A at Single Nucleotide Polymorphism-358 Is Required for G at -420 to Confer the Highest Plasma Resistin in the General Japanese Population

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Abstract

Insulin resistance is a feature of type 2 diabetes. Resistin, secreted from adipocytes, causes insulin resistance in mice. We previously reported that the G/G genotype of single nucleotide polymorphism (SNP) at -420 (rs1862513) in the human resistin gene (RETN) increased susceptibility to type 2 diabetes by enhancing its promoter activity. Plasma resistin was highest in Japanese subjects with G/G genotype, followed by C/G, and C/C. In this study, we cross-sectionally analyzed plasma resistin and SNPs in the RETN region in 2,019 community-dwelling Japanese subjects. Plasma resistin was associated with SNP-638 (rs34861192), SNP-537 (rs34124816), SNP-420, SNP-358 (rs3219175), SNP+299 (rs3745367), and SNP+1263 (rs3745369) in all cases. SNP-638, SNP-420, SNP-358, and SNP+157 were in the same linkage disequilibrium (LD) block. SNP-358 and SNP-638 were nearly in complete LD (r^2 = 0.98), and were tightly correlated with SNP-420 (r^2 = 0.50, and 0.51, respectively). The correlation between either SNP-358 (or SNP-638) or SNP-420 and plasma resistin appeared to be strong (risk alleles for high plasma resistin; A at SNP-358, r^2 = 0.5224, P = 4.94 × 10^{-13}; G at SNP-420, r^2 = 0.2616, P = 1.71 × 10^{-13}). In haplotypes determined by SNP-420 and SNP-358, the estimated frequencies for C-G, G-A, and G-G were 0.6700, 0.2005, and 0.1284, respectively, and C-A was rare (0.0011), suggesting that subjects with A at −358, generally had G at −420. This G-A haplotype conferred the highest plasma resistin (8.24 ng/ml difference/allele compared to C-G, P<0.0001). In THP-1 cells, the RETN promoter with the G-A haplotype showed the highest activity. Nuclear proteins specifically recognized one base difference at SNP-358, but not at SNP-638. Therefore, A at −358 is required for G at −420 to confer the highest plasma resistin in the general Japanese population. In Caucasians, the association between SNP-420 and plasma resistin is not strong, and A at −358 may not exist, suggesting that SNP-358 could explain this ethnic difference.

Introduction

In mice, resistin is secreted from adipocytes, which antagonizes the action of insulin both in vitro and in vivo [1,2]. While serum resistin levels are increased in obese diabetic mice, peroxisome proliferator-activated receptor γ (PPARγ) ligands reduce serum resistin [2]. Mice that overexpress the resistin gene (retn) in the liver are insulin-resistant through increased levels of serum resistin [3], while mice without retn show decreased fasting plasma glucose levels [4]. Therefore, elevated levels of serum resistin appear to be directly related to insulin resistance in mice. In humans, resistin appears to be mainly expressed in monocytes and macrophages [5,6,7].

Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance in insulin target tissues, and impaired insulin secretion from pancreatic β cells [8]. It has been reported that some single nucleotide polymorphisms (SNPs), such as PPARγ Pro12Ala, KCNJ11 E23K, and TCF7L2 are associated with T2DM [9]. Recent genome-wide association studies (GWAS) have successfully identified ~20 T2DM susceptibility SNPs, but the functional SNPs responsible for disease risk remain to be elucidated [10].
We previously carried out systematic analyses of SNPs in the human resistin gene (RETN), and found that the G/G genotype of a promoter SNP at -420 (rs1062513) was associated with T2DM susceptibility [11,12]. Functionally, Sp1/3 transcription factors specifically recognized G at -420, resulting in enhanced RETN promoter activity. In the general Japanese population, subjects with the G/G genotype had the highest plasma resistin levels, followed by C/G and C/C [13]. However, the specific cis-acting SNPs that are responsible for determining circulating resistin levels around RETN remains to be elucidated.

Mezler et al. reported that a SNP rs2431866, located at a distance of ~100 Kb from RETN, is associated with circulating resistin in a genome-wide protein QTL (quantitative trait locus) analysis [14]. In an analysis of human lymphocyte samples from 1,240 adults, Tejero et al. reported that a QTL that influences RETN mRNA is localized on chromosome 19p, where the strongest positional candidate is RETN itself [15]. In a previous study, we reported that monocyte mRNA was positively correlated with its simultaneous serum level [16].

