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Original Article

Association of resistin promoter polymorphisms with plasma resistin levels and type 2 diabetes in women and men

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Abstract: This study's objective was to examine the associations between resistin (*RETN*) polymorphisms, plasma resistin levels, and type 2 diabetes risk. We conducted two nested case-control studies in postmenopausal women (359 incident cases and 359 controls) and middle-aged/elderly men (170 incident cases and 170 controls). Controls were matched (1:1) to cases by age, race, duration of follow-up, and time of blood draw. Circulating resistin levels were higher among carriers of the variant allele for rs34861192 ($p<0.0001$ for women, $p=0.002$ for men) but not rs1862513 ($p=0.15$ for women, $p=0.14$ for men). Neither polymorphism was significantly associated with risk of type 2 diabetes after adjusting for diabetes risk factors (exercise, smoking status, alcohol intake, family history of diabetes, and matching factors) among women (rs1862513: OR=1.19, 95% CI=0.80-1.77; rs34861192: OR=0.41, 95% CI=0.14-1.19) and men (rs1862513: OR=1.05, 95% CI=0.57-1.95; rs34861192: OR=0.64, 95% CI=0.14-2.89). In conclusion, *RETN* promoter polymorphism rs34861192 was associated with elevated circulating resistin levels, but rs1862513 was not. Neither polymorphism was associated with an increased risk for type 2 diabetes.

Keywords: Resistin, RETN, type 2 diabetes, CRP, TNFα-RII, BMI

Introduction

The clinical and biological significance of the adipokine resistin in the development of type 2 diabetes remains uncertain. Current evidence from animal models indicates that a reduction in functional resistin protein (via gene knockout, anti-resistin immunoglobulin, and dominant inhibition) can lower blood glucose levels and improve insulin sensitivity [1-3]. In addition, elevated levels of serum resistin in diabetic *db/db* mice implicate resistin as a key mediator between adiposity and type 2 diabetes [3].

In humans, circulating resistin levels are higher among individuals with type 2 diabetes than among apparently healthy individuals [4-6]. Recently, two prospective cohort studies related baseline resistin levels to future development of type 2 diabetes, although this relation appeared to be mediated, in part, by BMI[4, 5] or inflammatory markers [4]. The suggestion of inflammatory pathway involvement is consistent with the fact that mononuclear cells are the primary source of resistin in humans [7]. In contrast, the causal relation between resistin levels and adiposity in humans remains unclear. Resistin lev-

els are elevated in obese individuals [8], however, adiposity (estimated by BMI) does not consistently correlate with resistin levels across studies [9].

Studies of *RETN* polymorphisms are advantageous in clarifying the directionality of the relation between inflammation, adiposity, and resistin levels. The most widely studied *RETN* polymorphism is the promoter polymorphism rs1862513, whose G-allele has been associated with elevated resistin levels and type 2 diabetes risk in some [6, 10] but not all studies [11-14]. Azuma, et al., observed that this polymorphism, in conjunction with another novel *RETN* promoter polymorphism (rs34861192), strongly influenced circulating resistin levels [15]. Based on these findings, we analyzed data from two well-characterized populations to further clarify the association between these *RETN* promoter polymorphisms, circulating resistin levels and type 2 diabetes risk.

Materials and methods

The Women's Health Study (WHS) is a randomized, controlled, 2x2 factorial trial which consisted of 39,876 female health professionals whose ages were 45 years or older and were free of cancer (except non-melanoma skin cancer) and cardiovascular disease at baseline [16, 17]. For the present analysis we conducted a nested case-control study using risk-set sampling. Cases and controls were selected from among original trial participants whose baseline blood samples were available (n=28,345) and who were postmenopausal and not using hormone replacement therapy at the time of blood collection (n=6,574). Cases included all women who developed type 2 diabetes by February 2005 (median follow-up of 10 years). Controls were matched to cases in a 1:1 ratio by age (within 1 year), follow-up duration (within 1 month), race, and fasting status at blood collection (72% provided fasting blood, defined as ≥10 hours since last meal). Based on these criteria, 359 cases and 359 controls were eligible for analysis in WHS.

