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RESEARCH ARTICLE

Unraveling the understudied influence of a lead variant in the 9p21 locus on the atherogenic index among type 2 diabetes patients with coronary artery disease

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Abstract

Introduction The region on chromosome 9p21 has consistently been identified in genome-wide association studies (GWAS) as the top locus for type 2 diabetes (T2D), however, genetic variations in this locus affecting both T2D and coronary artery disease (CAD) require further characterized. Our aim was to assess the effects of rs10811661, a variant validated in GWAS, on log (TG/HDL-C), which has been associated with an atherogenic lipid profile.

Methods A total of 121 patients with T2D who underwent coronary angiographic examination were included in this study. The patients were categorized into two groups, those with angiographically normal coronary arteries or less than 50% stenosis (non-CAD) and those having at least 70% stenosis in one of the main coronary arteries (severe CAD). The rs10811661 variant was genotyped using the restricted fragment length polymorphism (RFLP) analysis after PCR amplification.

Results When the data was divided into tertiles according to HbA1c, our findings revealed that in tertile 3 (HbA1c ≥ 7.8%), the frequency of TT genotypes was higher compared to CT + CC genotypes (37.1% vs. 27.8%). T2D patients with CAD who carried the TT genotype had higher concentrations of log (TG/HDL) ($p = 0.037$) and TG ($p = 0.003$) compared to those with the C allele (CC or CT genotypes). After adjustment for covariates, the T allele of rs10811661 indicated significant associations with TG (OR = 1.66, 95% CI: 1.22–2.33, $p = 0.002$) and log (TG/HDL-C) (OR = 1.12, 95% CI: 1.02–2.13, $p = 0.023$) levels.

Conclusion Our findings provide insight into how a GWAS-validated variant, rs10811661, can influence atherogenicity in patients with T2D and establish a link between this functional variant in the 9p21 locus and lipid factors associated with atherosclerosis. Further investigations are needed to understand the mechanisms by which this important variant influences lipid and lipoprotein levels, which could be useful in developing personalized medicine interventions.

Keywords Single nucleotide polymorphism · Biomarkers · Type 2 diabetes · Coronary artery disease

Introduction

Coronary artery disease (CAD) is the leading cause of mortality in individuals with diabetes, and it is the most prevalent cardiovascular disease, CVD [1, 2]. Its impact, along with stroke, extends beyond physical health, affecting socio-economic factors and emotional well-being in both patients and communities [3]. The interplay between environmental and genetic factors is crucial in understanding the development of CVD and type 2 diabetes (T2D), two significant diseases of our time [4, 5]. T2D is a chronic condition that gives rise to various complications, including retinopathy, blindness, nephropathy, neuropathy, and an elevated risk of cardiac diseases and stroke. Certain genetic

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variants have been identified as contributors to cardiovascular complications in individuals with diabetes [4, 6]. The 9p21 genetic locus has been identified as one of the most influential genetic markers of CAD and myocardial infarction in diverse populations, as evidenced by genome-wide association studies (GWAS) that have been replicated in multiple studies [4]. This locus contains several variants that have been linked to T2D [7–9] and range of vascular diseases, including stroke, CAD, peripheral artery disease, and abdominal aortic aneurysm [10–12]. This implies that this particular locus might confer a predisposition for both T2D and CAD through shared underlying biological mechanisms [13]. Investigating the role and impact of this locus in the development of these conditions can yield valuable insights into their pathogenesis and potentially open new avenues for prevention and treatment strategies.

Understanding the influence of variants on the risk of complex diseases like T2D is a significant focus in genetics research [14]. The 9p21 locus encompasses four genes: methylthioadenosine phosphorylase (MTAP), the cyclin-dependent kinase inhibitors (CDKN2A and CDKN2B) and a long non-coding RNA (lncRNA) known as ANRIL [15]. The variant rs10811661 has been validated as an important SNP located upstream of recognized genes within the 9p21 locus [16]. This particular SNP may exert a long-range effect on the activity of CDKN2A and CDKN2B, whose role is to inhibit the activity of CDK4 and CDK6 [8]. Notably, CDKN2A and CDKN2B are expressed in islet cells and adipocytes [8]. Additionally, the variant rs10811661 has been associated with alterations in the expression of ANRIL. ANRIL plays a role in regulating endothelial cell functions that are directly linked to the pathogenesis of CAD [1, 15, 17]. These findings suggest that this variant may have a functional role in the susceptibility to T2D and related diseases. Further investigation into the functional implications of this variant can provide valuable insights into the development and progression of T2D and associated conditions.