In view of this, to determine the SNPs responsible for circulating resistin around RETN, we cross-sectionally analyzed 2,019 Japanese subjects. Plasma resistin was found to be most strongly associated with SNP-358 and SNP-420, where the relevant allele, A at-358, was tightly linked to G at -420.

Table 1. Association between each SNP around RETN and plasma resistin in the general Japanese population.

| SNP (rs No.) | Genotype | n   | HWE | Plasma resistin (ng/ml) |
|-------------|----------|-----|-----|------------------------|
|             |          |     |     | mean ± SD               | F     | r²       | p       |
| −1093A>G    | A/A      | 1705| 0.310| 11.5±6.5                | 0.07  | 7.4*10⁻⁵ | 0.929  |
| (rs3760678) | G/G      | 17  | 0.043| 11.6±6.0                | 0.07  | 7.4*10⁻⁵ | 0.929  |
| −638G>A     | G/G      | 1304| 0.056| 8.2±3.3                 | 1125.489| 0.5275 | 2.75 x 10⁻²⁹a |
| (rs34861192) | G/A      | 620 | 0.529| 16.5±5.9                | 0.07  | 2.4 x 10⁻⁹ | 0.975  |
|             | A/A      | 95  | 0.417| 24.5±7.6                | 0.07  | 4.63 x 10⁻¹⁴a |
| −537A>C     | A/A      | 1855| 0.417| 11.8±6.7                | 31.18 | 0.0300 | 4.63 x 10⁻¹⁴a |
| (rs34124816) | C/C      | 159 | 0.529| 7.8±3.9                 | 0.07  | 2.4 x 10⁻⁹ | 0.975  |
| −420C>G     | C/C      | 921 | 0.241| 8.1±3.2                 | 357.13| 0.2616 | 1.71 x 10⁻¹³a |
| (rs1862513) | C/G      | 868 | 0.529| 13.3±6.5                | 0.07  | 2.4 x 10⁻⁹ | 0.975  |
|             | G/G      | 230 | 0.529| 18.0±9.1                | 0.07  | 4.94 x 10⁻¹²a |
| −358G>A     | G/G      | 1300| 0.073| 8.2±3.3                 | 1102.52| 0.5224 | 4.94 x 10⁻¹²a |
| (rs3219175) | G/A      | 624 | 0.529| 16.4±5.9                | 0.07  | 2.4 x 10⁻⁹ | 0.975  |
| +157C>T     | C/C      | 1804| 0.049| 11.6±6.6                | 1.62  | 0.0016 | 0.198  |
| (rs3219177) | C/T      | 204 | 0.529| 10.8±6.5                | 0.07  | 2.4 x 10⁻⁹ | 0.975  |
|             | T/T      | 11  | 0.529| 10.1±1.6                | 0.07  | 2.4 x 10⁻⁹ | 0.975  |
| +299G>A     | G/G      | 796 | 0.359| 8.5±3.9                 | 214.26| 0.1753 | 5.24 x 10⁻⁸a |
| (rs3745367) | G/A      | 928 | 0.529| 12.4±6.6                | 0.07  | 2.4 x 10⁻⁹ | 0.975  |
|             | A/A      | 295 | 0.529| 16.5±8.2                | 0.07  | 2.4 x 10⁻⁹ | 0.975  |
| +1263G>C    | G/G      | 1074| 0.037| 12.6±7.2                | 36.64 | 0.0351 | 2.33 x 10⁻¹⁶a |
| (rs3745369) | G/C      | 822 | 0.529| 10.4±5.7                | 0.07  | 2.4 x 10⁻⁹ | 0.975  |
|             | C/C      | 123 | 0.529| 9.2±4.4                 | 0.07  | 2.4 x 10⁻⁹ | 0.975  |

ANOVA is used for statistical analysis (n = 2,019). HWE, Hardy-Weinberg Equilibrium. *, these P values remained significant after Bonferroni’s correction (raw P value × 8).

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Results

SNP-358, and SNP-638 were in nearly complete linkage disequilibrium (LD), and were tightly correlated with SNP-420 in the general Japanese population

We first typed the 8 SNPs listed in Table 1. All of these SNPs were considered to be in Hardy-Weinberg equilibrium because P = 0.049 for SNP+157, and P = 0.037 for SNP+1263 did not remain significant after Bonferroni’s correction. Based on a confidence interval analysis, SNP-358, SNP-420, SNP-358, and SNP+157 were determined to be in the same LD block (Fig. 1). Four major haplotypes defined by SNP-368, SNP-420, SNP-358, and SNP+157 were found (haplotype, frequency; G-C-G-C, 0.670; A-G-A-C, 0.200; G-G-G-G, 0.073; G-G-G-T, 0.055). Approximately 94.5% of chromosomes can be captured by two tag SNPs, namely, SNP-358 (or SNP-638) and SNP-420. SNP-358 and SNP-638 were in nearly complete LD (r² = 0.90), and were tightly correlated with SNP-420 (r² = 0.50, and 0.51, respectively).

