA	26	22.8±1.7				
rs34124816	0.045	AA	456	11.5±0.3	0.035	1.49×10^{-3}	1.00	0.859
		AC	43	7.4±0.6				
		CC	1	2.6±0.0				
rs1862513	0.372	CC	195	8.1±0.3	0.200	8.94×10^{-24}	1.00	1.000
		CG	238	12.0±0.4				
		GG	67	17.0±1.0				
rs3219175	0.220	GG	306	8.0±0.2	0.428	6.14×10^{-60}	1.00	1.000
		AG	168	15.1±0.5				
		AA	26	22.8±1.7				
rs3219176	0.041	CC	460	11.2±0.3	0.003	1.00	1.00	0.032
		CT	39	10.4±1.5				
		TT	1	5.1±0.0				
rs3219177	0.080	CC	422	11.3±0.3	0.003	1.00	1.00	0.041
		CT	76	10.3±0.6				
		TT	2	9.1±3.5				
rs3745367	0.416	GG	177	8.7±0.3	0.137	1.20×10^{-15}	1.00	1.000
		AG	230	11.2±0.4				
		AA	93	15.7±0.9				
rs34788323	0.013	CC	487	11.2±0.3	0.007	0.70	1.00	0.162
		CT	13	7.8±1.2				
		TT	0					
rs10402265	0.314	CC	243	10.0±0.4	0.043	1.87×10^{-4}	1.00	0.941
		CG	200	11.6±0.5				
		GG	57	14.4±1.0				
rs3745368	0.094	GG	414	11.5±0.3	0.017	0.14	0.60	0.425
		AG	78	9.4±0.6				
		AA	8	8.1±1.7				
rs3833230	0.043	6/6	4	14.9±5.5	0.072	1.75×10^{-6}	1.00	0.995
		6/7	25	18.7±1.9				
		7/7	462	10.7±0.3				
		7/8	8	10.9±2.0				
		8/8	1	10.4±0.0				

Plasma resistin concentrations are means±SE

The p values for ANOVA have been corrected by the Bonferroni method by multiplying the raw p values by 11. The p values for identification of departure from HWE were calculated by the χ^2 test

Power at $\alpha=0.05$ is based on variables in the screening study including MAF, the observed effect, the number of polymorphisms genotyped ($n=11$) and the number of samples ($n=500$)

Fig. 1 Pairwise linkage disequilibrium r^2 values (white to black scales, low to high), as determined with Haploview software for the ten studied SNPs of *RETN*. The rs34861192 and rs3219175 polymorphisms were in perfect linkage disequilibrium



r^2 of ≥ 0.8 as revealed by the Tagger function in Haploview. The combination of these three polymorphisms accounted for 47.6% of the variance in plasma resistin level in the screening population.

Large-scale study On the basis of the multiple linear regression analysis in the screening study, we selected rs34861192, rs3745368 and rs3833230 polymorphisms for further study. We also examined the rs1862513 polymorphism in the large-scale study because this SNP has previously been associated with plasma resistin concentration or type 2 diabetes. We first evaluated the relation of plasma resistin concentration to the four selected polymorphisms in the remaining 2,633 participants of the overall study population, both by one-way ANOVA and by Scheffe’s multiple comparison test. The genotype distributions of these polymorphisms were also in HWE

in these participants. All four polymorphisms were significantly related to the plasma resistin concentration by one-way ANOVA and Scheffe’s test (Fig. 2), with rs34861192 and rs1862513 showing the lowest p values. Multiple linear regression analysis with a stepwise forward selection procedure revealed that only rs34861192 and rs3745368 polymorphisms were significantly and independently associated with plasma resistin concentration (Table 4). The combination of these two polymorphisms accounted for 37.9% of the variance in plasma resistin level among all ($n=3,133$) study participants (Table 4).