It is worth highlighting that the rs10811661 variant has been linked to reduced insulin secretion capacity following glucose challenges, whether administered orally or intravenously [18, 19]. Additionally, it has been associated with insulin sensitivity [20]. As adipose tissue is a significant target for insulin, it is expected that the function of this tissue will be affected by the variant. The precise mechanisms through which T2D risk variants influence the disease and its complications remain largely unknown for the majority of variants [15]. In light of this, we conducted genotyping of the rs10811661 SNP in T2D patients with and without CAD. Our aim was to assess the impact of rs10811661 on the logarithm of the ratio of triglyceride (TG) to high density lipoprotein cholesterol [log (TG/HDL-C)]. This ratio has been associated with an atherogenic lipid profile, LDL density and higher non-HDL cholesterol levels [21]. It can serve as an indicator of increased CVD risk, even in individuals who do not exhibit extremely abnormal lipid levels [22].

Materials and methods

Study population and design

The study enrolled a total of 121 patients diagnosed with T2D according to the criteria established by the World Health Organization (WHO). All participants underwent coronary angiographic examination. The patients were divided into two groups: those with angiographically normal coronary arteries or less than 50% stenosis (referred to as the non-CAD group, with a total of 41 patients) and those with at least 70% stenosis in one of the major coronary arteries (referred to as the severe CAD group, with a total of 80 patients). None of the patients had type 1 diabetes, liver diseases, renal failure, chronic diseases, or were pregnant. The assessment process included a comprehensive questionnaire to collect demographic information, medical history, and personal habits. Medications administered to the patients included metformin, statin, angiotensin-converting enzyme inhibitors, beta-blockers, calcium channel blockers, and aspirin. None of the patients received insulin therapy or oral antidiabetic drugs prior to their T2D diagnosis. The study was designed in accordance with the ethical guidelines outlined in the Declaration of Helsinki and obtained approval from the local ethics committee at the university. Participants provided informed consent after receiving a detailed explanation regarding the purpose and objectives of the investigation.

Biochemical analyses

Fasting glucose levels, triglycerides (TGs), HDL-C, and low density lipoprotein-cholesterol (LDL-C) were measured using standard enzymatic methods on an automated analyzer. The concentrations of HbA1c were determined using the immunoturbidimetric method. To calculate the log (TG/HDL-C) ratio, the ratio of TG to HDL-C was logarithmically transformed.

Genotyping

The rs10811661 variant was identified using the restricted fragment length polymorphism (RFLP) analysis after PCR amplification. The specific primers designed for this variant were as follows: F 5′- TATTGGCAGGGTTTCAAA AG −3′ and R 5′- ACCCGACTGGAAAACCTTAT −3′.

Genomic DNA samples were amplified in a 25 µL reaction mixture. The PCR protocol involved an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. A final extension step was performed at 72 °C for 5 min. The amplification products from each sample had a length of 388 base pairs (bp). These products were then subjected to cleavage by the restriction enzyme *BspHI*, resulting in fragments of 131 bp and 257 bp. The cleaved fragments were separated by electrophoresis on a 2.5% agarose gel for visual analysis and detection of the rs10811661 variant.

Statistical tests

Statistical calculations were performed using R software (version 3.0.1) and SPSS (version 16.0). For parametric variables, differences between groups were analyzed using the t-test, while nonparametric variables were assessed using the Mann–Whitney test. The association of categorical variables was evaluated using chi-square tests. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated to analyze the relationship between the T allele of rs10811661 and CAD. The genotype frequencies of the rs10811661 variant were tested for deviations from Hardy–Weinberg equilibrium expectations using a chi-square test. A logistic regression model was employed, incorporating age, gender, body mass index (BMI), duration of diabetes, hypertension, and HbA1c levels as covariates. A p-value < 0.05 was accepted as statistically significant.

