

Genetic variants at the resistin gene promoter might have a role in atherogenesis in patients with coronary artery disease

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Abstract

Introduction: Recent studies have demonstrated that resistin, an inflammatory adipokine, plays a key role in the pathogenesis of coronary artery disease (CAD). It is also known that the resistin gene (RETN) polymorphism, particularly in promoter region, is correlated with serum resistin levels and consequently an increased risk of CAD. This study investigates potential correlation between resistin gene polymorphism at -420C/G (rs1862513) and diabetes, as well as severity of CAD, in an Iranian cohort.

Methods: As a cross-sectional study, we recruited 113 subjects who were candidate for diagnostic coronary angiography. Laboratory measurements were FBS, OGTT, HbA₁C, hsCRP, and lipid profile. Genotyping for Single Nucleotide Polymorphism (SNP) was performed using the PCR-RFLP method.

Results: Our findings showed that CAD patients with diabetes had significantly higher FBS, HbA₁C and cholesterol levels and more severe coronary artery stenosis compared to non-diabetic subjects. Besides, the frequencies of the RETN -420C/G genotype in the diabetic group were significantly higher as compared to those in non-diabetic group ($P=0.009$). Moreover, the CC genotype carriers had more than twofold increased risk of Type 2 diabetes mellitus (T2DM) compared with the GG carriers. Although, there was no statistically significant correlation between RETN -420C/G polymorphism and severity of CAD ($P=0.3$), when we reanalyzed data with entering both the patients with one and two involved coronary arteries as one group, it was observed that patients with CC genotype more severely suffered from the coronary artery disease than the other genotypes ($P=0.04$). The Odds Ratio (OR) and relative risk for CAD patients with CC genotype were 4.33 (95%CI=1.02-18.38) and 2.25 (95%CI=0.97-5.19, $P=0.04$), respectively.

Conclusion: The results indicate that polymorphism in promoter region of RETN gene plays a role in the pathogenesis of coronary artery disease (probably due to its inflammatory characteristics) and the CC genotype is associated with an increased risk of diabetes and CAD compared to GG and CG.

Keywords: Resistin, Coronary Artery Disease, Polymorphism, Adipokine, RETN gene

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Introduction

A constellation of genetic and environmental factors including inflammation contribute to the pathogenesis of coronary artery disease (CAD). Cytokines and adipokines which provoke inflammatory signals could be potential candidates for investigation into the underlying mechanisms of CAD and its progression. Therefore, we considered resistin, a novel proinflammatory adipokine, to elucidate its potential role in the pathogenesis of CAD as previous studies have suggested its involvement (1, 2).

Resistin belongs to a family of cysteine-rich secretory proteins called resistin-like molecules (RELMs) or FIZZ (found in inflammatory zones) proteins (3, 4).

In murine models, it is demonstrated that resistin is mainly produced by adipocytes and plays a role in insulin resistance and obesity. Although, several studies in human have indicated the association between resistin and diabetes as other adipokines, some other studies had reported contrary findings (5-7). Unlike rodents, human resistin is produced by inflammatory cells such as macrophages. Moreover, it has been demonstrated that treatment of human endothelial cells with recombinant resistin increases cytokine and adhesion molecule levels (8-9). It is also demonstrated that resistin causes endothelial cell activation and induces smooth muscle cell proliferation. It also involves in angiogenesis and macrophage accumulation of cholesterol and triglyceride (10).

Although several studies have reported an association between high levels of serum resistin and CAD (11-13), to our knowledge, most of them have failed to show a positive relationship between those levels and different types of vascular diseases (14-16).

It is demonstrated that resistin plays a role in inflammatory pathway and influences the secretion of other cytokines (17-19). Therefore, the study of single nucleotide polymorphisms (SNPs) in the promoter region of RETN (resistin gene) could potentially result in elucidating the role of resistin in inflammatory response; particularly when it has been known that genetic variants of RETN promoter region affect serum levels of resistin (20, 21).

There is a large body of evidence on the RETN SNP -420C/G (rs1862513) polymorphism in

the promoter region (20) and its association with inflammation. However, because of contradicting results of some studies, the precise role of resistin in the inflammatory pathway remains unclear. Therefore, this study was designed to investigate the potential association between the RETN-420C/G polymorphism and CAD in an Iranian population.

Methods

Subjects

As a cross-sectional study, 113 patients with coronary artery disease (CAD) were recruited. Subjects were selected from hospitalized patients in Dr. Shariati Hospital (an affiliated teaching hospital of Tehran University of Medical Sciences, Tehran, Iran) from January to September 2009.