Both rs34861192 and rs1862513 are located in the promoter region of *RETN* and are in linkage disequilibrium ($r^2=0.47$) (Fig. 1). Of the nine possible combined genotypes for these two polymorphisms, we detected only six among all study participants (Fig. 3). No significant interaction between the two polymorphisms was apparent,

Table 4 Multiple linear regression analysis, with stepwise forward selection procedure, of the relation between plasma resistin concentration and *RETN* polymorphisms

Variables	Screening study ($n=500$)			Large-scale study ($n=2633$)			Total ($n=3133$)		
	MAF	Effect size \pm SE	p value	MAF	Effect size \pm SE	p value	MAF	Effect size \pm SE	p value
Selected polymorphism									
rs34861192	0.220	7.123 \pm 0.372	3.35×10^{-62}	0.201	7.757 \pm 0.202	$<1 \times 10^{-99}$	0.204	7.698 \pm 0.179	$<1 \times 10^{-99}$
rs3745368	0.094	-2.789 \pm 0.501	4.33×10^{-8}	0.092	-2.017 \pm 0.285	1.89×10^{-12}	0.092	-2.181 \pm 0.252	7.47×10^{-18}
rs3833230	0.043	-3.083 \pm 0.941	1.12×10^{-3}						
Constant value		11.429 \pm 0.956	1.53×10^{-15}		8.251 \pm 0.151	$<1 \times 10^{-99}$		8.285 \pm 0.134	$<1 \times 10^{-99}$
R^2			0.476			0.368			0.379

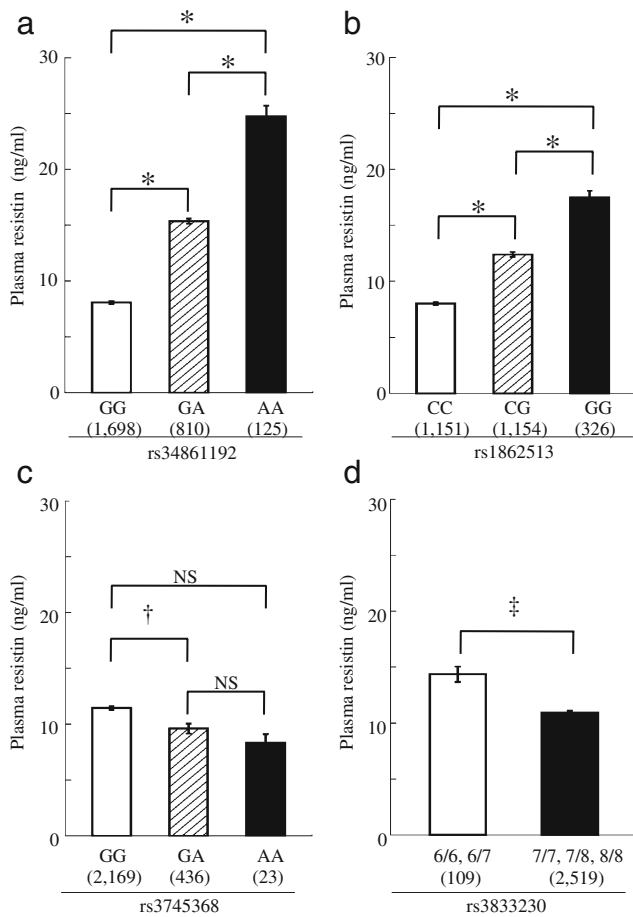


Fig. 2 Association between *RETN* polymorphisms and plasma resistin concentration. The plasma resistin concentration is shown for participants in the large-scale study with the different genotypes of rs34861192 (a), rs1862513 (b), rs3745368 (c) and rs3833230 (d) polymorphisms. The rs3833230 is an ATG repeat variation polymorphism including 6, 7 and 8 allele repeats. Numbers in horizontal axis of (d) indicate genotypes of this polymorphism (e.g. 6/7—participant with 6 repeats allele and 7 allele repeats). Numbers in parentheses indicate numbers of participants with the indicated genotypes. Data are means±SE. * $p < 1 \times 10^{-15}$, † $p = 2.26 \times 10^{-5}$, ‡ $p = 2.26 \times 10^{-6}$ by Scheffe’s test. The corrected p values from one-way ANOVA for the rs34861192, rs1862513, rs3745368 and rs3833230 polymorphisms were $p < 1 \times 10^{-99}$, $p < 1 \times 10^{-99}$, $p = 5.85 \times 10^{-5}$ and $p = 1.29 \times 10^{-3}$, respectively (corrected by the Bonferroni method by multiplying the raw p values by 11). The calculated powers based on the observed effect and sample sizes with $\alpha = 0.05/11 = 0.0045$ were 1.000, 1.000, 0.962 and 0.971 for rs34861192, rs1862513, rs3745368 and rs3833230 polymorphisms, respectively

