RAPID COMMUNICATION

Significant interaction between *RETN* -420 G/G genotype and lower BMI on decreased risk of type 2 diabetes mellitus (T2DM) in Japanese – the J-MICC Study

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Abstract. We examined the association of the *RETN* (*resistin*) -420 C>G polymorphism (rs1862513) with risk of diabetes mellitus (DM), considering lifestyle factors, in Japanese. Subjects were participants of J-MICC Study, where 2,651 participants aged 35–69 years provided their blood for genotyping and lifestyle data after informed consent. Odds ratio (OR) of DM for *RETN*-420 G/G genotype was estimated using unconditional logistic regression model. Statistically significant interaction on risk of DM was observed between *RETN*-420 G/G genotype and BMI<25 (OR for interaction = 0.12; *P* = 0.046), and when subjects with *RETN*-420 C/C+C/G and BMI ≥ 25 (*n* = 69 for DM and 544 for non-DM) were defined as the reference, the adjusted ORs for subjects with *RETN*-420 G/G genotype and BMI ≥ 25 (*n* = 10 for DM and 111 for non-DM), *RETN*-420 C/C+C/G and BMI < 25 (*n* = 81 for DM and 1,605 for non-DM), and *RETN*-420 G/G and BMI < 25 (*n* = 1 for DM and 230 for non-DM) were demonstrated to be 0.72 (95% confidence interval: 0.36–1.46), 0.40 (0.28–0.56) and 0.03 (0.005–0.25), respectively. The present study revealed the significant interaction of *RETN*-420 G/G genotype with lower BMI on the decreased risk of DM, but the direction was opposite to the reported ones in Japanese. We should be careful in interpretation of the present study results because of the limited sample sizes. Further investigation of this association as well as of the actual biological roles of RETN in the genesis of human metabolic disorders including DM will be required.

Key words: Resistin, Single nucleotide polymorphisms, Diabetes mellitus, BMI

WITH THE INCREASING prevalence of sedentary lifestyle in the recent years, lifestyle-related diseases such as coronary heart disease (CHD) or cerebrovascular diseases is placing an increasing burden on

populations both in developed and developing countries. Numbers of studies established that disruptions in the controls of blood glucose levels increase the risk of these diseases [1]. Resistin (RETN), a protein hormone produced both by adipocytes and immunocompetent cells including those residing in adipose tissue, has been implicated in the pathogenesis of obesity-mediated insulin resistance and type 2 diabetes mellitus (T2DM) [2]. In contrast, some human studies of late

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years demonstrated that resistin is mainly expressed in monocytes and macrophages, but not in adipocytes in humans [3]. One polymorphism of *RETN* in its 5' flanking region, *RETN* -420 C>G polymorphism (rs1862513) was reportedly associated with the risk of diabetes mellitus (DM) in several ethnicities [4, 5]. One recent study in Japanese demonstrated that the G/G genotype of a *RETN* single-nucleotide polymorphism at -420 increases T2DM susceptibility by inducing promoter activity through specific binding of Sp1/3 [6], while another study in Japanese suggested the role of this *RETN* -420C>G polymorphism as a determinant of serum resistin levels as well as a possible risk marker of stroke in patients with T2DM, although the genotype frequencies of *RETN* -420 C>G polymorphism were not significantly different between T2DM patients and controls in this study [7]. The gene-environment interactions between this *RETN* polymorphism and lifestyle factors on the risk of DM is yet to be investigated.

Considering that the DM is a significant contributor towards the increasing burden of chronic diseases as a result of its complications such as CHD or renal failures, finding effective ways to prevent this disease is in pressing need. This study aims to examine the association of the G/G genotype of the *RETN* -420 C>G polymorphism with the risk of DM diagnosed by blood glucose levels, considering lifestyle factors to elucidate gene-environment interaction in a Japanese sample using the cross-sectional data of the J-MICC Study.