The Physicians' Health Study II (PHS) is a randomized, controlled 2x2x2x2 factorial trial consisting of 14,641 male physicians aged 50.5 years or older who were free of cancer and cardiovascular disease at baseline [18]. In a similar manner as was done for WHS, cases and controls were selected from among original trial participants whose baseline blood samples were available (n=11,133). Cases included all men who developed type 2 diabetes during 8 years of follow-up. Controls were matched to cases in a 1:1 ratio by age (within 2 years), follow-up duration (within 1 month), race, and time of blood draw. Among the PHS cases and controls selected for the present analysis, 57% provided fasting blood (defined as ≥10 hours since their last meal). Based on these criteria, 170 cases and 170 controls were eligible for analysis in PHS.

Written informed consent was obtained from all participants in both WHS and PHS. Both studies were conducted according to the ethical guidelines of Brigham and Women's Hospital, Harvard Medical School, and the UCLA institutional review boards.

Methods used to ascertain incident type 2 diabetes in the present analyses have been described in detail previously [19]. Briefly, participants with type 2 diabetes at baseline were excluded. The remaining participants were asked annually whether and when they had been diagnosed with type 2 diabetes during follow-up. Self-reported cases of type 2 diabetes were confirmed by supplementary questionnaires. A small validation study in WHS compared self-reported type 2 diabetes to physician-led telephone interviews, supplementary questionnaires, and medical record reviews; the positive predictive value of self-reported type 2 diabetes was at least 91% in each comparison [20].

Centrifuged blood samples were stored in liquid nitrogen freezers. Case-control pairs were handled identically. Laboratory personnel were blinded to case-control status. Each matched pair was assayed in random order and in the same analytical run. Plasma levels of resistin were measured by an enzymatically amplified "two-step" sandwich-type ELISA immunoassay (R&D Systems, Minneapolis, MN). TNFα-receptor II (TNF-RII) was also measured by ELISA (R&D Systems, Minneapolis, MN). C-reactive protein (CRP) was measured at a core laboratory facility using a validated, high-sensitivity assay (Denka Seiken) [21]. The CRP assay was conducted only in WHS.

Two *RETN* SNPs—rs1862513 (*RETN* -420C>G)

and rs34861192 (*RETN* -638G>A)—were successfully genotyped in >98% of the samples in both WHS and PHS (providing 96% of the total case-control pairs for genetic analysis). Polymorphism selection was based on a review of the past literature and HapMap. Priority was given to polymorphisms that were associated with differences in plasma resistin levels, associated with type 2 diabetes, had a relatively high minor allele frequency, and found in the *RETN* gene promoter region. All DNA samples were genotyped using the ABI Taqman system (Applied Biosystems, Foster City, CA). Endpoint fluorescence of PCR-amplified DNA was read by the ABI PRISM 7900HT Sequence Detection System (SDS). Genotypes were assigned using SDS 2.2.2 Allelic Discrimination Software (Applied Biosystems, Foster City, CA) by two independent technicians blinded to the identification number and case-control status of each sample. Predesigned Taqman SNP genotyping probes were used for rs1862513, and custom probes were designed for rs34861192 (Applied Biosystems, Foster City, CA).

Biomarker values with skewed distributions were log-transformed to enhance compliance with normality assumptions. McNemar's χ^2 test (for categorical variables) and Wilcoxon signed rank test (for continuous variables) were used to compare baseline characteristics between cases and controls. We calculated geometric means for biomarkers and arithmetic means for BMI by genotype. Arithmetic means for BMI were multivariably adjusted for physical activity levels, smoking status, alcohol intake, and ever having a first-degree relative with diabetes. Geometric means for biomarkers were further adjusted for BMI. Differences in genotype-specific means were assessed by generalized linear models. Conditional logistic regression provided odds ratios (OR) and 95% CI on the association between *RETN* SNPs and type 2 diabetes. ORs in the present analyses yield unbiased estimates of relative risks (RR), specifically rate ratios, since risk-set sampling was used. Estimates from the WHS and PHS were pooled using a random-effects meta-analysis model [22] (Stata 10.0, StataCorp, Texas Station, TX). All other analyses were conducted in SAS 9.1.3 (SAS Institute Inc., Cary, NC).

