Original Article

The Regulatory Variant -108C/T in the Promoter of Paraoxonase 1 (PON1) Gene has a More Important Role in Regulating PON1 Activity Compared to rs3735590 in 3′-UTR in Patients with Coronary Artery Disease

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Abstract

Background: This study aimed to assess the serum activity of paraoxonase 1 (PON1) in patients with coronary artery disease (CAD) based on two genetic variants including the -108C/T variant in the promoter region and the rs3735590 variant in the binding site of miR-616 at the 3′-UTR of the PON1 gene.

Materials and Methods: A total of 140 subjects who exhibited clinical symptoms of CAD underwent diagnostic coronary angiography. The patients with CAD were further categorized into two groups: single-vessel disease (SVD) and multi-vessel disease (MVD). The study variants were genotyped using the restriction fragment length polymorphism (RFLP) technique after polymerase chain reaction amplification.

Results: After adjusting for age, gender, body mass index, metformin, and statin usage, a significant association was observed between the -108C/T variant and PON1 activity ($P < 0.001$). In the sub-groups of both SVD and MVD, individuals with the TC+CC genotypes exhibited significantly higher PON1 activity compared to TT homozygotes ($P = 0.001$ for SVD and $P = 0.01$ for MVD). As for the rs3735590 variant, individuals with the A allele (GA+AA genotypes) had higher PON1 activity compared to those with the GG genotype in both the SVD and MVD groups, although the results did not reach statistical significance.

Conclusions: Our study findings indicate a significant decrease in PON1 activity among patients with obstructive CAD. Notably, our results suggest that the -108C/T variant exerts a greater influence on PON1 activity compared to the rs3735590 variant. These findings highlight the crucial role of the -108C/T variant in modulating PON1 activity within the context of atherosclerosis.

Keywords: Coronary artery disease, PON1, single-nucleotide variation

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Submitted: 17-Nov-2022; **Revised:** 20-Feb-2024; **Accepted:** 02-Mar-2024; **Published:** 29-Jul-2024

Introduction

Human paraoxonase 1 (PON1) is an esterase/lactonase synthesized mainly in the liver. It is carried on high-density lipoprotein (HDL) in the circulation and is responsible for an important part of the anti-oxidative activity of this critical lipoprotein.[1,2] PON1 function is essential for the protective effect of HDL on low-density lipoprotein (LDL) and for

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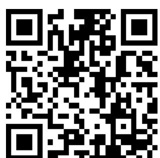

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DOI: 10.4103/abr.abr_391_22

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How to cite this article: Zargari M, Maadi N, Rezapour M, Bagheri A, Fallahpour S, Nosrati M, *et al*. The regulatory variant -108C/T in the promoter of paraoxonase 1 (PON1) gene has a more important role in regulating PON1 activity compared to rs3735590 in 3′-UTR in patients with coronary artery disease. Adv Biomed Res 2024;13:38.

preventing LDL oxidation. Oxidized-LDL particles are well known to be key factors in atherosclerosis development.[3] PON1-deficient mice were unable to impede LDL oxidation and were prone to accelerated atherosclerosis.[4] The dysfunction of PON1 can influence HDL dysfunction and lead to elevating the risk of coronary artery disease (CAD).[1] Furthermore, low PON1 activity is related to an increase in CAD risk in patients with type 2 diabetes.[5]

PON1 gene is polymorphic in human populations and is located on the long arm of chromosome 7.[6] It is known that single-nucleotide variations in the PON1 gene influence the activity and expression of PON1.[7,8] These genetic variations can contribute to different responses to substrates and help explain, in part, the significant variability observed in PON1 levels among individuals.[9] In addition to common variations in the coding region (Q192R and L55M), the variations in the promoter region, particularly the variant located at position -108C/T, affect PON1 gene expression.[10,11] The -108C/T variant has a binding site for the transcription factor specificity protein 1 (SP1) that affects PON1 gene expression, and as a result, it changes its activity.[12] The TT genotypes of this variant are found to be related to decreased serum PON1 activity and an increased CAD risk.[13] Moreover, another variant in the 3' untranslated region (3′-UTR) of the PON1 gene is found to affect its expression.[8,14]