All the patients underwent diagnostic coronary angiography. Patients with malignancy, myocardial infarction, unstable angina, or previous coronary intervention (PCI) were excluded. Coronary artery disease was defined as the presence of a visible luminal narrowing of 50% or more in at least 1 coronary artery detected by angiography.

The severity of CAD was defined as one vessel disease, two vessels disease, and three vessels disease, based on the number of the affected coronary arteries. Type 2 diabetes was diagnosed according to the World Health Organization (WHO) criteria for diagnosis of the disease (22).

The study protocol was approved by the research ethics committee of Endocrinology and Metabolism Research Center (EMRC) and the ethics committee of the Iran Ministry of Health and Medical Education and informed consent was obtained from all the participants.

Laboratory measurements

Venous blood was collected following an overnight fasting and before angiography. Then, samples were centrifuged and serum separated and stored at -80 °C. All samples were run in the same assay. All measurements were performed in the EMRC laboratory of Shariati hospital.

HbA₁C was measured using HPLC¹ exchange ion method (DS5 England), FBG² by GOD/PAP method, TG³ by GPO-PAP method, total cholesterol by Enzymatic Endpoint method and direct high-density lipoprotein-cholesterol by enzymatic clearance assay. All tests were performed using Randox laboratories kit (Hitachi 902). Serum levels of hsCRP⁴, a known marker of inflammation, were measured by immunoturbidometric assay (High sensitivity assay, by Hitachi 902). Following an overnight fasting, the subjects were administered a standard dose of glucose (a solution of 75 grams of glucose in 250 ml of water). Then, blood samples were drawn after 120 min to measure plasma glucose concentrations using the GOD/PAP and Randox method laboratory kits.

Genotyping

Genomic DNA was extracted from whole blood, using FlexiGen Kit (QIAGEN Inc. Valencia, CA), according to its protocol. The -420C/G polymorphism in the 5' flanking region of the RTN gene was detected by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The target region amplified by the PCR using the forward primer 5'-TGT CAT TCT CAC CCA GAG ACA- 3' and the reverse primer 5' - TGG GCT CAG CTA ACC AAA TC- 3. PCR was performed in a total volume of 20 containing 200 ng genomic DNA, 0.5 pM of each primer, 0.2 mM dNTP, 2 mM MgCl₂, 2 ml of 10 X buffer and 1 U of *Taq* DNA polymerase (MBI Fermentas, Vilnius, Lithuania). PCR conditions were as followed: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. The resulting fragment was 534 bp in length. The PCR products were digested with 1 U BbsI (Fermentas, Vilnius, Lithuania) for 16 h at 37°C using the recommended buffer. Then the digestion products were separated by 2.5% agarose gel electrophoresis stained with ethidium bromide and visualized under

ultraviolet. Digestion of the PCR product produced two fragments, with lengths of 327 and 207 bp in the presence of the C homozygote, and three fragments for the heterozygote (CG), while G homozygotes remained uncleaved.

Statistical analysis

Numerical variables were reported as the mean \pm SD and categorical variables were presented as percentage. All of the statistical analyses were performed using the SPSS version 15 software. Comparisons of quantitative variable between cases and controls were carried out using student's T-test. We used Chi-square test to compare the qualitative variables and ANOVA to compare the quantitative variable in different genotypes. P-values less than 0.05 were considered to be statistically significant.

Results

Of all participants 83 (73.8%) were male and the prevalence of T2DM was 42%. The clinical features and biochemical characteristics of diabetic and non-diabetic subjects who were suffering from CAD are presented in Table 1.

There were no significant differences in age distribution and BMI between the diabetic and non-diabetic patients, but diabetic patients had significantly higher FBG, HbA₁C and cholesterol and more severe coronary involvement than the non-diabetic subjects.

The genotype frequencies of the -420C/G polymorphism of resistin gene in non-diabetic and diabetic subjects demonstrated significant differences ($P=0.009$) (Table 2). We also observed CC genotype carriers had more than twofold increased risk of T2DM as compared with the GG carriers. The OR for diabetic patients with CC genotype was 3.78 (95%CI=1.65-8.66) and the relative risk for patients with CC genotype was 2.99 (95% CI=1.34-6.68, $P= 0.009$). Due to the smaller proportion of GG carriers, subjects with CG and GG genotype were considered as a single group.

Table 3 shows genotype distribution of subgroups of CAD patients according to the severity of coronary involvement. There was no statistically significant association between RETN -420C/G polymorphism and severity of

¹ High pressure liquid chromatography

² fasting blood glucose

³ triglycerides

⁴ Hypersensitive C-reactive protein

CAD ($P=0.3$), and therefore, patients with one or two vessel disease were considered as a single group and defined as "mild or moderate" condition. Patients with three vessel disease were classified as "severe group"; then after reanalysis, there was an explicit statistical significant association between the RETN - 420C/G genotypes and CAD severity ($P=0.04$).