Table 1 Characteristics of study parameters in participants

Parameter	Non-CAD	CAD	p value
Age (year)	64.3 ± 8.2	64.04 ± 8.06	0.875
Women (%)	80.5	70.7	0.248
Duration of diabetes (year)	16.54 ± 6.81	16.88 ± 7	0.799
BMI (kg/m^2)	30.11 ± 5.47	30.27 ± 4.91	0.881
Current smoking (%)	9.7	6.2	0.489
Hypertension (%)	53.6	57.5	0.684
HbA1c (%)	7.37 ± 1.42	7.53 ± 1.59	0.588
Fasting glucose (mmol/L)	7.8 ± 2.4	7.7 ± 2.3	0.824
Triglyceride (mmol/L)	1.46 ± 0.66	1.65 ± 0.71	0.156
HDL-C (mmol/L)	1.22 ± 0.25	1.14 ± 0.24	0.089
LDL-C (mmol/L)	1.88 ± 0.48	1.99 ± 0.63	0.328
Medications (%)			
Metformin	95	86	0.135
Sulfonylurea	49	42	0.465
Pioglitazone	22	19	0.698
Angiotensin receptor blocker	41	47	0.532
Beta blocker	36	50	0.145
Aspirin	54	67	0.164
Statin	93	92	0.845

Results

Table 1 presents the clinical and biochemical characteristics of the study patients. Age, gender, and BMI did not significantly differ between the non-CAD group and CAD patients. The genotype distributions in both groups were in accordance with the Hardy-Weinberg equilibrium ($p > 0.05$). The non-CAD group had a genotype frequency of 68.29% TT, 29.27% CT, and 2.44% CC, while the CAD group had a frequency of 76.25% TT, 21.25% CT, and 2.5% CC. The CAD group had a higher frequency of the TT genotype, resulting in a higher T allele frequency compared to the non-CAD group (86.88% vs. 82.93%).

The results of PCR-RFLP for two study variants have been presented in Fig. 1. As shown in Table 2, when the data was divided into tertiles according to HbA1c, our findings revealed that in tertile 3 (HbA1c ≥ 7.8%), the frequency of TT genotypes was higher compared to CT + CC genotypes (37.1% vs. 27.8%).

After adjustment for covariates, the presence of the T allele in rs10811661 was found to be significantly associated with higher TG levels. The OR for this association was 1.66 (95% CI: 1.22 to 2.33). The p-value for this association was 0.002, indicating statistical significance. Additionally, the T allele of rs10811661 was also found to have a significant association with the ratio log (TG/HDL-C). The OR for this association was 1.12 (95% CI: 1.02 to 2.13) and the p-value was 0.023, indicating statistical significance as well (Table 3). As shown in Fig. 2A, T2D patients with CAD who carried the TT genotype had significantly higher log (TG/HDL-C) levels compared to those with the CT + CC genotype ($p = 0.037$). Additionally, T2D subjects with CAD who had the TT genotype had significantly higher TG concentrations ($p = 0.003$) compared to those with the C allele (CC or CT genotypes) (Fig. 2B).

Discussion

The region on chromosome 9p21 has consistently been identified in GWAS as the top locus for both CVD and T2D [11–13]. Within this locus, the tumor suppressor genes CDKN2A and CDKN2B play a role in cell proliferation, apoptosis, and tumorigenesis [23]. Studies on a mouse model of atherosclerosis have shown that the loss of CDKN2B promotes the advanced development of atherosclerotic plaques [10]. Variants in the 9p21 genetic locus may contribute to the risk of T2D by affecting beta-cell proliferation and islet gene expression [15]. A significant variant in the CDKN2A/B gene, rs10811661, has been linked to an elevated risk of developing diabetes by up to 40% in diverse populations [16]. This particular variant has been