with the plasma resistin level appearing to depend more on the genotype of rs34861192 than on that of rs1862513 ($R^2 = 0.359$ and 0.180 , respectively) (Fig. 3).

Relation of RETN polymorphisms to metabolic traits We also evaluated the relation between the selected polymorphisms and metabolic traits in the entire study population ($n = 3133$). We did not detect any significant association between the selected polymorphisms and

metabolic traits by multiple linear or logistic regression analysis (Tables 5 and 6). Finally, the results of triangulation analysis revealed that the direction of *RETN* polymorphism–clinical variable effects differed between observed and expected (Table 7).

Discussion

We examined the relation of plasma resistin concentration to 11 polymorphisms of *RETN* as well as to various physical and clinical variables. Our study, with 3133 aged Japanese participants, revealed that, among the *RETN* polymorphisms examined, the rs34861192 (−638G→A) and rs3745368 (+1084G→A) SNPs had the most significant effect on plasma resistin concentration. Indeed, the combination of these two polymorphisms accounted for 37.9% of the variation in plasma resistin level among all study participants. Although SNPs in the promoter region of *RETN* have previously been associated with the circulating resistin level [21, 23, 25, 29], only one previous study has demonstrated an association between resistin level and rs34861192 [29]; in that study, the number of participants was smaller than that in the present study. The rs3745368 polymorphism (also known as +62G→A) in the 3′ untranslated region of *RETN* has previously been associated with hypertension and type 2 diabetes [18, 19, 22].

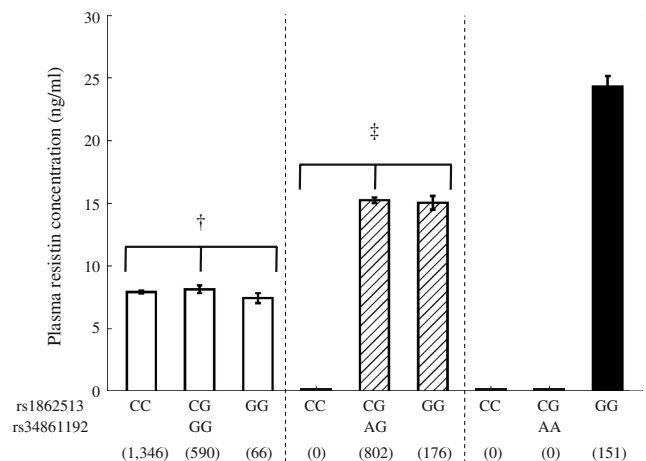


Fig. 3 Effects of combined genotypes of rs34861192 and rs1862513 polymorphisms of *RETN* on plasma resistin concentration for all study participants. Numbers in parentheses indicate numbers of participants with the indicated genotypes. With the GG genotype of rs34861192, the plasma resistin levels were similar in each genotype of rs1862513. In the same way, when the genotype of rs34861192 was AG, a comparison between CG and GG genotypes of rs1862513 showed no significant difference in plasma resistin level. All participants with AA genotype of rs34861192 have only GG genotype of rs1862513. Data are means±SE. † $p = 0.475$, ‡ $p = 0.690$ by one-way ANOVA for each genotype of rs34861192

Table 5 Multiple linear regression analysis of the relation between physical or clinical variables and selected polymorphisms of *RETN* with adjustment for age and sex (model 1) and for age, sex and BMI (model 2) in the entire study population