Within severe group, the frequency of genotypes was CC: 72.2% vs. GG+CG: 27.8% as compared to mild to moderate group which were CC: 37.5% vs. GG+CG: 62.5%. The OR and relative risk for CAD patients with CC genotype were 4.33 (95%CI=1.02-18.38) and 2.25 (95%CI=0.97-5.19, $P=0.04$), respectively.

Table 1- Clinical characteristics of diabetic and non-diabetic subjects suffering CAD

Variables	Non-diabetic subjects (n=66)	Diabetic patients (n=47)
Age(years)	58±8	58±9
BMI(kg/m ²)	27.7±5	28.8±6.2
FBG(mg/dl)*	86.2±18.3	129±53.8
Total cholesterol(mg/dl)*	171.2±71.5	218.4±39.9
Triglyceride(mg/dl)	148±52	166±94
hsCRP(mg/dl)*	2±1.1	2.4±1.8
HbA1c (%)	5.3±0.7	6.8±1.7
HDL-cholesterol(mg/dl)	47±12	43±8
Number of diseased* vessels		
Single vessel, n (%)	33(50%)	3(6.1%)
Double vessel, n (%)	17(25%)	17(36.7%)
Triple vessel, n (%)	16(25%)	27(57.1%)

All variables are presented as mean±SD.

Difference was statistically significant ($P<0.05$)*

Table 2-Allele and genotype frequencies of RETN -420C/G polymorphism in control subjects and type 2 diabetic patients

RETN -420C/G	Control subjects (n=66)	Diabetic subjects (n=47)	Relative risk(95%CI)
CC*	16(24.2%)	23(48.9%)	2.99 (1.34-6.68)
CG*	41(62.1%)	16(34%)	0.31 (0.14-0.68)
GG	9(13.6%)	8(17%)	1.29 (0.46-3.66)
C-allele	73(55%)	62(66%)	1.56 (0.9-2.7)

Percent of subjects is given in parentheses.

*Difference was statistically significant ($P<0.05$)

Table 3- Genotype distribution of subgroups of CAD patients, according to coronary involvement severity

CAD severity*	Genotype, n (%)		
	CC	CG	GG
SVD (n=15)	6(40%)	9(60%)	0(0%)
DVD (n=36)	12(33.3%)	15(41.7%)	9(25%)
TVD (n=62)	38(61.9%)	15(23.8%)	9(14.3%)

CAD, coronary artery disease; SVD, single-vessel disease ; DVD, double-vessel disease; TVD, triple-vessel disease

Percent of subjects is given in parentheses.

Differences were not statistically significant ($P>0.05$)*

Discussion

It has recently been suggested that resistin as a novel inflammatory adipokine plays a role in the pathogenesis of atherosclerosis. Due to its

proinflammatory characteristics and the fact that functional polymorphisms of promoter region of RETN gene alter resistin secretion, our study was designed to investigate RETN -

420C/G polymorphism and presumptively to help elucidating possible role of resistin in the pathogenesis of atherosclerosis. To our knowledge, this is the first study for investigation the association between the RETN -420C/G polymorphism and severity of CAD in an Iranian population.

Perhaps the most interesting finding of our study was that among CAD patients with type 2 diabetes, the frequency of CC genotype was twofold greater compared to patients without diabetes. This could potentially indicate a correlation between the aforementioned genotype and diabetes; which, in turn, contributes to coronary artery disease.

Our results are consistent with those reported by Ukkola *et al.* on a Finnish cohort of non-diabetic and hypertensive subjects. Ukkola *et al.* demonstrated that subjects with CC genotype showed increased fasting blood glucose, HbA₁C and LDL as compared to other genotypes. Besides, they showed that the subjects with CC genotype had the most severe insulin resistance and higher plasma triglyceride levels (23). Moreover, Wang *et al.* reported the CC genotype of SNP -420C/G was associated with increased insulin resistance in Caucasian population (24). The results of these two investigation and our study consistently indicates that the C allele in -420C/G resistin gene is associated with an increased risk for atherosclerosis and diabetes. Nonetheless, various studies in China (25), Japan (26, 27) and Quebec (28) showed different results. The results of their studies indicated that G allele was associated with higher blood glucose levels, disturbed lipid profile and increased susceptibility to type 2 diabetes, findings which explicitly are in contradiction to our results. Moreover, Tang *et al.* revealed that patients with GG or CG genotypes had more severe CAD involvement as compared with CC carriers; although, this association was not significant (29). On the other hand, the results of a study conducted by Tsukahara *et al.* showed that individuals with the CG and GG genotypes had the highest prevalence of stroke within their study subjects (30).