Results

Demographic characteristics, circulating biomarker levels, and genotypic frequencies for rs1862513 and rs34861192 are presented in **Table 1**. The minor allele frequencies among the controls were 30% and 34% for rs1862513, and 3% and 5% in rs34861192 among women and men, respectively. Among control participants, Hardy-Weinberg equilibrium was observed for rs1862513 ($p<0.0001$ among women, $p=0.0001$ among men) but not for rs34861192 ($p=0.89$ among women, $p=0.73$ among men). Still, allele frequencies for both SNPs were consistent between the men and women in our analysis and those of HapMap CEU Europeans and the Environmental Genome Project (EGP) CEPH Europeans.

The adjusted means of resistin levels, BMI, TNF-RII, and CRP did not differ substantially between rs1862513 genotypes in either men or women (**Table 2**). Compared to non-carriers, rs34861192 variant A-allele carriers were similar in most measures, but had higher resistin levels ($p<0.0001$ among women, $p=0.002$ among men) and lower BMI ($p=0.003$ among men). Additive and dominant genetic models led to similar results, while the recessive model possessed weaker discriminatory ability (data not shown).

The risk of type 2 diabetes did not differ significantly between carriers and non-carriers of the rs1862513 variant allele in any of our statistical models (Final model: pooled RR=1.15, 95% CI=0.82-1.60) (**Table 3**). The crude and BMI-adjusted models for rs34861192 suggested a lower risk of type 2 diabetes among minor allele carriers; however, this association was no longer statistically significant after controlling for additional type 2 diabetes risk factors and BMI in the final model (pooled RR=0.48, 95% CI=0.20-1.13). Further adjustment for TNF-RII (or CRP, when available) in the final models did not alter estimates substantially (data not shown).

Discussion

The present analysis examined two SNPs with putative function in the promoter sequence of the *RETN* gene in relation to plasma resistin levels and risk of type 2 diabetes. In the widely studied rs1862513 no significant differences in circulating resistin levels nor risk of type 2 diabetes were observed across genotypes. However, elevated resistin levels were observed

Table 1. Baseline characteristics of case and control participants from the Women's Health Study (n=718) and Physicians' Health Study II (n=340)

	WOMEN			MEN		
	Cases	Controls	p-value*	Cases	Controls	p-value*
n	359	359		170	170	
Age (mean ± SD)	60.3 ± 6.1	60.3 ± 6.1	(matched)	63.7 ± 7.6	63.7 ± 7.6	(matched)
Race, White	332 (93.5%)	332 (93.5%)	(matched)	145 (85.3%)	145 (85.3%)	(matched)
BMI (mean ± SD)	30.9 ± 6.1	26.0 ± 5.0	<0.0001	28.9 ± 3.9	25.5 ± 3.4	<0.0001
Current multivitamin use	82 (22.9%)	88 (25.2%)	0.47	47 (27.7%)	46 (27.1%)	0.90
Current smoker	52 (14.5%)	49 (13.7%)	0.74	11 (6.5%)	2 (1.2%)	0.01
Exercise, ≥4 times/week	32 (8.9%)	41 (11.4%)	0.27	16 (9.4%)	21 (12.4%)	0.38
Family history of diabetes	174 (48.5%)	86 (24.0%)	<0.001	56 (32.9%)	29 (17.1%)	0.0007
Alcohol consumption, ≥1 drink/day	18 (5.0%)	41 (11.4%)	0.002	49 (28.8%)	46 (27.1%)	0.72
hs-CRP (mg/L)			<0.0001†			-
Median	4.0	1.6		-	-	
Interquartile range	2.3-7.0	0.7-3.6		-	-	
TNF-RII (pg/mL)			0.002†			0.24†
Median	2825	2645		2298	2215	
Interquartile range	2334-3331	2275-3125		1985-2647	1946-2562	
Resistin (ng/mL)			<0.0001†			0.09†
Median	11.7	10.4		10.6	9.1	
Interquartile range	8.7-17.6	7.5-13.9		6.6-16.0	6.4-12.8	
rs1862513			0.99			1.00
GG	175 (49.3%)	174 (49.3%)		71 (43.0%)	71 (43.0%)	
CG	149 (42.0%)	147 (41.6%)		76 (46.1%)	76 (46.1%)	
CC	31 (8.7%)	32 (9.1%)		18 (10.9%)	18 (10.9%)	
Minor allele frequency	30%	30%		34%	34%	
rs34861192			0.10			0.12
GG	348 (98.3%)	338 (95.5%)		162 (96.4%)	151 (91.0%)	
GA	5 (1.4%)	13 (3.7%)		5 (3.0%)	12 (7.2%)	
AA	1 (0.3%)	3 (0.9%)		1 (0.6%)	3 (1.8%)	
Minor allele frequency	1%	3%		2%	5%	