Single-nucleotide changes within the binding sites of microRNAs (miRNAs, miRs) can have an impact on their functions.[15] These non-coding RNAs play a role in regulating gene expression at the post-transcriptional level, leading to the degradation of target mRNA or the repression of mRNA translation.[16] Understanding the detailed molecular mechanisms by which miRNAs and other non-coding RNAs operate could potentially contribute to the development of therapeutic strategies and improved diagnostic approaches for various diseases.[17] The variant rs3735590 is located in the binding site of miR-616 at the 3′-UTR of the PON1 gene and plays a role in regulating the binding ability of this miRNA to the PON1 gene.[8,14] The A allele of rs3735590 contributes to inducing an elevation in the expression of PON1 levels.[14] Based on previous findings, this variant may affect susceptibility to ischemic stroke and asymptomatic carotid atherosclerosis[18] and calcific aortic valve stenosis.[8] It was also presented as a prognostic biomarker in patients with chronic obstructive pulmonary disease treated by coronary artery bypass grafting.[19] This variant as a functional variant in the 3′-UTR of the PON1 gene may influence the risk of CAD by interfering with the binding of miR-616. According to the regulatory role of miR-616 in PON1 gene expression,[14,19] rs3735590 may be of importance for PON1 activity. The presence of the T (A) or C (G) allele at the rs3735590 position can influence PON1 gene expression and as a result impact its activity.[20]

There are a few data on rs3735590, particularly its effects on PON1 activity in patients with CAD. Also, its roles concerning other important variants of the PON1 gene have not been investigated in previous studies. This study was thus conducted to evaluate PON1 activity in patients with CAD according to a variant in the promoter region (-108C/T) and another in the binding site of miR-616 at the 3′-UTR of the PON1 gene (rs3735590) and also to compare their impacts on PON1 activity based on the number of obstructive coronary vessels. This will help to develop further knowledge on PON1 status in cardiovascular disease in which PON1 has been known as an important player.

Materials and Methods

Subjects

The present study comprised 140 subjects who had a positive exercise test and underwent diagnostic coronary angiography for showing clinical symptoms of CAD (70 without significant CAD and 70 with severe CAD). These subjects were categorized into two groups: non-CAD (no major vessels with stenosis over 50%) and severe CAD (having at least one epicardial stenosis ≥70%). The CAD patients were further classified into the following categories: single-vessel disease (SVD; ≥70% stenosis in one of the major coronary arteries) and multi-vessel disease (MVD; ≥70% stenosis in at least two of the three coronary arteries).[21,22] Medical history, demographic characteristics, personal habits, and medication were collected by a questionnaire. A list of medications is shown in Table 1. Criteria for exclusion were having a history of myocardial infarction or stroke (hemorrhagic or ischemic),

Table 1: Participant characteristics

Parameter	Non-CAD (n=70)	CAD (n=70)	P
Age, year	54.3±11.5	58.1±7.8	<0.001
Women, %	58.2	50	0.245
Diastolic blood pressure, mmHg	76.1±8	78.1±10.01	0.509
Systolic blood pressure, mmHg	121.4±15.4	124.5±20.5	0.057
Smoking, %	18.2	24.6	0.378
Hypertension, %	60.4	54.6	0.409
Diabetes, %	40.7	53.7	0.066
BMI, kg/m^2	28.5±4.6	28.2±4.7	0.760
Fasting glucose, mmol/L	6.92±3.01	8.61±4.09	<0.001
HDL-C, mmol/L	1.01±0.22	0.97±0.21	0.317
Total cholesterol, mmol/L	4.21±1.06	4.22±1.01	0.992
Triglyceride, mmol/L	1.69±0.74	1.83±0.84	0.292
LDL-C, mmol/L	2.42±0.86	2.43±0.79	0.883
PON1 activity, μmol/min/mL	58.1±18.9	52±24.1	0.092
Medications (%)			
Angiotensin II receptor blocker	24.2	21.3	0.629
ACE inhibitor	11	8.3	0.525
Calcium channel blocker	5.5	10.2	0.225
Diuretics	3.3	3.7	0.877
Aspirin	61.5	55.6	0.394
Metformin	17.6	29.6	0.048
Statin	46.2	59.3	0.065
Fibrate	2.2	3.7	0.536

liver diseases, renal failure, auto-immune diseases, and cancer. The research protocol was approved by the university's local ethics committee and was planned in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects.