In view of this, to determine the SNPs responsible for circulating resistin around RETN, we cross-sectionally analyzed 2,019 Japanese subjects. Plasma resistin was found to be most strongly associated with SNP-358 and SNP-420, where the relevant allele, A at-358, was tightly linked to G at -420.

Nuclear proteins specifically recognized a difference in one base at SNP-358 but not at SNP-638

To determine which SNP, i.e., SNP-358 or SNP-638, is potentially functional, we examined whether nuclear proteins
Resistance SNP and Plasma Levels

Figure 1. SNP-358, and SNP-638 were nearly in complete LD, and were tightly correlated with SNP-420 in the general Japanese population. The 8 SNPs around RETN were genotyped in 2,019 subjects in the general Japanese population, and LD between each pair of these SNPs was analyzed by Haploview [28]. One LD block consisting of SNP-638, SNP-420, SNP-358, and SNP+157 was defined by the confidence interval analysis. Each square represents a pairwise value of $D'$, with the standard gradation (black indicates LOD$>2$ and $D'=1$; none indicates, LOD<2 and $D'=1$; white indicates LOD<2 and $D'<1$, gray indicates LOD$>2$ and $D'<1$). The $r^2$ between SNP-420 and SNP-358 or SNP-638 was 0.50, and 0.51, respectively. doi:10.1371/journal.pone.0009718.g001

specifically bound to DNA sequences using electrophoretic mobility shift assay (EMSA) (Fig. 2). Nuclear proteins were found to bind to a labeled probe with G at SNP-358 (-358G probe)(1st lane from the left), which was reduced by a cold competitor with G at SNP-358, but not by that with A at SNP-358 (2nd and 3rd lanes, respectively). This suggests that nuclear proteins specifically recognized a difference in one base at SNP-358. Consistent with this, no specific proteins bound to a labeled probe with A at SNP-358 (4th to 6th lanes). Regarding SNP-638, non-specific protein binding was found with a $-638G$ probe (lane 7th), which was evenly reduced by a cold competitor with G at $-628$ (8th lane) and that with A at $-628$ (9th lane). The same non-specific protein binding was observed for a $-638A$ probe (10th to 12th lanes). In addition, a computer search (MatInspector program (http://www.genomatix.de/)) revealed the presence of transcription factor binding sites for SNP-358 but not for SNP-638. Therefore, we tentatively concluded that SNP-358 was a more functional SNP than SNP-638, and used SNP-358 when necessary in this study.

Plasma resistin was associated with SNP-638, SNP-537, SNP-420, SNP-358, SNP+299, and SNP+1263 in the general Japanese population

We next examined the relation between plasma resistin levels and each of the 8SNPs (Table 1). Plasma resistin levels were associated with SNP-638 ($P=2.75\times10^{-29}$), SNP-537 ($P=4.63\times10^{-11}$), SNP-420 ($P=1.71\times10^{-14}$), SNP-358 ($P=4.94\times10^{-22}$), SNP+299 ($P=5.24\times10^{-15}$), and SNP+1263 ($P=2.33\times10^{-16}$). These $P$ values remained significant after Bonferroni’s correction. A low $P$ value and high $r^2$ were found for SNP-358 (or SNP-638), and SNP-420 ($r^2=0.5224$ (0.5275), and 0.2616, respectively). The difference in plasma resistin levels between each genotype appears to be highest for SNP-358 (or SNP-638), followed by SNP-420 ($P<0.001$, and Scheffe, $P<0.001$ compared to subjects with each of the other genotypes)(Table 3). Since only 4 of the 921 subjects with the C/C genotype of SNP-358 had A/A genotype of SNP-420 and SNP-358 in this order (Table 4). The estimated frequencies for C-G, G-A, and G-G were 0.6700, 0.2005, and 0.1295, respectively. Since the G-A haplotype was rare (0.0011), subjects having A at $-358$, generally had a G at $-420$. Conversely, $\sim$60% of chromosomes having G at $-420$ had A at