* Chi-square test for comparing cases and controls in categorical variables. Wilcoxon signed rank test for comparing cases and controls in continuous variables.
† Wilcoxon signed rank test comparing log-transformed biomarker levels by case-control status.

Table 2. Adjusted means and geometric means* (95% CI) by *RETN* genotype (dominant genetic model) among control women (n=359) and men (n=170)

rs1862513		GG	CC+CG	p-value†
Women	Resistin (ng/mL)	11.2 (10.4-12.1)	10.3 (9.6-11.2)	0.15
	BMI (kg/m^2)	26.1 (25.4-26.9)	25.8 (25.0-26.5)	0.52
	TNF-RII (pg/mL)	2700 (2601-2802)	2634 (2538-2733)	0.36
	CRP (mg/L)	1.6 (1.3-1.8)	1.6 (1.4-1.9)	0.87
Men	Resistin (ng/mL)	9.0 (7.9-10.3)	10.3 (9.2-11.6)	0.14
	BMI (kg/m^2)	25.4 (24.6-26.3)	25.7 (24.9-26.4)	0.70
	TNF-RII (pg/mL)	2291 (2175-2413)	2206 (2109-2307)	0.29
rs34861192		GG	AA+GA	p-value†
Women	Resistin (ng/mL)	10.4 (9.9-11.0)	21.9 (17.0-28.2)	<0.0001
	BMI (kg/m^2)	25.9 (25.4-26.4)	26.9 (24.3-29.5)	0.45
	TNF-RII (pg/mL)	2679 (2608-2751)	2402 (2116-2726)	0.10
	CRP (mg/L)	1.6 (1.4-1.8)	1.4 (0.8-2.3)	0.59
Men	Resistin (ng/mL)	9.3 (8.5-10.2)	15.4 (11.5-20.7)	0.002
	BMI (kg/m^2)	25.8 (25.3-26.4)	23.0 (21.2-24.8)	0.003
	TNF-RII (pg/mL)	2222 (2146-2300)	2434 (2168-2734)	0.14

* All means were adjusted for BMI (except in computing the mean of BMI itself), exercise, smoking status, alcohol intake, and family history of diabetes.
† Tests of differences in adjusted (geometric) means between carriers and non-carriers of the minor allele.

among carriers of the rs34861192 variant allele. Interestingly, the risk of type 2 diabetes among rs34861192 variant allele carriers did not differ significantly from non-carriers. In addition, circulating inflammatory markers levels were similar across *RETN* genotypes.

RETN -420C>G (rs1862513) has been studied extensively in regards to circulating resistin levels and type 2 diabetes risk. While the majority of studies found elevated resistin levels [13, 23, 24] among carriers of the variant G-allele, our analysis corroborates the findings of a recent longitudinal study [25] showing no substantial differences in resistin levels nor in type 2 diabetes risk across rs1862513 genotypes. Racial/ethnic differences may explain these differences. Studies that showed such associations were exclusively in Asian populations [9]; whereas, studies of predominantly Caucasian populations showed little to no differences between rs1862513 genotypes [25].