There are studies, however, which have reported no significant association between

resistin gene polymorphism and the risk of diabetes and coronary artery disease mentioned earlier.

In the present study we also examined a potential association between the RETN-420C/G polymorphism and the severity of coronary artery disease. We observed a statistically significant difference between various RETN-420C/G genotypes and CAD severity after clustering patients with single or double vessel disease as a single group.

Notwithstanding aforementioned controversies, our results indicate that RETN-420C/G polymorphism might be of some importance in diabetes risk and CAD severity. Moreover, our findings are in accordance with the existing literature indicating that diabetic patients are at a higher risk for more severe coronary artery disease compared to non-diabetic subjects.

In conclusion, our results revealed that CC genotype, was associated with an increased risk of CAD and susceptibility to diabetes compared with CG and GG genotypes. However, as we discussed, there is a significant inconsistency about the role of genetic variants of RETN in the pathogenesis of inflammatory-related phenotypes reported by different studies conducted in different populations. This inconsistency may be due to several factors including the study design, sample size, ethnicity differences, and age distribution of subjects.

Although, our findings are consistent with the well documented literature on the role of diabetes as a major risk factor for CAD, the precise role of CC genotype at -420 position of RETN and its potential effects on the pathogenesis of coronary artery disease remains obscure. Therefore, we believe that more study is warranted to investigate the role of SNP -420C/G in evolving atherosclerosis, and consequently myocardial infarction.

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References

1. Reilly MP, Lehrke M, Wolfe ML, Rohatgi A, Lazar MA, Rader DJ. Resistin is an inflammatory marker of atherosclerosis in humans. *Circulation* 2005; 111: 932-939.
2. Lehrke M, Reilly MP, Millington SC, Iqbal N, Rader DJ, Lazar MA. An inflammatory cascade leading to hyperresistinemia in humans. *Plos Med* 2004; 1:e45.
3. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee R, Wright CM, et al. The hormone resistin links obesity to diabetes. *Nature* 2001; 409: 307-312.
4. Steppan CM, Brown EJ, Wright CM, et al. A family of tissue-specific resistin-like molecules. *Proc Natl Acad Sci* 2001; 98: 502-506.
5. Kusminski CM, McTernan PG & Kumar S. Role of resistin in obesity, insulin resistance and Type II diabetes. *Clinic Sci* 2005; 109: 243-256.
6. Utzschneider KM, Carr DB, Tong J, Wallace TM, Hull RL, Zraika S, et al. Resistin is not associated with insulin sensitivity or the metabolic syndrome in humans. *Diabetologia* 2005; 48: 2330-2333.
7. Mirzaei K, Hossein-Nezhad A, Hosseinzadeh-Attar MJ, Jafari N, Najmafshar A, Mohammadzadeh N, Larijani B. Visfatin genotype may modify the insulin resistance and lipid profile in type 2 diabetes patients. *Minerva Endocrinologica* 2009; 34: 273-279.
8. Jung HS, Park KH, Cho MY, Chung SS, Cho HJ, Cho SY, et al. Resistin is secreted from macrophages in atheroma and promotes atherosclerosis. *Cardiovasc Res* 2006; 69:76-87.
9. Verma S, Li SH, Wang CH, Fedak PW, Li RK, Weisel RD, Mickle DA. Resistin promotes endothelial cell activation: further evidence of adipokine- endothelial interaction. *Circulation* 2003; 108: 736-740.
10. Rae C, Graham A. Human resistin promotes macrophage lipid accumulation. *Diabetologia* 2006; 49:1112-1114.
11. Burnett MS, Lee CW, Kinnaird TD, Stabile E, Durrani S, Dullum MK, et al. The potential role of resistin in atherogenesis. *Atherosclerosis* 2005; 182: 241-248.
12. Ohmori R, Momiyama Y, Kato R, Taniguchi H, Ogura M, Ayaori M, et al. Associations between serum resistin levels and insulin resistance, inflammation, and coronary artery disease. *J Am Coll Cardiol* 2005; 46:379-380.
13. Hu WL, Qiao SB, Hou Q, Yuan JS. Plasma resistin is increased in patients with unstable angina. *Chin Med J* 2007; 120(10):871-875.
14. Pischon T, Bamberger CM, Kratzsch J, Zyriax BC, Algenstaedt P, Boeing H, Windler E. Association of plasma resistin levels with coronary heart disease in women. *Obstet Res* 2005; 13:1764-1771.
15. Hoefle G, Saely CH, Risch L, Koch L, Schmid F, Rein P, et al. Relationship between the adipose-tissue hormone resistin and coronary artery disease. *Clin Chim Acta* 2007; 386: 1-6.
16. Qasim AN, Metkus TS, Tadesse M, Lehrke M, Restine S, Wolfe ML, et al. Resistin gene variation is associated with systemic inflammation but not plasma adipokine levels, metabolic syndrome or coronary atherosclerosis in non-diabetic Caucasians. *Clin Endocrinol (Oxf)* 2009; 70(5):698-705.
17. Kawanami D, Maemura K, Takeda N, Harada T, Nojiri T, Imai Y, et al. Direct reciprocal effects of resistin and adiponectin on vascular endothelial cells: a new insight into adipocytokine endothelial cell interactions. *Biochem Biophys Res Commun* 2004; 314:415-419.
18. Xu W, Yu L, Zhou W, Luo M. Resistin increases lipid accumulation and CD36 expression in human macrophages. *Biochem Biophys Res Commun* 2006; 351: 376-382.
19. Osawa H, Yamada K, Onuma H, Murakami A, Ochi M, Kawata H, et al. The G/G genotype of a resistin single-nucleotide polymorphism at -420 increases type 2 diabetes mellitus susceptibility by inducing promoter activity through specific binding of Sp1/3. *Am J Hum Genet* 2004; 75: 678-686.
20. Cho YM, Youn B S, Chung S S, Kim K W, Lee H K, Yu K Y, et al. Common genetic polymorphisms in the promoter of resistin gene are major determinants of plasma resistin concentrations in humans. *Diabetologia* 2004; 47: 559-565.
21. Silswal N, Singh AK, Aruna B, Mukhopadhyay S, Ghosh S, Ehtesham NZ.