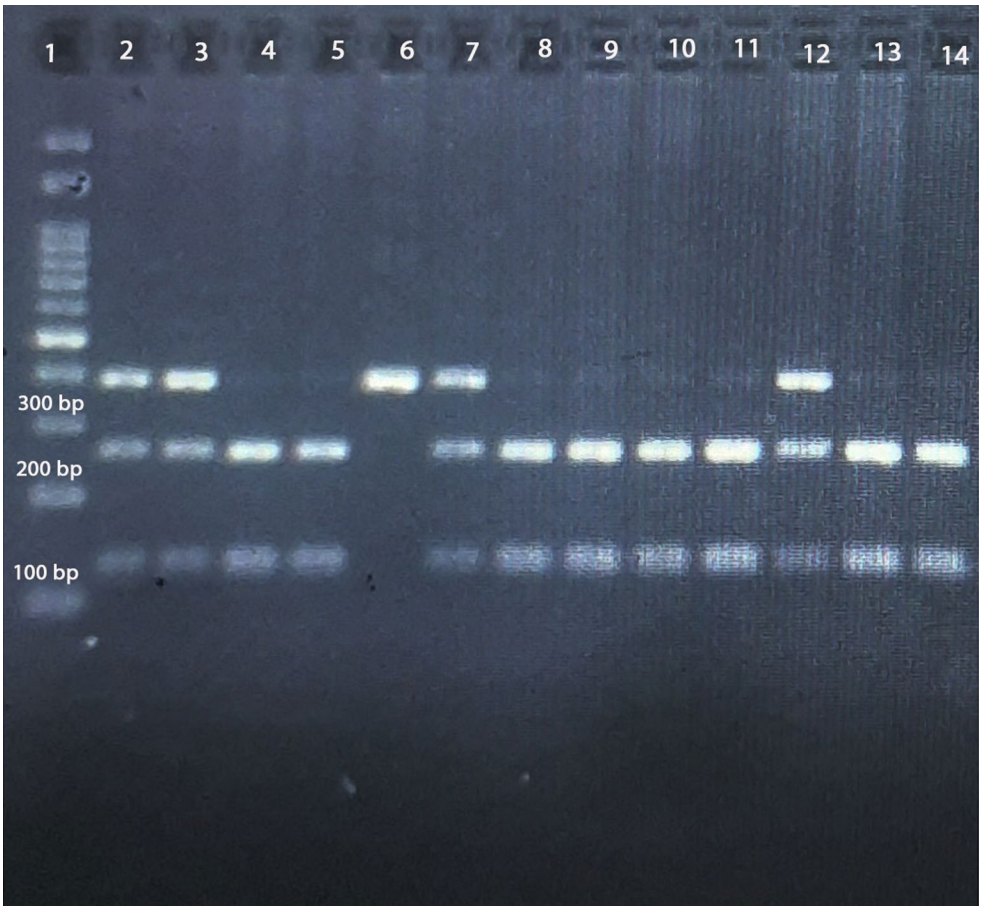

Fig. 1 Restriction fragment length polymorphism (RFLP) analysis of PCR products for the study variant. Amplicons of 388 bp were subjected to the restriction enzyme *BspHI*. TT genotypes (lanes 4, 5, 8–11, 13 and 14) displayed fragments of 257 and 131 bp. CT heterozygotes were identified by the presence of three fragments of 388, 257, and 131 bp (lanes 2, 3, 7 and 12). Non-digested fragments of 388 bp (lane 6) indicated CC genotypes. A 100 bp DNA ladder was loaded in lane 1

Table 2 Genotype distribution of rs10811661 according to HbA1c in the study subjects

Tertile	TC + CC	TT
Tertile 1 (HbA1c < 6.8%)	38.9%	38.7%
Tertile 2 (HbA1c ≥ 6.8 − 7.8%)	33.3%	24.2%
Tertile 3 (HbA1c ≥ 7.8%)	27.8%	37.1%

extensively investigated and found to influence insulin resistance and glucose metabolism. In a study of 38 fasting plasma glucose-related variants, rs10811661, along with rs6943153 and rs2293941, showed an association with longitudinal changes in fasting plasma glucose [24]. This further supports the role of this variant in the development of T2D.

In our study, we investigated how a T2D risk variant could impact the concentrations of log (TG/HDL-C) and TG in T2D patients with CAD. Even after adjusting for factors such as age, gender, BMI, duration of diabetes, hypertension, and HbA1c concentrations, the association between the risk allele (T allele) of rs10811661 with this ratio and TG remained significant. Our findings provide insight into how a GWAS-validated variant can influence atherogenicity in patients with T2D and establish a link between a functional variant in the 9p21 locus and lipid factors associated with atherosclerosis and CAD, the leading cause of mortality in patients with diabetes. The importance of the association between rs10811661 and the ratio TG/HDL-C becomes more apparent when you realize that an elevated TG/HDL-C has been identified as an independent predictor of long-term all-cause mortality in patients with CAD and is strongly associated with an increased risk of major adverse cardiac events [25]. In addition, this ratio may be a warning

Table 3 Association of rs108116c61 with coronary artery disease in the current study and other studies