PON1 activity measurement

Phenylacetate was used as a substrate for assaying the arylesterase activity of PON1 in serum. The initial rate of substrate hydrolysis was measured spectrophotometrically at 270 nm with an extinction coefficient of 1310 M^{-1} cm^{-1}. The assay cuvette contained 1mM $CaCl_2$ and 1 mM phenylacetate in Tris/HCL buffer (100 mM, pH 8.0). This coefficient was used for determining the units of enzyme activity, which is expressed as micromoles of phenol produced per minute. The inter- and intra-assay coefficients of variation were 4.3% and 3.1%, respectively.

Genotype determination

Genomic DNA was extracted using a phenol-chloroform method. Two study variants were genotypes by PCR amplification, followed by the restriction fragment length polymorphism (RFLP) and gel electrophoresis. The primer pairs used for the variant -108C/T were F 5' GACCGCAAGCCACGCCTTCTGTGCACC 3' and R 5' TATATTTAATTGCAGCCGCAGCCCTGCTGGG GCAGCGCCGATTGGCCCGCCGC 3'.[10] The primers for the rs3735590 variant were designed using the program Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The specificity of the designed primers was confirmed by BLAST. For this variant, F primer 5' CATTTTGGCAATAAATCCCTCT 3' and R primer 5' CCCATTGACATAGGATTTCCA 3' were used.

DNA was amplified with an initial melting temperature of 93°C for 3 min and 35 cycles of denaturation, annealing, and extension. The annealing temperatures used were 64°C for -108C/T and 59°C for rs3735590. PCR products (639 bp) were analyzed by 1% gel electrophoresis. The genotypes of the variant -108C/T and rs3735590 were determined by *BstUI* and *BsrI* digestion, respectively. For the -108C/T variant, the digested fragments were 67 and 52 bp in size, which were analyzed by electrophoresis on a 3% agarose gel. For rs3735590, the produced fragments were 343 and 296 bp in size, which were electrophoresed through 3% agarose.

Statistics

Statistical calculations were performed using the software R (version 3.0.1) and SPSS (version 16.0). The Kolmogorov–Smirnov test was used to test the normality of the variables. Differences between the study groups were compared by *t*-test or Mann–Whitney test for continuous variables and Chi-square or Fisher exact tests for categorical variables. Logistic regression analysis was performed to estimate the independent association of the study variants with PON1 activity. The concordance of genotype frequencies with Hardy–Weinberg's equilibrium expectations was analyzed by the Chi-square test. A value of $P < 0.05$ on the two-tailed test was accepted as statistically significant.

Results

Figure 1 presents the results of PCR-RFLP for genotyping two study variants. For the -108C/T variant [Figure 1a], the amplicons (119 bp) were subjected to restriction analysis with *BstUI*, leading to 67 and 52 bp fragments. The presence of 119, 67, and 52 bp fragments belongs to TC genotypes. An undigested fragment with 119 bp length was referred to as TT genotypes. CC homozygotes were identified by the presence of two fragments with lengths of 67 and 52 bp. For rs3735590 [Figure 1b], GA genotypes were identified by the presence of 639, 343, and 296 bp fragments, while the presence of fragments with the length of 343 and 296 bp was the basis for identifying GG genotypes. A non-digested fragment with 639 bp length was for AA homozygotes. According to our analyses, the genotypes of both study variants were in Hardy–Weinberg equilibrium in both CAD patients and the non-CAD group ($P > 0.05$).