Bonferroni’s correction. A low $P$ value and high $r^2$ were found for SNP-358 (or SNP-638), and SNP-420 ($r^2=0.5224$ (0.5275), and 0.2616, respectively). The difference in plasma resistin levels between each genotype appears to be highest for SNP-358 (or SNP-638), followed by SNP-420 ($P<0.001$, and Scheffe, $P<0.001$ compared to subjects with each of the other genotypes)(Table 3). Since only 4 of the 921 subjects with the C/C genotype of SNP-420 appeared to be heterozygotes of A at $-358$, and none was this homozygote, it was not possible to conclude the existence of an effect of A at $-358$, independent of G at $-420$. It is noteworthy that findings reported in two previous studies suggest that the effect of G at $-420$ is independent of G at SNP-358 in vivo [12,17]. Therefore, the G of SNP-420 and the A of SNP-358 are both possible primary SNPs with the potential function of determining high plasma resistin levels around RETN in the general Japanese population.

The G-A haplotype defined by SNP-420 and SNP-358 conferred the highest plasma resistin in the general Japanese population

To determine which combination of these potentially functional SNPs is most responsible for plasma resistin, we examined the relation between plasma resistin and haplotypes defined by SNP-420 and SNP-358 in this order (Table 4). The estimated frequencies for C-G, G-A, and G-G were 0.6700, 0.2005, and 0.1294, respectively. Since the G-A haplotype was rare (0.0011), subjects having A at $-358$, generally had a G at $-420$. Conversely, $\sim$60% of chromosomes having G at $-420$ had A at
Table 2. Multiple regression analysis involving plasma resistin (ng/ml) as a dependent variable and each SNP as an independent variable (adjusted for age, gender, and BMI).

| SNP | Reference | Independent variable | Unstandardized regression coefficient | Standard error | Standardized regression coefficient | P |
|-----|-----------|----------------------|---------------------------------------|----------------|-------------------------------------|---|
| −1093A>G (rs3760678) | AA | A/G | 0.17 | 0.41 | 0.01 | 0.688 |
| | A/G | G/G | 0.38 | 1.60 | 0.01 | 0.813 |
| | A/A | A/C | 16.43 | 0.47 | 0.53 | 7.66×10⁻²⁰⁷ |
| | C/C | −7.09 | 2.89 | −0.05 | 0.114 |
| −638G>A (rs34861192) | GG | G/A | 8.31 | 0.22 | 0.58 | 2.19×10⁻⁰⁴⁰ |
| | G/A | A/G | 0.10 | 0.03 | 0.01 | 0.913 |
| −357A>C (rs34124816) | AA | A/C | −4.11 | 0.53 | −0.17 | 1.82×10⁻¹⁴ |
| | C/C | −7.09 | 2.89 | −0.05 | 0.114 |
| −420C>G (rs1862513) | CC | C/G | 5.16 | 0.27 | 0.39 | 3.08×10⁻⁷⁷ |
| | C/G | G/G | 9.85 | 0.41 | 0.47 | 1.08×10⁻¹¹⁰ |
| −358G>A (rs3219175) | GG | G/A | 8.25 | 0.22 | 0.58 | 1.80×10⁻²³⁶ |
| | G/A | A/A | 16.40 | 0.48 | 0.53 | 1.55×10⁻²⁰⁴ |
| +157C>T (rs3219177) | CC | C/T | 0.40 | 2.02 | 0.02 | 0.843 |
| | C/T | T/T | 1.14 | 1.98 | 0.05 | 0.565 |
| +299G>A (rs3745367) | GG | G/A | 3.93 | 0.29 | 0.30 | 5.05×10⁻⁴¹ |
| | G/A | A/A | 8.05 | 0.40 | 0.43 | 8.97×10⁻⁸¹ |
| +1263G>C (rs3745369) | GG | G/C | −2.22 | 0.30 | −0.17 | 1.43×10⁻¹³ |
| | G/C | C/C | −3.57 | 0.61 | −0.13 | 6.05×10⁻⁹ |

Multiple regression analyses involving plasma resistin (ng/ml) as a dependent variable and each SNP genotype, age (years), gender (male = 0, female = 1), and BMI as independent variables were performed as described in Methods. For each SNP genotype, 5/5 was compared to N/N, and 5/S was compared to N/N, respectively where the major and minor alleles based on the allele frequency were defined by normal (N), and susceptibility (S) alleles, respectively.