Dependent variable per polymorphism	Model 1		Model 2	
	Effect size±SE	<i>p</i> value	Effect size±SE	<i>p</i> value
rs34861192				
BMI (kg/m ²)	0.074±0.091	1.00		
Abdominal circumference (cm)	0.066±0.253	1.00	-0.112±0.126	1.00
Systolic BP (mmHg)	0.577±0.581	1.00	0.464±0.565	1.00
Diastolic BP (mmHg)	0.325±0.336	1.00	0.268±0.329	1.00
Serum total cholesterol (mmol/l)	-0.027±0.026	1.00	-0.028±0.026	1.00
Serum triacylglycerol (mmol/l)	-0.008±0.023	1.00	-0.012±0.022	1.00
Serum HDL-cholesterol (mmol/l)	0.003±0.013	1.00	0.006±0.012	1.00
Serum LDL-cholesterol (mmol/l)	-0.027±0.025	1.00	-0.029±0.025	1.00
FPG (mmol/l)	-0.062±0.035	0.96	-0.066±0.035	0.65
IRI (pmol/l)	-2.423±1.374	0.94	-2.737±1.319	0.42
HOMA-IR	-0.125±0.062	0.53	-0.138±0.060	0.24
HbA _{1c}	-0.027±0.022	1.00	-0.030±0.022	1.00
rs3745368				
BMI (kg/m ²)	0.117±0.129	1.00		
Abdominal circumference (cm)	0.220±0.357	1.00	-0.061±0.178	1.00
Systolic BP (mmHg)	0.637±0.82	1.00	0.461±0.797	1.00
Diastolic BP (mmHg)	0.412±0.474	1.00	0.322±0.463	1.00
Serum total cholesterol (mmol/l)	0.024±0.037	1.00	0.022±0.037	1.00
Serum triacylglycerol (mmol/l)	0.032±0.032	1.00	0.026±0.031	1.00
Serum HDL-cholesterol (mmol/l)	-0.024±0.018	1.00	-0.019±0.017	1.00
Serum LDL-cholesterol (mmol/l)	0.034±0.035	1.00	0.031±0.035	1.00
FPG (mmol/l)	0.040±0.050	1.00	0.034±0.049	1.00
IRI (pmol/l)	-1.532±1.940	1.00	-2.029±1.862	1.00
HOMA-IR	-0.034±0.088	1.00	-0.055±0.085	1.00
HbA _{1c}	-0.001±0.031	1.00	-0.005±0.031	1.00

All *p* values have been corrected by the Bonferroni method by multiplying the raw *p* values by 12 (model 1) or 11 (model 2)
Effect size is the partial regression coefficient

Table 6 Multiple logistic regression analysis of the relation between various disorders and selected polymorphisms of *RETN* with adjustment for age and sex (model 1) or for age, sex and BMI (model 2) in the entire study population

Dependent variable per polymorphism	Model 1		Model 2	
	OR (95% CI)	<i>p</i> value	OR (95% CI)	<i>p</i> value
rs34861192				
Hypertension	0.95 (0.84–1.08)	1.00	0.94 (0.82–1.07)	1.00
Type 2 diabetes	1.01 (0.84–1.22)	1.00	1.01 (0.83–1.22)	1.00
Dyslipidaemia	0.97 (0.85–1.09)	1.00	0.96 (0.85–1.09)	1.00
Metabolic syndrome	1.01 (0.86–1.17)	1.00	0.98 (0.82–1.17)	1.00
Myocardial infarction	0.84 (0.45–1.57)	1.00	0.84 (0.45–1.57)	1.00
Angina pectoris	0.78 (0.52–1.16)	1.00	0.78 (0.52–1.16)	1.00
Cerebral infarction	1.00 (0.63–1.61)	1.00	1.00 (0.63–1.61)	1.00
rs3745368				
Hypertension	0.97 (0.82–1.16)	1.00	0.95 (0.80–1.14)	1.00
Type 2 diabetes	1.24 (0.96–1.59)	0.69	1.23 (0.95–1.58)	0.79
Dyslipidaemia	1.01 (0.85–1.21)	1.00	1.01 (0.85–1.20)	1.00
Metabolic syndrome	1.22 (0.99–1.50)	0.42	1.26 (1.00–1.59)	0.38
Myocardial infarction	NC	NC	NC	NC
Angina pectoris	0.74 (0.41–1.32)	1.00	0.73 (0.41–1.32)	1.00
Cerebral infarction	0.82 (0.40–1.67)	1.00	0.82 (0.40–1.68)	1.00

