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ORIGINAL RESEARCH

Single Nucleotide Polymorphism in the 3' Untranslated Region of *PRKAA2* on Cardiometabolic Parameters in Type 2 Diabetes Mellitus Patients Who Received Metformin

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Video abstract

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Purpose: This study aimed to explore the association of *rs857148* A>C as 3′UTR variants with blood pressure, HbA1c profile, and lipid profiles as cardiometabolic parameters among patients with T2DM receiving metformin.

Patients and Methods: This cross-sectional analytic research was conducted with 114 consecutively selected patients with T2DM. Polymerase chain reaction-restriction fragment length polymorphism was conducted to determine *rs857148*. A total of 108 patients fulfilled inclusion and exclusion criteria.

Results: Genotype distribution agreed with the Hardy Weinberg Equation for Equilibrium ($p>0.05$) but wildtype allele was found as the minor allele. Subjects with CC genotype and C allele had enhanced HbA1c levels (OR=7.12; 95% CI=1.05–48.26; $p=0.04$; OR=1.66; 95% CI=1.06–2.60; $p=0.03$, respectively). It was confirmed by dominant model whereas subjects with AA tended to have reduced HbA1c compared to AC+CC genotype (OR=0.15; 95% CI=0.02–0.97; $p=0.047$). AC genotype had significant correlation to total cholesterol (OR=1.05; 95% CI=1.01–1.10; $p=0.03$) compared to AA genotype.

Conclusion: We conclude that polymorphism of *rs87148*, specifically CC genotype and C allele, has a significant association with HbA1c and total cholesterol after considering oral hypoglycemia agent dose, age, gender, and combination therapy, compared to AA genotype. Future studies that involve a larger sample population and more rigorous selection criteria are required.

Keywords: *PRKAA2*, rs857148, 3′UTR, cardiometabolic, type 2 diabetes mellitus

Introduction

The prevalence of type 2 diabetes mellitus (T2DM) is regarded as a serious public health problem, especially in developing countries.[1] Metformin is recommended as a cornerstone for initial therapy of T2DM. However, additional therapy may be added in combination when the target HbA1c has not been achieved.[2,3] Remarkably, the T2DM prevalence in Indonesia has been rising in the last two decades,[4] especially among the urban population.[5] Studies related to metformin efficacy either monotherapy or combination in Indonesian population found that our patients tend to have poor glycemic control.[6–8]

Several publications have declared glycemic response variations of metformin therapy, either monotherapy or combination therapy.[9–11] Furthermore, a group of patients could not tolerate the metformin's side effects.[12] A study among patients with T2DM who were covered national health insurance in Indonesia confirmed that the patients reported

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metformin side effects in the gastrointestinal tract.[13] On the other hand, it is well known that the main pharmacological mechanism of metformin is reducing gluconeogenesis in the liver besides sensitizing insulin and improving GLP-1.[14,15] Notably, adenosine monophosphate protein kinase (AMPK) contributed as the main target of the metformin mechanism.[16,17]

Individual genetic imprints are one of the causes of metformin effectiveness variability. The previous study confirmed that genetic variations affect metformin efficacy regarding pharmacokinetics and pharmacodynamics of metformin.[18–21] Recently, AMPK is the focused enzyme related to metformin pharmacodynamic. Xiao et al in 2021 reported that one of the subunits of AMPK, which is *PRKAG2*, is associated with metformin response among Chinese patients with T2DM.[21] AMPK has 3 subunits whereas AMPKα2, encoded by *PRKAA2*, plays a pivotal role during Thr-172 phosphorylation.[22,23]

Metformin has been reported to have a beneficial effect on blood pressure and lipid profiles. Hypertension risk could be reduced among metformin users who are newly diagnosed with T2DM.[24] Metformin has proven blood pressure reduction in patients with T2DM compared with insulin therapy.[25] Monotherapy metformin could significantly reduce triglyceride and low-density lipoprotein cholesterol (LDL-c) levels, and enhance (high-density lipoprotein cholesterol) HDL-c.[26] The various international guidelines recommend detecting and monitoring cardiometabolic parameters in addition to HbA1c level in patients with T2DM.[2,3] Therefore, it is important to assess cardiometabolic parameters to reduce cardiovascular disease risk in patients with T2DM.[27,28] Virginia et al in 2021 demonstrated that the *PRKAA2* genetic variation (rs9803799) had a significant association to cardiovascular risk among patients with T2DM receiving monotherapy metformin.[29]

However, few studies have observed the association of cardiometabolic parameters and AMPK subunit genetic variations. Single nucleotide polymorphism (SNP) rs857148 is a 3' untranslated region (UTR) of *PRKAA2* and located in chromosome 1:5,670,948.[30] Although this SNP is not located in the exon area, the 3' UTR has been confirmed to affect DNA stability and the micro RNA/mRNA interaction, mRNA stability, localization, translation, and degradation.[31–33] Therefore, this present study aimed to observe the influence of 3' UTR *PRKAA2* variants, especially rs857148 A>C, on blood pressure, HbA1c profile, and lipid profiles as cardiometabolic parameters among patients with T2DM consuming metformin.