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The RETN promoter with the G-A haplotype defined by SNP-420 and SNP-358 showed the highest activity in THP-1 cells

Finally, to determine the functional relevance of haplotypes defined by SNP-420 and SNP-358, we performed transfection experiments using THP-1 human monocytes. The RETN promoter activity with G at −420 and A at −358 showed the higher activity than the reference control promoter with C at −420 and G at −358 (n = 8, mean ± SE, 1.62 ± 0.16 fold; ANOVA, P = 0.002; Scheffe, P = 0.008) (Fig. 3). The promoter activity with the other two haplotypes, namely, G-G and C-A was not different from that with C-G. Since SNP-638 was not included in these promoter constructs, the observed effect was independent of A at SNP-638. Therefore, from statistical analyses of genetic data, A at SNP-638 is required for G at −420 to confer the highest plasma resistin in the general Japanese population.

Discussion

These findings herein indicate that plasma resistin levels are associated with SNP-638, SNP-357, SNP-420, SNP-358, SNP-299, and SNP+1263 around RETN in the general Japanese population. The lowest P value was observed for SNP-358 (or SNP-638), followed by SNP-420. SNP-420, and SNP-358 were in the same LD block, and a risk allele, A of SNP-358, was tightly linked with a risk allele, G of SNP-420. The G-A haplotype defined by SNP-420, and SNP-358, which included these risk alleles, conferred the highest plasma resistin. Functionally, the RETN promoter with this haplotype showed the highest activity in THP-1 cells. Nuclear proteins specifically recognized a difference in one base at SNP-358, but not at SNP-638.
We found that SNP-358 was associated with plasma resistin in the order of A/A > G/G > A/G in a large number of Japanese subjects. Subjects having the relevant allele for high plasma resistin, i.e., an A at −358, generally had a G at −420. In four independent studies, the activity of the mutant RETN promoter including G at −420 was higher than that of the wild type including C at −420 in vitro [12,17,18,19]. Of these, two studies have suggested that the effect of G at −420 is independent of A at SNP-358. In a previous study, we showed that Sp1/3 enhanced RETN promoter activity with G at −420 and G at −358 in the absence of SNP-638 in SL2 cells [12]. The findings reported herein indicate that RETN promoter activity with G at −420 and A at −358 without SNP-638 was ∼60% higher than that with the other combinations. Therefore, both the A of SNP-358 and the G of SNP-420 appear to be required for the highest RETN promoter activity.

During the preparation of this manuscript, Asano et al. reported on-line that rs34861192 (SNP-638G) and rs3745268 (3’ UTR) are significant determinants of plasma resistin in an aged Japanese population [20]. Whereas their SNP typing data were similar to our results, the analysis and interpretation were different from those reported here. Based exclusively on statistical analyses of genetic data, they concluded that SNP-638 was a more promising candidate than SNP-420. However, SNP-638, SNP-420, and SNP-358 were all located in the same LD block, and all of these three SNPs were strongly associated with circulating resistin levels. It should be pointed out that it is difficult to identify a causal variant from highly correlated SNPs by the exclusive use of statistical analyses of genetic data without other information [21]. Therefore, a haplotype analysis and functional data were taken into account in assessing promising causal variants in the present study.

Since the A at −358 was tightly linked with the G at −420, the issue of whether the A of SNP-358 itself is largely responsible for high plasma resistin levels, or whether both the A of SNP-358 and the G of SNP-420 are required remains to be elucidated. Also, the possible relevance of SNP-358 is suggested, since the sequences surrounding SNP-358 are similar to the NF-κB consensus sequences based on the MatInspector program (http://www.genomatix.de/).

The induction of RETN expression by lipopolysaccharides is inhibited by an NF-κB inhibitor in human macrophages [22]. Although no transcription factor binding sites were detected around SNP-638 by this program, it should be noted that SNP-638 had the same statistical association as SNP-358. The lack of apparent nuclear protein binding to the DNA sequence around SNP-638 may have resulted from inappropriate reaction conditions in detecting specific binding in the present study. SNP-638, and other SNPs including SNP+299 which show low \( P \) values clearly merit further investigation.

We previously reported that a functional promoter SNP-420 was tightly associated with plasma resistin, its final gene product, in Japanese subjects [12,13,16]. This association is also supported by a study reported by Cho et al. in an analysis of Korean subjects [18], but not that by Menzaghi et al. in an analysis of Caucasian subjects [23]. Most recently, Hivert et al. also reported that SNP-420 was not associated with circulating resistin in Caucasian subjects [24]. In their meta-analysis, which included Caucasians, Japanese, and Korean subjects, SNP-420 was found to be associated with plasma resistin with significant heterogeneity among the groups studied, suggesting that ethnic differences may be a factor [24]. In fact, whereas the A allele of SNP-358, which is tightly linked with the G allele of SNP-420, showed the strongest association with high plasma resistin in the present study, the A allele of SNP-358 was not found in the HapMap CEU population (http://www.hapmap.org/index.html.en). Therefore, SNP-358 is likely to be monomorphic in Caucasians, which could account for the ethnic differences in the association between plasma resistin and SNP-420.