Subjects and Methods

Study subjects

We leveraged the data repository of Japan Multi-Institutional Collaborative Cohort Study (J-MICC Study), launched in 2005 in 10 areas of Japan, in which about 70,000 voluntarily enrolled participants aged 35-69 years provided their blood and their lifestyle data based on the questionnaire after informed consent [8].

The subjects for this analysis were 4,519 randomly selected J-MICC study participants (about 500 subjects from each of the 10 areas) for whom genotyping of 108 selected polymorphisms were conducted by multiplex polymerase chain reaction-based Invader assay [9]. From these 4,519 subjects, 1,089 were excluded due to fasting blood sugar (FBS) data (1,084 were from the 2 areas without FBS measurement, 5 were from the oth-

ers), 1 excluded due to genotyping error, 727 excluded due to taking meals within 3 hours before the blood drawings, and 50 excluded due to the implausible values of estimated energy intake (< 1,000 kcal/day or > 4,000 kcal/day) and 1 excluded due to missing information on smoking status, leaving 2,651 subjects eligible for the analyses. Genotype call rate was 99.97% for those with FBS data ($n = 3,430$). Missing data were considered independent of the exposure-outcome (E-O) relationship. Intakes of energy, carbohydrate and fat were estimated using a food-frequency questionnaire [10, 11], and the proportions of energy from carbohydrate and fat were calculated as energy from each nutrient divided by total energy intake (shown in percentage: %energy). Smoking status, alcohol consumption and BMI were assessed based on the questionnaire. Informed consent was obtained from all the subjects and the study protocol was approved by the Ethics Committees of Nagoya University School of Medicine and the other participating institutions.

Samples and diagnostic criteria

The FBS levels were measured routinely for health check-ups or for research in participating institutions. The diagnostic criterion for DM was $FBS \geq 126$ mg/dL and/or taking DM medications.

Model building

We selected the logistic regression model in the present study because its parameter estimates have a convenient interpretation in standard study designs. We adopted the strategy of backwards elimination to pair-down model with the goal of getting the most unbiased estimate of the relationship between *RETN* -420 G/G genotype and risk of DM using the most parsimonious model. First of all, we extracted all possible variables for consideration using the directed acyclic graph (DAG) [12]. Age, gender, smoking status, alcohol consumption, total energy intake, carbohydrate intake, fat intake and BMI were extracted based on the DAG. We next evaluated the interaction term for effect measure modification by using the Breslow-Day test of homogeneity with $\alpha = 0.05$. Only BMI fulfilled this criterion of all the covariates. Then all the covariates as potential confounders were evaluated. For each covariate, the results of likelihood ratio test (LRT) for each covariate are considered to extract the covariates that can contribute significantly to the improvement of predictability of E-O relationship. The criteria for the signifi-

cance of LRT is $\alpha = 0.10$. As a result of this, age (as continuous variable), BMI (binary variable: BMI < 25 or BMI $\geq$ 25) and smoking status (recorded as the indicator variables: never smokers, current smokers & former smokers) remained in the final model. All the calculations were done using the STATA version 10 (Stata Corp, College Station, TX).