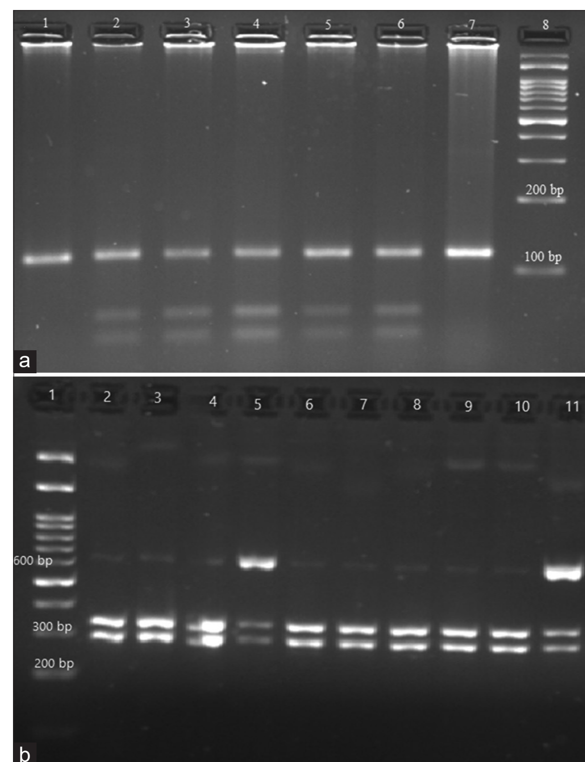

Figure 1: PCR-based RFLP for the variants -108C/T and rs3735590. For the -108C/T variant (a), TT genotypes: the presence of an undigested fragment with the length of 119 bp (lanes 1 and 7); TC genotypes: the presence of three fragments with the lengths of 119, 67, and 52 bp (lanes 2–6); CC homozygotes: the presence of two fragments with the lengths of 67 and 52 bp (data not shown). DNA ladder (100 bp) is presented in lane 8. For rs3735590 (b), GA genotypes: the presence of three fragments with the lengths of 639, 343, and 296 bp (lanes 5 and 11); GG genotypes: the presence of fragments with the lengths of 343 and 296 bp (lanes 2–4 and 6–10); AA homozygotes: a non-digested fragment with the length of 639 bp (data not shown). Lane 1 corresponds to DNA ladder 100 bp

Table 1 compares values of the study parameters in CAD patients and the non-CAD group. As expected, CAD patients had a lower PON1 activity compared with non-CAD subjects, although the values were not statistically significant. There was no statistically significant difference between the two groups with respect to diastolic and systolic blood pressure, gender, hypertension, diabetes mellitus, smoking, BMI, LDL-C, HDL-C, TC, and TG levels. Fasting glucose and age were significantly higher in patients with CAD than in the non-CAD group. No significant differences were found between the two groups with respect to medications, including calcium channel blockers, angiotensin II receptor blockers, angiotensin-converting enzyme (ACE) inhibitors, diuretics, aspirin, statins, and fibrates. However, metformin use was significantly higher in CAD patients compared with non-CAD subjects.

As could be seen in Figure 2, both variants affect PON1 activity in CAD patients. Accordingly, for the -108C/T variant, compared with the TT genotypes, PON1 activity was significantly higher in TC+CC group ($P < 0.001$). Also, for rs3735590, the GA+AA group had higher PON1 activity than GG homozygotes, although significant differences were not observed among the groups. No significant differences were observed according to the genotypes of both variants in the non-CAD group.

Linear regression analyses were conducted using PON1 activity as the dependent variable to further analyze the relationship between PON1 activities with two studied variants. As shown in Table 2, for the -108C/T variant, there was a significant relationship with PON1 activity after performing an adjustment for age, gender, BMI, metformin, and statin ($P < 0.001$). Compared to patients with TT genotype, those containing C-allele genotypes had a higher PON1 activity with an amount average of 20.39. Also, the rs3735590 variant (A-allele containing genotypes) had an increasing effect on PON1 activity, although significant differences were not found ($P = 0.326$).