### Table 4. Estimated frequency and plasma resistin in each haplotype defined by RETN SNP-420, and SNP-358 genotypes.

| SNP-420 | SNP-358 | Estimated frequency | Difference of plasma resistin (95% CI) (ng/ml) | \( P \) |
|---------|---------|---------------------|-----------------------------------------------|-------|
| C       | G       | 0.6700              | 0.00 = reference                               | –     |
| G       | A       | 0.2005              | 8.24(7.89–8.59)                               | <0.0001|
| G       | G       | 0.1284              | −0.05(−0.47–0.37)                             | 0.8000 |
| C       | A       | 0.0011              | 2.47 (−1.89–6.83)                             | 0.2700 |

Frequency and plasma resistin for each haplotype defined by RETN SNP-420 and SNP-358 genotypes were estimated by SNPstats [29]. Estimated differences of plasma resistin compared to a reference haplotype, C-G, are shown in each haplotype.

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![Figure 3. The RETN promoter with the G-A haplotype defined by SNP-420 and SNP-358 showed the highest activity in THP-1 cells. The RETN promoter reporter including −450/206 of RETN with each haplotype defined by SNP-420 and SNP-358 was transiently transfected into THP-1 human monocytes. Luciferase activity was measured as described in Methods. Relative luciferase activities are shown as the mean fold induction ± SE relative to the activity of the reference promoter reporter with the C-G haplotype. The data represent eight independent experiments with triplicate wells for each condition. ANOVA, \( P = 0.002 \). * Scheffe, \( P = 0.008 \) compared to C-G. The base numbers were shown where the translation initiation site was defined as +1.

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![Resistin SNP and Plasma Levels](http://www.plosone.org)
Most recently, in “humanized resistin mice”, where human resistin is specifically expressed in macrophages and endogenous *retn* is disrupted, a high fat diet was found to induce insulin resistance with inflammation of adipose tissue [25]. Although this supports the pathophysiological relevance of human resistin in insulin resistance, it remains controversial whether circulating resistin is associated with insulin resistance, T2DM, or adiposity in humans [1,13]. The discrepancy among previous reports may be resolved by analyzing SNPs that are correlated with circulating resistin. The lower power with a smaller numbers of subjects may also explain this difference. The broader range of the assay used in this study could also be a contributing factor. In addition, serum resistin probably exists as a hexamer (major form) or trimer (a more biologically active form) in mice [26]. The presence of multimers in human serum has been suggested, and this may also have affected the assay results [27].

In summary, of the potentially functional SNPs, SNP-358 appears to be most strongly associated with plasma resistin in the general Japanese population, followed by SNP-420. Subjects having the responsible allele for the highest plasma resistin, A at −358, generally had G at −420. The G-A haplotype defined by SNP-420 and SNP-358 conferred the highest plasma resistin. Functionally, the RETN promoter with this G-A haplotype showed the highest activity. It is not clear why A of SNP-358 is required for G of SNP-420 to confer the highest plasma resistin, and whether the A of SNP-358 indeed accounts for ethnic differences in the association between plasma resistin and SNP-420. Further studies will be required to clarify these points.

**Methods**

**Ethics Statement**

All subjects were informed of the purpose of the study and their written consent was obtained. The study was approved by the ethics committee of the Ehime University Graduate School of Medicine.

**Subjects**

This cross-sectional study included community-dwelling Japanese subjects who were attending an annual medical check-up in a rural town located in Ehime prefecture in Japan. We previously summarized in Table 5.

**SNP typing**

The seven SNPs were selected, based on our systemic analysis of SNPs in RETN [11,12], and one SNP (rs3745369) in the 3′ flanking region was added from HapMap (http://www.hapmap.org/index.html.en). These SNPs included all of the 4SNPs reported in HapMap around RETN. SNPs were typed by TaqMan analysis using assay on demand probes and primers sets (Applied Biosystems) except rs3760678, rs34861192, rs34124816, and rs3219177 which were generated as assay by design. The baseline characteristics of these subjects are investigated by an individual interview using a structured questionnaire. The clinical characteristics of these subjects are summarized in Table 5.