Results

The characteristics of the study subjects are described in Table 1. The genotype frequency for *RETN*-420 C>G polymorphism were 42.6% (1,062/2,490) for C/C, 43.7% (1,087/2,490) for C/G and 13.7% (341/2,490) for G/G among those without DM, while 47.2% (76/161), 46.0% (74/161) and 6.8% (11/161), respectively, among those with DM. The genotype frequencies among the non-DM subjects were significantly different from the Hardy-Weinberg's equilibrium ($\chi^2 = 5.502$, $P = 0.019$); the expected genotype frequency was 42.7% for C/C, 45.8% for C/G, and 13.7% for G/G. Odds ratio (OR) of DM for *RETN*-420 G/G genotype was estimated using crude and stratified tabular analysis, and unconditional logistic regression adjusting for age, smoking, and BMI. The crude OR was 0.46 (95% confidence interval [CI]: 0.25-0.86). In stratified analysis with the final model by BMI, the adjusted OR (aOR) was 0.08 (0.01-0.61) among BMI<25 and 0.72 (0.36-1.45) among BMI $\geq$ 25. Statistically significant interaction on risk of DM was observed between *RETN*-420 G/G genotype and BMI < 25 (OR for interaction = 0.12; $P = 0.046$), and when subjects with *RETN*-420 C/C+C/G and BMI $\geq$ 25 ($n = 69$ for DM and 544 for non-DM) were defined as the reference, the ORs for subjects with *RETN*-420 G/G genotype and BMI $\geq$ 25 ($n = 10$ for DM and 111 for non-DM), *RETN*-420 C/C+C/G and BMI<25 ($n = 81$ for DM and 1,605 for non-DM), and *RETN*-420 G/G and BMI<25 ($n = 1$ for DM and 230 for non-DM) were demonstrated to be 0.72 (95% CI: 0.36-1.46), 0.40 (0.28-0.56) and 0.03 (0.005-0.25), respectively (Table 2). We also conducted the stratified analysis of the effect of *RETN*-420 C>G polymorphism on DM risk by gender, which revealed the aOR of 0.23 (95% CI: 0.08-0.63) in men and 0.84 (0.37-1.92) in women, the interaction of which didn't reach the statistical significance (likelihood-ratio test: $P = 0.13$).

Discussion

The present study revealed the significant interaction of *RETN*-420 G/G genotype with lower BMI on the decreased risk of DM, where the direction of the association was opposite to the reported ones in Japanese. Although *RETN* is known to be a protein hormone produced by adipocytes and has been implicated in the pathogenesis of obesity-mediated insulin resistance and T2DM [2], the actual biological roles of *RETN* in the genesis of DM and insulin resistance in humans remain still controversial. Some recent studies demonstrated that circulating *RETN* levels are not associated with obesity or insulin resistance in humans and are not regulated by fasting or administration of leptin [13], whose main biological role is an indicator of energy balance as well as a neuromodulator that inhibits appetite by counteracting the effect of neuropeptide Y. The receptor of *RETN* is yet unknown. The insulin-responsive tissues affected are reported to be highly dependent on the model being studied; the human and mouse promoter sequences are demonstrated to be quite divergent, which makes it no wonder that the adipose-specific gene expression of rodent is not recapitulated in humans [14]. In addition, one research from U.S. demonstrated that serum *RETN* level is not a significant predictor of insulin resistance in humans [15], and recent meta-analysis did not observe any association between the polymorphism of *RETN*-420 C>G and the risk of type 2 DM [16]. Some of the recent studies also suggest the possibility of racial/ethnic differences in the effect of *RETN*-420 C>G on the risk of type 2 DM in humans, where the association between this *RETN*-420 C>G polymorphism and plasma resistin was found in Japanese, but not in Caucasians [17, 18]. To date, interaction between the *FTO* rs9939609 polymorphism and BMI on metabolic syndrome risk was reported [19], and our finding points towards the novel evidence that variation in the *RETN* gene may also interact with BMI to decrease the risk of metabolic disorder. Recent research also clarified the roles of inflammatory pathways in the induction of *RETN* in causing insulin resistance in mice [20]. The analysis of the interaction between *RETN* and inflammatory pathways in the causation of T2DM in the actual human epidemiological data might also help provide more detailed insights into the personalized prevention of T2DM in the near future. According to the literature, *RETN*-420G allele was shown to have stronger

Table 1 Characteristics of the study subjects and the stratified analyses of the odds ratio of T2DM for *RETN* -420 G/G vs. C/C+C/G