Human resistin stimulates the proinflammatory cytokines TNF-alpha and IL-12 in macrophages by NF-kappa B-dependent pathway. *Biochem Biophys Res Commun* 2005; 334: 1092-1101.

22. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. I. Diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998; 15:539-53.

23. Ukkola O, Kunnari A, Kesäniemi YA. Genetic variants at the resistin locus are associated with the plasma resistin concentration and cardiovascular risk factors. *Regul Pept* 2008; 149:56-59.

24. Wang H, Chu WS, Hemphill C, Elbein SC. Human resistin gene: molecular scanning and evaluation of association with insulin sensitivity and type 2 diabetes in Caucasians. *J Clin Endocrinol Metab* 2002; 87, 2520-2524.

25. Xu JY, Sham PC, Xu A, Tso AW, Wat NM, Cheng KY, et al. Resistin gene polymorphisms and progression of glycaemia in southern Chinese: a 5-year prospective study. *Clin Endocrinol* 2007; 66:211-217.

26. Osawa H, Yamada K, Onuma H, Murakami A, Ochi M, Kawata H, et al. The G/G genotype of a resistin single-nucleotide polymorphism at -420 increases type 2 diabetes mellitus susceptibility by inducing promoter activity through specific binding of Sp1/3. *Am J Hum Genet* 2004; 75:678-686.

27. Osawa H, Tabara Y, Kawamoto R, Ohashi J, Ochi M, Onuma H, et al. Plasma resistin, associated with single nucleotide polymorphism -420, is correlated with insulin resistance, lower HDL cholesterol, and high-sensitivity C-reactive protein in the Japanese general population. *Diabetes Care* 2007; 30: 1501-1506.

28. Engert JC, Vohl MC, Williams SM, Lepage P, Loredo-Osti JC, Faith J, et al. 5' flanking variants of resistin are associated with obesity. *Diabetes* 2002; 51(5):1629-1634.

29. Tang NP, Wang LS, Yang L, Zhou B, Gu HJ, Sun QM, et al. A polymorphism in the resistin gene promoter and the risk of coronary artery disease in a Chinese population. *Clin Endocrinol (Oxf)* 2008; 68: 82-87.

30. Tsukahara T, Nakashima E, Watarai A, Hamada Y, Naruse K, Kamiya H, et al. Polymorphism in resistin promoter region at S420 determines the serum resistin levels and may be a risk marker of stroke in Japanese type 2 diabetic patients. *Diabet Res Clin Prac* 2009; 84: 179-186.

31. Norata GD, Ongari M, Garlaschelli K, Tibolla G, Grigore L, Raselli S, et al. Effect of the -420C/G variant of the resistin gene promoter on metabolic syndrome, obesity, myocardial infarction and kidney dysfunction. *J Intern Med* 2007; 262: 104-112.