**Measurement of plasma resistin**

Fasting plasma resistin was determined using a human resistin ELISA kit (LINCO Research Inc., St. Charles, MO), according to the manufacturer’s protocol [12]. The assay was linear below 0.16 ng/ml. Inter- and intra-assay coefficients of variation (CVs) were 6.9 and 1.7% (low levels), and 7.2 and 8.1% (high levels), respectively.

**Electrophoretic mobility shift assay (EMSA)**

THP-1 cells were grown in RPM1 medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO2 incubator. Nuclear extracts of THP-1 cells were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo SCIENTIFIC). The following oligonucleotides were used for EMSA as probes or competitors, sense hRE-358, 5′-CAGCTGGTGACGGTAC-3′; antisense hRE-358G, 5′-GATCTGGGCAAGGAAAGCTCTTGAAGCAGACAGTCGTTGGAAGCTCCT-3′; antisense hRE-358A, 5′-GATCTGGGCAAGGAAAGCTCTTGAAGCAGACAGTCGTTGGAAGCTCCT-3′; antisense hRE-358G, 5′-CAGCTGGTGACGGTAC-3′; antisense hRE-358G, 5′-CAGCTGGTGACGGTAC-3′; antisense hRE-358G, 5′-CAGCTGGTGACGGTAC-3′; antisense hRE-358G, 5′-CAGCTGGTGACGGTAC-3′.

The lower power with a smaller numbers of subjects may also explain this difference. The broader range of the assay used in this study could also be a contributing factor. In addition, serum resistin probably exists as a hexamer (major form) or trimer (a more biologically active form) in mice [26]. The presence of multimers in human serum has been suggested, and this may also have affected the assay results [27].

In summary, of the potentially functional SNPs, SNP-358 appears to be most strongly associated with plasma resistin in the general Japanese population, followed by SNP-420. Subjects having the responsible allele for the highest plasma resistin, A at −358, generally had G at −420. The G-A haplotype defined by SNP-420 and SNP-358 conferred the highest plasma resistin. Functionally, the RETN promoter with this G-A haplotype showed the highest activity. It is not clear why A of SNP-358 is required for G of SNP-420 to confer the highest plasma resistin, and whether the A of SNP-358 indeed accounts for ethnic differences in the association between plasma resistin and SNP-420. Further studies will be required to clarify these points.

**SNP typing**

The seven SNPs were selected, based on our systemic analysis of SNPs in RETN [11,12], and one SNP (rs3745369) in the 3′ flanking region was added from HapMap (http://www.hapmap.org/index.html.en). These SNPs included all of the 4SNPs reported in HapMap around RETN. SNPs were typed by TaqMan analysis using assay on demand probes and primers sets (Applied Biosystems) except rs3760678, rs34861192, rs34124816, and rs3219177 which were generated as assay by design. The baseline characteristics of these subjects are investigated by an individual interview using a structured questionnaire. The clinical characteristics of these subjects are summarized in Table 5.

| Table 5. Characteristics of the population studied (n = 2,019). |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Sex (male/female)** | **883/1136** |
| **Age (years)** | 62.1±12.6 |
| **Body mass index (kg/m²)** | 23.4±3.2 |
| **Systolic blood pressure (mmHg)** | 138.8±22.4 |
| **Diastolic blood pressure (mmHg)** | 81.9±11.9 |
| **Total cholesterol (mg/dl)** | 203.4±34.7 |
| **Glucose (mg/dl)** | 98.4±21.7 |
| **Insulin (µU/ml)** | 6.8±5.0 |
| **HOMA-IR** | 1.6±1.4 |
| **Resistin (ng/ml)** | 11.5±6.6 |
| **Current smoking (%)** | 27.8 |
| **Current drinking (%)** | 57.2 |
| **History of cardiovascular diseases (%)** | 7.3 |
| **Medication (%)** | 25.9 |
| **Type 2 diabetes** | 3.4 |
| **Hyperlipidemia** | 5.8 |

Values are means±SD, or n (%). Number of subjects who measured immunoreactive insulin was 1,958. HOMA-IR (homeostasis model assessment insulin resistance index) was calculated as glucose (mg/dl) x insulin (µU/ml)/405. CVD indicates cardiovascular disease, including stroke, myocardial infarction, and angina pectoris.