All *p* values have been corrected by the Bonferroni method by multiplying the raw *p* values by seven
NC, not calculable

Table 7 Associations of clinical variables with *RETN* polymorphisms and with plasma resistin concentration

Trait per polymorphism	Expected change in trait ^a	<i>p</i> value (resistin vs trait)	Observed change in trait per A allele	<i>p</i> value ^b	<i>p</i> value observed vs expected
rs34861192					
BMI (kg/m ²)	0.161±0.055	3.72×10 ⁻³	0.074±0.091	0.42	0.42
Abdominal circumference (cm)	0.408±0.154	8.17×10 ⁻³	0.066±0.253	0.79	0.25
Systolic BP (mmHg)	0.480±0.354	0.18	0.577±0.581	0.32	0.89
Diastolic BP (mmHg)	0.320±0.204	0.12	0.325±0.336	0.33	0.99
Serum total cholesterol (mmol/l)	-0.005±0.016	0.74	-0.027±0.026	0.32	0.50
Serum triacylglycerol (mmol/l)	0.067±0.014	1.30×10 ⁻⁶	-0.008±0.023	0.74	5.15×10 ⁻³
Serum HDL-cholesterol (mmol/l)	-0.042±0.008	3.43×10 ⁻⁸	0.003±0.013	0.82	2.19×10 ⁻³
Serum LDL-cholesterol (mmol/l)	0.005±0.015	0.72	-0.027±0.025	0.28	0.27
FPG (mmol/l)	-0.008±0.022	0.72	-0.062±0.035	0.08	0.19
IRI (pmol/l)	3.171±0.835	1.49×10 ⁻⁴	-2.423±1.374	0.08	5.04×10 ⁻⁴
HOMA-IR	0.108±0.038	4.32×10 ⁻³	-0.125±0.062	0.04	1.36×10 ⁻³
HbA _{1c} (%)	0.011±0.013	0.41	-0.027±0.022	0.22	0.14
rs3745368					
BMI (kg/m ²)	-0.036±0.013	3.72×10 ⁻³	0.117±0.129	0.36	0.24
Abdominal circumference (cm)	-0.092±0.035	8.17×10 ⁻³	0.220±0.357	0.54	0.39
Systolic BP (mmHg)	-0.108±0.080	0.18	0.637±0.820	0.44	0.37
Diastolic BP (mmHg)	-0.072±0.046	0.12	0.412±0.474	0.39	0.31
Serum total cholesterol (mmol/l)	0.001±0.004	0.74	0.024±0.037	0.52	0.54
Serum triacylglycerol (mmol/l)	-0.015±0.003	1.30×10 ⁻⁶	0.032±0.032	0.31	0.14
Serum HDL-cholesterol (mmol/l)	0.010±0.002	3.43×10 ⁻⁸	-0.024±0.018	0.19	0.06
Serum LDL-cholesterol (mmol/l)	-0.001±0.003	0.72	0.034±0.035	0.33	0.31
FPG (mmol/l)	0.002±0.005	0.72	0.040±0.050	0.42	0.45
IRI (pmol/l)	-0.716±0.189	1.49×10 ⁻⁴	-1.532±1.94	0.43	0.68
HOMA-IR	-0.024±0.009	4.32×10 ⁻³	-0.034±0.088	0.70	0.91
HbA _{1c} (%)	-0.003±0.003	0.41	-0.001±0.031	0.96	0.97

Expected changes were calculated by multiplying *RETN* polymorphism–plasma resistin level effect sizes by plasma resistin level–clinical variable effect sizes

Observed changes were *RETN* polymorphism–clinical variable effect sizes

^a Per 7.65 ng/ml (rs34861192) and 1.73 ng/ml (rs3745368) resistin increase

^b Polymorphism vs trait

The *p* values for difference between observed and expected changes were calculated with the *Z* statistic

However, we did not detect a significant association between this polymorphism or rs34861192 and physical or clinical variables.