We further analyzed the effects of the variants on PON1 activity according to SVD and MVD among CAD patients as shown in Figures 3 and 4. For rs3735590, PON1 activity was higher in individuals with A allele (GA+AA) compared to GG genotypes in both SVD and MVD groups, but results did not reach a statistically significant level [Figure 3]. When such analyses were performed for -108C/T variant, the findings showed that PON1 activity was significantly increased in TC+CC group than in TT homozygotes in both the sub-groups of SVD ($P = 0.001$) and MVD ($P = 0.01$).

Discussion

Based on studies, the anti-oxidant properties of HDL and its anti-inflammatory capacity mostly depend on its PON1 activity.[23,24] This enzyme plays an important role in the prevention of LDL oxidation, which contributes to the pathogenesis of CAD.[25] The risk of CAD has been closely associated with the decreased activity of PON1 in several studies.[26-28] Based on meta-analyses, PON1 variants have been associated with human diseases, including CAD and ischemic stroke.[28,29] Therefore, the regulation of PON1 activity by these variations is of importance in CAD development. One of these variants that has recently received attention is rs3735590 in

Table 2: Linear regression analysis of the relationship between PON1 activity and the variants -108C/T and rs3735590 in patients with CAD

	Beta	SE	*P*
rs3735590	6.46	6.53	0.326
Age	0.14	0.37	0.711
Gender	6.86	6.11	0.266
BMI	0.63	0.72	0.382
Metformin	-3.75	6.95	0.592
Statin	0.15	6.27	0.98
-108C/T	20.39	4.94	<0.001
Age	-0.21	0.30	0.481
Gender	-0.01	4.51	0.999
BMI	0.54	0.52	0.297
Metformin	-2.40	4.94	0.628
Statin	0.39	4.66	0.934

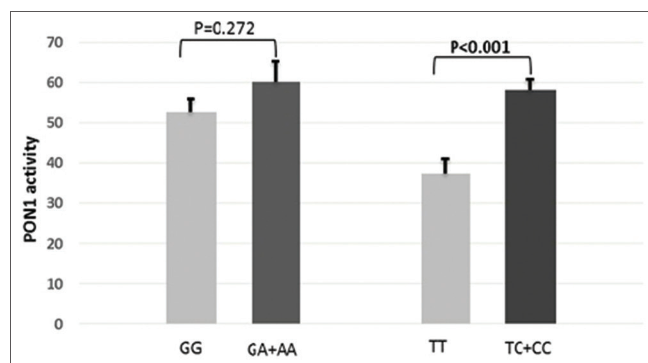

Figure 2: Change in paraoxonase 1 (PON1) activity according to the genotypes of the variant rs11558471 (GG and GA+AA) and those of -108C/T (TT and CT+CC) in patients with CAD

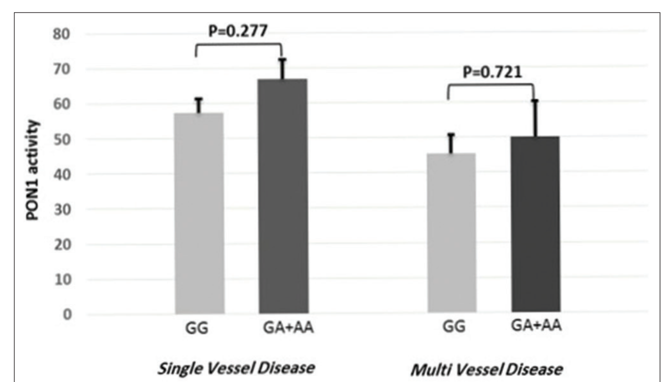

Figure 3: Change in PON1 activity according to the genotypes of the variant rs11558471 (GG and GA+AA) in CAD patients with single-vessel disease and multi-vessel disease