Study population	Risk allele (T) frequency		Odds ratio	95% CI	p value	Ref
	Controls	Cases				
Current study	0.829 (non-CAD)	0.869	1.35	0.66–2.79	0.419	
Iceland	0.819	0.811	0.94	0.87–1.02	0.12	[13]
Atlanta	0.826	0.827	1.01	0.84–1.21	0.93	[13]
Durham	0.837	0.840	1.03	0.85–1.24	0.77	[13]
China	0.570	0.611	1.19	1.06–1.33	0.002	[27]

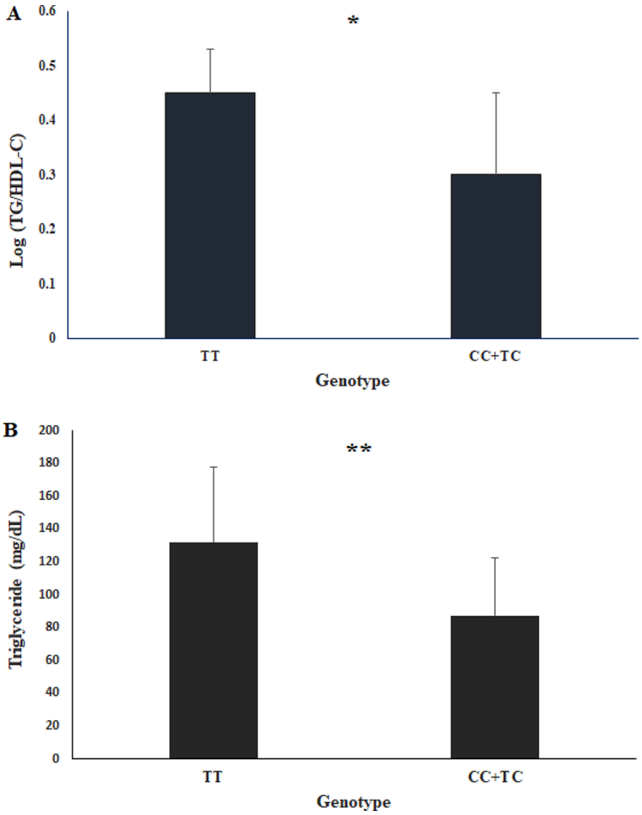

Fig. 2 Change in log (TG/HDL-C) (**A**) and triglyceride levels (**B**) among patients with type 2 diabetes with coronary artery disease, stratified by genotypes of rs10811661. *$p = 0.037$; **$p = 0.003$

Table 4 Adjusted association of rs10811661 (T allele) with lipid factors in type 2 diabetes patients with coronary artery disease

Parameter	Odds ratio (OR)	95% CI	P value
TG	1.66	1.22–2.33	0.002
Log (TG/HDL-C)	1.12	1.02–2.13	0.023
LDL-C	1.041	0.753–1.41	0.791

for the increased cardiovascular risk in subjects who do not yet have tremendously altered lipid concentrations [26].

The association between the T allele (risk allele) of rs10811661 with CAD has been compared in our study and different populations (Table 4). The results show that there were no significant associations between this allele and CAD in our population, as well as in Iceland, Atlanta, and Durham [13]. However, a significant association was found between the T allele and CAD in a Chinese population [27].

Our results are consistent with those of Mehramiz et al., who found that rs10811661 was associated with dyslipidemia and cardiovascular risk factors and could be used to predict CVD. The researchers suggested that a low-energy diet and high physical activity could mitigate the unfavorable effects of the risk allele of rs10811661 in the CDKN2A/B locus [28]. It should be noted that the risk allele of rs10811661 has been linked to reduced insulin secretory capacity, higher HbA1c levels, a higher prevalence of T2D, and increased transcription of CDKN2A in the human pancreas [29]. Additionally, a study revealed a notable variation in insulin resistance among different genotypes of rs10811661 [30]. Furthermore, the log (TG/HDL-C) ratio, which serves as an atherogenic index, displayed a positive correlation with insulin resistance [31].