Covariates	Non-DM	DM	Total	OR*	95% CI*	Test of Homogeneity (Breslow-Day)		
	N (%)	N (%)	N (%)			χ^2 (df)	P-value	
RETN -420 C>G genotype								
C/C	1,062 (42.6)	76 (47.2)	1,138 (42.9)					
C/G	1,087 (43.7)	74 (46.0)	1,161 (43.8)					
G/G	341 (13.7)	11 (6.8)	352 (13.3)					
G/G vs. C/C+C/G	- -	- -	- -	0.46	0.25-0.86	-	-	
Age								
35-39	121 (4.9)	1 (0.6)	122 (4.6)	0	-			
40-49	511 (20.5)	19 (11.8)	530 (20.0)	0.38	0.01-2.50			
50-59	885 (35.5)	55 (34.2)	940 (35.5)	0.55	0.17-1.40			
60-69	973 (39.1)	86 (53.4)	1,059 (39.9)	0.42	0.13-1.06	0.26 (3)	0.968	
Gender								
Male	1,249 (50.2)	107 (66.5)	1,356 (51.2)	0.26	0.07-0.71			
Female	1,241 (49.8)	54 (33.5)	1,295 (48.8)	0.87	0.33-1.98	3.44 (1)	0.064	
Smoking								
Never	429 (17.2)	27 (16.8)	456 (17.2)	0.33	0.01-2.10			
Former	567 (22.8)	60 (37.3)	627 (23.7)	0.31	0.06-0.98			
Current	1,494 (60.0)	74 (46.0)	1,568 (59.1)	0.63	0.24-1.39	1.15 (2)	0.563	
Alcohol intake								
Non-drinker	1,083 (43.5)	65 (40.4)	1,148 (43.3)	0.72	0.27-1.63			
Drinker (current)								
<23g/day	1,110 (44.6)	64 (39.8)	1,174 (44.3)	0.21	0.02-0.82			
≥23g/day	297 (11.9)	32 (19.9)	329 (12.4)	0.43	0.05-1.81	2.35 (2)	0.309	
Energy intake								
<30kcal/kg/day	919 (36.9)	69 (42.9)	988 (37.3)	0.77	0.29-1.73			
≥30kcal/kg/day	1,571 (63.1)	92 (57.1)	1,663 (62.7)	0.27	0.07-0.74	2.52 (1)	0.112	
Carbohydrate intake								
<60% energy	1,756 (70.5)	111 (68.9)	1,867 (70.4)	0.47	0.20-0.99			
≥60% energy	734 (29.5)	50 (31.1)	784 (29.6)	0.43	0.08-1.39	0.02 (1)	0.902	
Fat intake								
<15% energy	225 (9.0)	18 (11.2)	243 (9.2)	0	0-1.83			
15-20% energy	641 (25.7)	53 (32.9)	694 (26.2)	0.28	0.03-1.08			
20-25% energy	797 (32.0)	50 (31.1)	847 (32.0)	0.69	0.21-1.78			
25-30% energy	531 (21.3)	28 (17.4)	559 (21.1)	0.69	0.13-2.33			
≥30% energy	296 (11.9)	12 (7.5)	308 (11.6)	0.48	0.01-3.47	2.54 (4)	0.638	
BMI								
<25	1,835 (73.7)	82 (50.9)	1,917 (72.3)	0.09	0.002-0.50			
≥25	655 (26.3)	79 (49.1)	734 (27.7)	0.71	0.32-1.44	4.81 (1)	0.028	

DM, diabetes mellitus; OR, odds ratio; 95%CI, 95% confidence interval.

*OR and 95% CI of T2DM for *RETN* -420 G/G stratified by each covariate, unadjusted for any other covariates.

Table 2 Combined effects of the *RETN* -420 C>G polymorphism and BMI on the risk of T2DM

<i>RETN</i> -420 C>G genotype	Body Mass Index (BMI)	
	BMI $\geq$ 25	BMI < 25
<i>RETN</i> -420 C/C + C/G (DM/non-DM) [#]	OR* = 1 (Referent) (69/544)	OR* = 0.40 (0.28-0.56) (81/1,605)
<i>RETN</i> -420 G/G (DM/non-DM) [#]	OR* = 0.72 (0.36-1.46) (10/111)	OR* [†] = 0.03 (0.005-0.25) (1/230)

DM, diabetes mellitus; OR, odds ratio.

*All ORs are adjusted for age and smoking status. Numbers in the parentheses indicates the 95% confidence intervals (95% CIs).

[#]DM/non-DM indicates the number of subjects with DM and that of non-DM subjects, respectively (the numbers of the subjects are described in the parentheses).