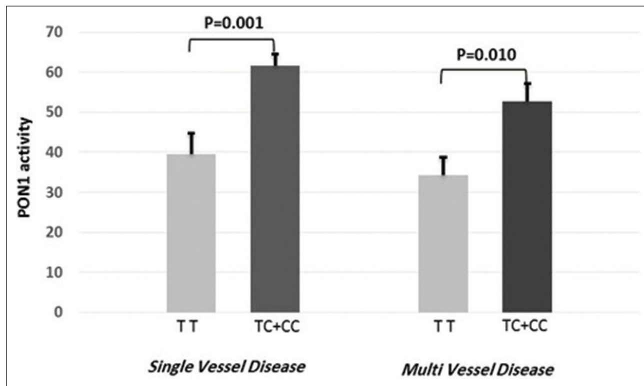

Figure 4: Change in PON1 activity according to the genotypes of the variant -108C/T (TT and CT+CC) in CAD patients with single-vessel disease and multi-vessel disease

the 3′-UTR of the PON1 gene. To the best of our knowledge, this study is the first to evaluate the effects of rs3735590 on PON1 activity *in vivo* and to compare it with -108C/T as the most important regulatory variant in the promoter region of the PON1 gene in patients with CAD.

Based on our findings, PON1 activity was higher in individuals with the A allele compared to GG genotypes. Such results were also obtained in the sub-groups of SVD and MVD. Our findings are consistent with previous research, indicating that the substitution of G by A at rs3735590 contributes to increased complementarity between miR-616 and PON1 mRNA, leading to enhanced expression of the PON1 gene.[8,18] It appears that the A allele reduces the binding affinity of miR-616 to the 3′-UTR compared to the G allele, potentially contributing to a decrease in CAD risk. A luciferase reporter assay conducted by Liu *et al*.,[14] demonstrated that the G allele of rs3735590 leads to decreased expression of PON1 in the presence of miR-616. However, these researchers did not analyze the effects of this variant on PON1 levels at the protein level (activity or concentration). In another study, Wang *et al*.[8] confirmed that PON1 is a validated target gene of miR-616, and PON1 expression and serum protein levels were influenced by rs3735590 in patients with calcific aortic valve stenosis. They used paraoxon as the substrate to assay PON1 activity (paraoxonase activity). It is worth noting that paraoxonase activity can be influenced by other PON1 gene variants, particularly those located in the coding region,[30] which may have an impact on the obtained results. In the current study, we used phenylacetate as the substrate and measure the arylesterase activity of PON1, which is minimally influenced by PON1 variants and thus would have minor inter-individual variability.[24] Our study supports the suggestion that the minor allele A of rs3735590 may have a protective impact on CAD risk, which can exert via elevated PON1 activity. However, further studies still require to fully understand the roles of this variant, particularly CVD.

About the -108C/T variant, our results indicated that PON1 activity significantly increases in the C-carrying genotypes, which is confirmed by the fact that the binding activity of Sp1 to the -108 site is weaker in the presence of allele T compared to allele C.[12,31] Moreover, according to regression analysis, this variant indicated a significant association with PON1 activity in CAD patients after adjustment for gender, age, BMI, metformin, and statin therapy. Importantly, the modified binding of Sp1 to the upstream region of the PON1 gene influences the promoter activity, causing changes in PON1 protein expression, which in turn affects its activity. Our findings align with the data from Mackness *et al*.,[32] who reported significant differences in PON1 activity and concentration among -108C/T genotypes in coronary heart disease. Additionally, Kim *et al*.[33] provided further support by demonstrating that the -108C/T variant accounts for approximately 12% of the variation in PON1 activity, even when combined with other nutritional factors. Moreover, PON1 activity was significantly lower in C allele-containing people regardless of the number of obstructive vessels that have not been shown in previous studies. In patients with CAD, it appears PON1 activity was increased concerning the C allele in both patients with SVD and MVD.

In conclusion, our study findings indicate a significant decrease in PON1 activity among patients with obstructive CAD. Notably, our results suggest that the -108C/T variant exerts a greater influence on PON1 activity compared to the rs3735590 variant. These findings highlight the crucial role of the -108C/T variant in modulating PON1 activity within the context of atherosclerosis. Further investigation is warranted to explore the potential impact of the rs3735590 variant on PON1 levels.

Financial support and sponsorship

This study was supported by Mazandaran University of Medical Sciences.

Conflicts of interest

There are no conflicts of interest.

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