Heterogeneity between European and Japanese populations

A recent analysis of the Framingham Offspring Study, which included 2,531 white participants, revealed that two SNPs in the 3' untranslated region of *RETN* (rs4804765 and rs1423096) showed independent associations with the circulating resistin level, accounting for 1.5% of variance in this variable [33]. Moreover, meta-analysis did not detect an association between the rs1862513 (-420C→G) SNP of *RETN* and resistin level in two populations of European descent, the Framingham Offspring Study and a cohort from

Italy [23, 33]. This apparent discrepancy with our present results might be explained if (1) whites do not harbour polymorphisms in the promoter region of *RETN* that influence plasma resistin level; or (2) other polymorphisms located downstream of rs3745368 have a greater effect on plasma resistin in such individuals. Indeed, rs34861192 is monomorphic (all samples having the GG genotype) in the EGP-CEPH Panel (European) of dbSNP. In contrast, the MAF of rs34861192 was 0.204 in our participants, and the minor (A) allele was related to a higher plasma resistin level in an additive manner. Furthermore, the minor allele of rs3745368 was less frequent in the Framingham Offspring cohort [33] than in our population (MAF 0.002 vs 0.092). Although this previous study found that SNPs in the 3'

untranslated region of *RETN* were associated with resistin level, these SNPs are located downstream of rs3745368, which was significantly related to plasma resistin in our study. We did not genotype these downstream SNPs, linkage data for which are not available in the public database. We were therefore not able to correlate our results for rs3745368 with those for the SNPs reported in the Framingham Offspring Study. The rs1862513 polymorphism has been investigated in several studies [21, 23–25, 29, 33, 34]. It was significantly associated with the circulating resistin level, with a MAF of 0.33, in another Japanese cohort [25]. This MAF is similar to that in our study (0.35); moreover, rs1862513 was also related to plasma resistin concentration by one-way ANOVA and Scheffe's test in our large-scale study. The *RETN* polymorphisms that influence plasma resistin level may thus differ among ethnic groups.

Resistin level and polymorphisms in the promoter region of RETN The rs34861192 polymorphism in the upstream region of *RETN* was in perfect linkage disequilibrium with the rs3219175 (–358G→A) polymorphism and affected the plasma resistin level to a greater degree than the rs1862513 polymorphism, which was also in linkage disequilibrium with rs34861192. At least three putative sterol regulatory element (SRE) motifs, which serve as preferential binding sites for SRE-binding protein 1c (SREBP1c, also known as ADD1), have been identified in the genomic region located between 760 and 600 bp upstream of the transcription initiation site of *RETN* [35]. SREBP1c was shown to regulate expression of *RETN* in a luciferase reporter assay [35]. The rs1862513 polymorphism of *RETN* was previously identified as a determinant of the plasma resistin concentration in Japanese, an effect mediated at the level of binding of the transcription factors Sp1 and Sp3 and promoter activity [21]. However, our results suggest that other transcription factors, such as SREBP1c, that bind to the resistin gene promoter in the vicinity of the rs34861192 polymorphism may play a greater role in determining the plasma resistin concentration than Sp1 and Sp3. If this is the case, it might explain why the rs1862513 SNP, which is in linkage disequilibrium with rs34861192, was not associated with resistin level in white cohorts [23, 33]. The rs3219175 polymorphism, which was in perfect linkage disequilibrium with rs34861192, was not included in our large-scale analysis because a MOTIF search (<http://motif.genome.jp>, accessed 9 November 2008) for binding sites of transcription factors using the TRANSFAC library [36] detected no such sites in the vicinity of nucleotide position –358.