[†]OR for interaction = 0.12, *P*-value for interaction = 0.046.

promoter activity [6]; considering this, and given that the function of *RETN* is to increase the risk of DM by antagonizing insulin as described by some researchers [6], the risk of DM would be higher in those with *RETN* -420 G allele, which speculation was contrary to our observation. This discrepancy observed may stem from the fact that the function of *RETN* itself is still controversial, or simply from the random error caused by the relatively small sample size of the present study. Further investigation will be required to verify this association. The rs (reference SNP)-number for *RETN* -420 C>G is rs1862513, and the C-allele is major allele here, because it is complementary to the sequence that the genotype frequency of rs1862513 in HapMap is based on (ss14703397) in the dbSNP Build 37.3 database [21]. We confirmed that our interpretation is correct by comparing our primer-probe sequences with that of dbSNP 37.3 database.

It has been reported that the association of *RETN* -420 C>G polymorphism and T2DM may be stronger in younger-onset T2DM [22], but lack of information for the onset age of DM makes it impossible to investigate this association, which might be one potential limitation of the present study. As gender reportedly affects the association between plasma *RETN* level and insulin resistance, we conducted the stratified analysis by gender, which suggested the possible trend for the differential effect of *RETN* polymorphism on the risk of DM by gender, although the interaction did not reach statistical significance. Further verification on this interaction will also be needed.

Consideration of technical aspects is as follows: the statistical power for the entire study subjects of 161 DM and 2,490 non-DM was more than 90% for an OR of 2 or 0.5 with a two-sided α error of 0.05, when a

genotype frequency among controls was between 30% and 70%. It was more than 50% for subjects with BMI $\geq$ 25 ($n=79$ [DM] and $n=655$ [non-DM]), and more than 65% for subjects with BMI < 25 ($n=82$ [DM] and $n=1,835$ [non-DM]), under the same conditions. The statistical power for this sample may be somewhat weak especially for the subgroup analyses (e.g., subgroup of BMI<25) because of the low frequency of the variant allele in the Japanese population, which may require careful interpretation of the results as well as necessitate further investigation of this association in the near future. In this study, the genotype frequencies among the non-DM subjects were significantly different from the Hardy-Weinberg's equilibrium, but the absolute differences between the actual and expected frequencies were minimal (within $\pm 2.5\%$), and this departure from the Hardy-Weinberg's equilibrium may be explained by the type-I error randomly caused by the relatively large sample size. Another reason for the significant difference from Hardy-Weinberg's equilibrium in non-diabetic subjects might be speculated as a result of population stratification caused by the variation in genotype frequencies between the areas; we conducted the analyses adjusted for institutions to exclude this effect, which were not substantially different from the unadjusted results, suggesting that the variation in genotype frequencies between the areas did not affect the main results. In any case, as the absolute differences between actual and expected genotype frequencies in non-cases are minimal, the potential bias caused by this deviation from Hardy-Weinberg's equilibrium would also be substantially minimal. Adjustments for multiple comparisons may be another issue. There are also number of criticisms suggesting that correction of multiple comparisons by Bonferroni procedures is some-

times too conservative and aggravate the researchers' tendency not to conduct or present more tests [23, 24], and considering that most of the analyses in the present study were conducted under the exploratory context—mainly for the purpose of determining the best statistically fit model, we decided not to adopt these adjustments in this study.

In summary, we found significant statistical interaction between *RETN* -420 G/G genotype and lower BMI (BMI < 25) on the decreased risk of T2DM in Japanese, suggesting the further need for investigating the actual function of *RETN* -420 G/G genotype in the genesis of human metabolic disorders including DM. We should also be careful in interpretation of the present study results because of the limited sample sizes. This association observed might potentially help provide novel evidence for the individualized prevention of T2DM in humans in the future, and further investigation of this association in other ethnicities/groups or with much larger populations, as well as of the actual biological roles of *RETN* are also required to confirm our findings.

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Conflict of Interest

The authors declared no conflict of interests.

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