The variant rs10811661 has been shown to be associated with insulin secretion and beta-cell proliferation [15, 19], suggesting that it may also have an impact on adipose tissue, an important target tissue of insulin. It is worth noting that the 9p21 locus is active in tissues such as adipose, and the effects of rs10811661 on TG and atherogenic lipid ratio may be related to its role in this tissue. Our findings are consistent with the idea that the risk alleles of variants located in 9p21 contribute to the risk of CVD by promoting the accumulation of ectopic fat, leading to higher postprandial TG levels, as reported by Svensson et al. Svensson et al. demonstrated that CDKN2B plays a significant role in regulating the expandability of subcutaneous adipose tissue, which is a crucial factor in lipotoxicity and may contribute to the development of atherosclerosis [32]. Furthermore, previous research has shown that increased serum TG levels are associated with higher expression of CDKN2B in adipose tissue [33]. Additionally, carriers of the rs10811661 G (C) allele exhibited a decrease in fat mass ranging from 0.4 to 0.8 kg [34]. Moreover, the association between rs10811661 and adipose tissue was further emphasized by the finding that rs10811661 displayed substantial interaction effects with multiple obesity indices, including body mass index (BMI), waist circumference, and waist-hip ratio [35].

The research conducted by Wei et al. provides insights into the impact of rs10811661 as a functional genetic variant on lipid profile and the risk of CAD. The study findings suggest that the C allele of rs10811661 may have a protective effect, as it is associated with lower levels of LDL-C and total cholesterol in Asian individuals and patients with T2D. This suggests that individuals carrying this allele may have a lower risk of developing CAD. The researchers proposed that rs10811661, along with other genetic variants located in the 9p21 locus, may indirectly influence lipid profiles by impacting the expression of lipid metabolism-associated genes, CDKN2A/2B and ANRIL. These genes are involved in various cellular processes and have been implicated in the development of atherosclerosis [36].

Research has shown that ANRIL promotes lipid uptake and intracellular lipid accumulation by altering lipid metabolism, while also inhibiting macrophage reverse cholesterol transport, which is involved in the development of atherosclerosis [37]. A lipidomics study conducted by Meckelmann et al. demonstrated that a genetic variant associated with increased risk in the 9p21 region remodels lipid

metabolism by affecting the expression of lysophospholipids, lysophosphatidic acid, and autotaxin. The authors concluded that lipid metabolites and genomic pathways related to coronary heart disease pathogenesis in the 9p21 locus and ANRIL-associated diseases [38]. ANRIL is also known to inhibit phospholipase D, an enzyme involved in the production of phosphatidic acid, which regulates the mammalian target of rapamycin (mTOR) pathway that influences lipid synthesis [39, 40]. ANRIL interacts with two polycomb proteins to regulate histone modification in the CDKN2A/B locus [41]. The expression of ANRIL is inhibited by RAS, potentially through a protein called RREB1 (Ras responsive element binding protein), suggesting a regulatory feedback loop between ANRIL and the RAS signaling pathway [41]. This pathway is closely associated with various metabolic pathways, including those involved in lipid metabolism [42].

Therefore, the protective effect observed for the C allele of rs10811661 may be mediated through its influence on the expression of CDKN2A/2B and ANRIL, as their expression is largely determined by this variant as well as other variants such as rs1333049, rs4977574, rs10757274, and rs10757278 [36]. To gain further insights into the mechanisms involved, conducting functional studies to validate the functional role of the rs10811661 variant in modulating cellular processes and lipid metabolism pathways would be valuable. Such data could help establish a connection between genetic associations and their biological effects.

One of the limitations of our study is the small sample size. However, it is important to highlight that the genotype distributions of the variant under investigation were consistent with the expectations of the Hardy-Weinberg equilibrium. Moreover, despite our best efforts to account for and minimize the probable effects of important confounders, it is possible that unmeasured or unaccounted factors could have influenced the results. To address these limitations and gain a more comprehensive understanding, future studies with larger sample sizes in diverse populations are warranted.

Conclusions

Our results suggest that the risk allele of rs10811661 (T) is associated with increased TG levels and a higher atherogenic ratio log (TG/HDL-C) in T2D patients with CAD, highlighting the importance of this variant in the development of atherosclerosis, in addition to its role in T2D. Our findings provide insight into how a GWAS-validated variant, rs10811661, can influence atherogenicity in patients with T2D and establish a link between this functional variant in the 9p21 locus and lipid factors associated with atherosclerosis. Further investigations are needed to understand the mechanisms by which this important variant influences lipid and lipoprotein levels, which could be useful in developing personalized medicine interventions.

Declarations

Conflict of interest All authors state that they have no conflicts of interest.

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