Resistin level and polymorphisms in the 3' untranslated region of RETN We also found that the rs3745368 polymorphism in the 3' untranslated region of *RETN* was

significantly associated with plasma resistin concentration independently of rs34861192, with the minor (A) allele being associated with a lower plasma resistin level ($R^2=0.010$). The 3' untranslated region of human genes plays an important role in formation of the 3' end of mRNAs as well as in regulation of the stability, nuclear export, subcellular localisation and translation of mRNAs [37]. The 3' end of fully processed eukaryotic mRNAs consists of a poly(A) tail that is synthesised by poly(A) polymerase and is important for regulating mRNA stability and translation. In mammalian cells, the AAUAAA hexamer is thought to define the core polyadenylation signal [37]. The rs3745368 polymorphism is located ten nucleotides upstream of the AATAAA hexamer in the 3' untranslated region of *RETN* and may therefore affect polyadenylation of resistin mRNA.

Resistin level and physical or metabolic variables We found that the plasma resistin concentration was inversely related to the serum HDL-cholesterol level and positively related to the serum concentration of triacylglycerol, IRI and BMI, with all of these relations being independent of age and sex. Obesity, especially visceral obesity, has been associated with conventional cardiovascular risk factors such as insulin resistance, dyslipidaemia (hypertriacylglycerolaemia and suboptimal HDL-cholesterol levels) and hypertension [38]. Our results now suggest that the plasma resistin concentration is related to obesity as well as to obesity-associated cardiovascular risk factors including hyperinsulinaemia and dyslipidaemia. Numerous studies have revealed a positive correlation between circulating resistin level and inflammatory markers [5–7, 14, 15, 39]. Moreover, inflammatory cytokines including TNF- α and interleukin-6 have been found to be necessary and sufficient for the induction of resistin expression [5]. Obesity and insulin resistance are also associated with chronic low-grade inflammation, as reflected by increased plasma levels of C-reactive protein, interleukin-6, interleukin-8 and TNF- α , in humans or animal models [40]. Hyperresistinaemia due to hereditary factors or to obesity-induced chronic inflammation may thus result in the development of insulin resistance.

Study limitations We selected 11 polymorphisms of *RETN* to achieve dense coverage of the gene from its promoter region to its 3' untranslated region. However, the previous analysis of the Framingham Offspring Study [33] included SNPs spanning the 20-kb upstream region to the 10-kb downstream region of *RETN* and showed that SNPs located 1.4 to 7.5 kb downstream of the 3' end of the gene were strongly associated with resistin levels. We may thus have failed to detect other causal SNPs because of non-coverage of the upstream and downstream regions of *RETN*. Furthermore, we selected polymorphisms with a MAF of

>1% in order not to exclude relatively uncommon and influential SNPs. With a sample size of 500, our screening study had a power of 80% to detect 2.7% of the variance in plasma resistin concentration, assuming $\alpha=0.05/11$ and a MAF of ≥ 0.01 . For relatively uncommon alleles, the required sample size would thus be quite large. We may therefore have missed smaller effect sizes in the genotype–phenotype relation.

Conclusions We have shown that the plasma resistin concentration was strongly influenced by the rs34861192 and rs3745368 polymorphisms of *RETN* in an aged Japanese population. The plasma resistin level was also associated with serum concentrations of HDL-cholesterol and triacylglycerol, IRI and BMI. However, straightforward relations between polymorphisms of *RETN* and metabolic traits were not detected. Although plasma resistin concentration was determined to a large extent by polymorphisms of *RETN*, the factors that explain the relation between circulating resistin and metabolic traits remain unclear. The mechanisms by which these two polymorphisms of *RETN* affect the plasma resistin concentration remain to be clarified.

Acknowledgements This study was supported in part by Grants-in-Aid for Scientific Research to M. Yokota, including those of Category (A) from the Japan Society for the Promotion of Science (17209021) and of Priority Area ‘Applied Genomics’ (1601223, 17019028, 18018020, 20018026) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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