RETN -638G>A (rs34861192) is a novel *RETN* polymorphism that has only been examined recently for its association with type 2 diabetes. Previously, Azuma, et al., reported that, in conjunction with rs1862513, the rs34861192 polymorphism strongly influenced resistin levels, potentially by eliminating recognition sites for heat shock transcription factors [15]. In our analysis the variant A-allele was associated with increased levels of circulating resistin, yet conferred slightly lower risk of type 2 diabetes, although this result should be interpreted with caution due to low minor allele frequency in our population. Asano, et al., observed rs34861192 to be strongly associated with plasma resistin levels but not with diabetes-associated traits in a Japanese population [23].

Table 3. Association between resistin genotype (dominant genetic model) and risk of type 2 diabetes*

rs1862513 (GG vs. CC+CG)	WOMEN RR (95%CI)	MEN RR (95%CI)	POOLED RR (95%CI)
n (GG:CC+CG)	349:359	142:188	491:547
Crude model	0.98 (0.72, 1.32)	1.06 (0.66, 1.72)	1.00 (0.77, 1.29)
BMI-adjusted model	1.03 (0.71, 1.48)	1.10 (0.61, 1.98)	1.05 (0.77, 1.43)
Multivariable model†	1.18 (0.84, 1.66)	1.08 (0.65, 1.81)	1.15 (0.87, 1.53)
Final model‡	1.19 (0.80, 1.77)	1.05 (0.57, 1.95)	1.15 (0.82, 1.60)
rs34861192 (GG vs. GA+AA)			
n (GG:GA+AA)	686:22	313:21	999:43
Crude model	0.38 (0.15, 0.96)	0.31 (0.10, 0.94)	0.35 (0.17, 0.71)
BMI-adjusted model	0.34 (0.12, 0.97)	0.65 (0.16, 2.67)	0.43 (0.19, 0.99)
Multivariable model†	0.37 (0.13, 1.00)	0.34 (0.11, 1.10)	0.36 (0.17, 0.76)
Final model‡	0.41 (0.14, 1.19)	0.64 (0.14, 2.89)	0.48 (0.20, 1.13)

* All models adjusted for matching factors (age, race, fasting time).
† Multivariable model adjusted for matching factors and diabetes risk factors (exercise, smoking status, alcohol intake, and family history of diabetes).
‡ Final model adjusted for matching factors, diabetes risk factors, and BMI.

The lack of association between *RETN* promoter polymorphisms and type 2 diabetes risk in our study may be due, in part, to the fact that we lacked the statistical power to detect modest genetic associations. While the promoter polymorphisms that we examined were not significantly associated with type 2 diabetes risk, we could exclude the possibility that other rare SNPs across the *RETN* gene may influence risk of insulin resistance and related phenotypes [26]. Fine mapping of the *RETN* gene revealed that circulating resistin levels may be more influenced by polymorphisms in the 3' region than by promoter SNPs [25]. Nevertheless, these prospective data are consistent with previous experimental findings [27], which suggest the resistin protein as a marker of adipose tissue inflammation and not a significant mediator in the development of type 2 diabetes. This may explain the observed correlations between BMI, inflammatory markers, and resistin levels in our previous analysis [4] and the lack of association between BMI, inflammatory markers, and *RETN* SNPs in the current analysis.

Limitations from this study included the lack of data on *RETN* 3'UTR polymorphisms, low minor allele frequency for rs34861192, and inadequate statistical power that may have precluded the detection of modest genetic effects and/or gene-environment interactions. While population stratification could not be ruled out completely, matching of cases and controls by race in these two prospective studies of well-characterized, relatively homogeneous populations minimize such a bias. The strength of this study included the use of *RETN* promoter polymorphisms, as surrogate measures of circulating resistin levels, reducing the possibility of reverse causation in examining the influence of resistin levels on adiposity and inflammatory markers.

In conclusion, we did not find any significant relation between the two *RETN* polymorphism with risk of type 2 diabetes in two prospective cohorts of men and women. However, the rs34861192 promoter polymorphism was associated with elevated circulating resistin levels whose functional role warrants further investigation.

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