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**Measurement of plasma resistin**

Fasting plasma resistin was determined using a human resistin ELISA kit (LINCO Research Inc., St. Charles, MO), according to the manufacturer’s protocol [12]. The assay was linear below 0.16 ng/ml. Inter- and intra-assay coefficients of variation (CVs) were 6.9 and 1.7% (low levels), and 7.2 and 8.1% (high levels), respectively.
200-fold molar excess of unlabelled double-stranded oligonucleotides to the reaction mixture. After the binding reaction, the mixture was subjected to 6% PAGE in 45 mM Tris, 45 mM boric acid, 1 mM EDTA at 100V for 2 h at 4°C. The gel was dried and exposed to a FujiFilm imaging plate. Signals were detected with the FujiFilm FLA-5100 image analyzing system.

Plasmid construction

The 5' flanking region between −450 and −206 of RETN was amplified by PCR using DNA from either a subject homozygous for a wild-type allele (−420C) or a subject homozygous for a mutant allele (−420G). These products were inserted into pGL3 basic vector (Promega, Madison, WI) to generate pGL3(−420G) and pGL3(−420C), both of which harbor G at the −358 locus, as described previously[12]. These constructs were modified by using QuickChange II Site-Directed Mutagenesis Kit (STRATAGENE, La Jolla, CA) to generate a point mutation at −358 from C to A. The primers used were 5′-CTTACGTGTCTGCTAGGAGGCTCGCTCTGACCAGC-3′ and 5′-CGGGCCAAAGGAGAACGCTTCCCTGAGCAGACAGTAAG-3′. The DNA fragments between −450 and −206 of RETN with or without this point mutation were subsequently cloned into a luciferase reporter vector pGL4.11 [Promega] at the site between Kpn I and Bgl II. The sequence of the generated four plasmids, pGL4(−420C/−358G), pGL4(−420C/−358A), pGL4(−420G/−358G) and pGL4(−420G/−358A), were confirmed by DNA sequencing. All plasmids used for the transfection were prepared by use of a Qiagen plasmid kit, followed by CsCl density-gradient ultracentrifugation as described previously [12].

Transient transfection and luciferase assay

THP-1 human monocyes purchased from Human Science Research Resources Bank (Osaka, Japan) were grown in RPMI1640 containing 10% FCS. One day before transfection, THP-1 cells were plated on a 6 well plate at 4.0×10⁵ cells/well. Trans IT-Jurkat Reagent (Mirus, Madison, WI) was used for transient transfection following the manufacturer’s protocol. Each of the pGL4(−420C/−358G), pGL4(−420C/−358A), pGL4(−420G/−358G) and pGL4(−420G/−358A) vectors was transfected into these cells along with pGL4.11 basic vector (control for basal activity), pGL4.11 promoter vector (positive control), and pRL-SV40 internal control vector. The THP-1 cells were then incubated with RPMI1640 containing 10% FCS for 48 hours. Luciferase assay were performed using the Dual-Luciferase™ Reporter Assay System according to the manufacturer’s protocol (Promega).

Statistical analysis

In the human population study, values are expressed as the mean±standard deviation. Differences in plasma resistin levels among genotypes were assessed by analysis of variance (ANOVA). Bonferroni’s correction was applied to Table 1 (raw P value ×8). To assess isolated effects of each SNP on plasma resistin, multiple regression analyses involving each SNP, age (year), gender (male = 1, female = 0), and BMI as independent variables, and plasma resistin (ng/ml) as a dependent variable were performed. In these analyses, we found that the major and minor alleles based on the allele frequency are normal (N), and susceptibility (S) alleles, respectively. Each SNP genotype, N/N, N/S, and S/S, were denoted by two dummy variables (c1, c2) = (0, 0), (1, 0), and (0, 1), respectively. First this dummy variable estimates the difference between N/S and N/N, and the second between S/S and N/N. These analyses were performed using a commercially available statistical software package (JMP ver. 7, SAS Institute, Cary, NC). Linkage disequilibrium (LD) was assessed using the Haploview software [28]. Frequency and plasma resistin in each haplotype defined by SNP-420 and SNP-358 genotypes were estimated using SNPStats [29]. ANOVA followed by Scheffe as a post-hoc test was used where indicated. Null hypotheses were rejected at P<0.05.

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Author Contributions

Conceived and designed the experiments: HO YT RK TT JO WN YT MO KY RK KK TM HM HO. Performed the experiments: HO YT RK TT RK KK. Analyzed the data: HO YT RK TT JO MO HM HO. Contributed reagents/materials/analysis tools: HO YT RK TT RK KK TM HM HO. Wrote the paper: HO YT RK JO HM HO.

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