Materials and Methods

This observational analytic research using a cross-sectional study design was conducted at the Bethesda Lempuyangwangi Hospital, Yogyakarta, Indonesia. The study included patients with T2DM who were 35–75 years old using national health coverage and have been consuming metformin either monotherapy or combination therapy for a minimum of 3 months consecutively. We excluded patients with other types of diabetes mellitus, including type 1, monogenic, and gestational, had eGFR<30 mL/min, and refused to sign informed consent. The minimum sample size was 98 participants according to our study design and using type 1 error rate 0.05, power 0.8, predicted risk ratio 2.0, and proportion 0.3. A total of 114 patients with T2DM were consecutively recruited in this study, and only 6 participants were excluded because they did not receive metformin.

Patient's data related to age, gender, blood pressure, and medication information were collected from medical records. Biochemical and genotyping data were obtained from blood samples were which collected during appointments. This study was approved by the Medical and Health Research Ethics Committee of Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada (KE/FK/0520/EC/2021) and data were complied with the principles established by Declaration of Helsinki and promoted by the Committee on Publication Ethics (COPE). All enrolled participants signed an informed written consent before participating.

Biochemical Analysis

Blood samples were collected in the morning after 8–10 h fasting by venipuncture. Clinical biochemical analyses were performed at the Laboratory of Bethesda Lempuyangwangi Hospital. Laboratory tests included HbA1c and lipid profiles (total cholesterol, HDL-c, LDL-c, and triglycerides), which were measured in fresh samples. HbA1c was assayed using tetradecyl trimethyl ammonium bromide. Total cholesterol levels were determined by cholesterol oxidase method/CHOD

PAP. HDL-c and LDL-c were analyzed using direct methods. Glycerol-3-phosphate oxidase was applied to measure triglyceride levels.

DNA Extraction and Genotyping Analysis

Genomic DNA was extracted from peripheral blood in the K3EDTA tube by FavorPrep™ Genomic DNA Extraction Mini Kit following manufacturing procedures. The quality of DNA was evaluated using electrophoresis. Extracted DNA samples were stored at −70°C until genotyping analysis. Single nucleotide polymorphism (SNP) rs857148 was selected according to the International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/). Genotyping was performed using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). One set of primers of forward 5'- GACTAAGTTTCTCCTGTGTTAGTGG-3' and reverse 5'-TTCCCAAAAGAGGTATGGACCC-3' was applied for the amplification 369 bp. Each individual sample contained 5 µL of DNA which was mixed with 12.5 µL of the GoTaq Green Master Mix® in a PCR tube. Following an initial denaturation step at 95 °C for 5 min. We conducted thirty five cycles of amplification including, denaturation (95°C for 20s), annealing (56.1°C for 30s) and extension (72°C for 30s), with a final extension step at 72°C for 5 min. The PCR products were electrophoresed on a 3% agarose gel to check PCR product size. *BSuRI* restriction enzyme (ThermoScientific) was applied to detect rs857148 in the *PRKAA2*. The reaction mixtures were incubated at 37°C for 2 h, were inactivated at 37°C for 20 min, then were electrophoresed on 1.5% agarose gel.

We did PCR condition optimization, including annealing temperature and primer concentration. Incubation duration during the restriction process was also optimized. Validation of genotyping procedure was done through replication of several samples randomly using the same method which was PCR-RFLP.

Statistical Analysis

Data are expressed as frequency (percentage) for categorical variables or as mean±standard deviation (SD) for continuous variables. We performed Anova or Kruskal Wallis as appropriate to compare clinical characteristics between *rs857148* genotypes. Multinomial logistic regression analysis was used to determine the association of *rs857148* with hypertension, HbA1c level, and lipid profile, where hypertension categorized as blood pressure >130/>80 mmHg. Multiple inherited models including dominant, recessive, and allelic models were applied to estimate the influence of *rs857148* on cardiometabolic parameters. A two tailed p-value < 0.05 was considered significant statistically. Two regression models were constructed to adjust potential confounders. Model 1 was adjusted for metformin dose, combination either sulfonylurea or insulin. Additionally, model 2 was adjusted for age, gender, metformin dose, glimepiride dose, and combination either sulfonylurea or insulin. We conducted all statistical procedures using SPSS software version 25.0 (IBM Corp., Armonk, NY)).

Results

Table 1 presents participants' characteristics with an average age 60.58±8.16 years old and predominately female subjects. Interestingly, the mean of HbA1c was considered poor glycemic control, but the mean of lipid profiles was adequate. Metformin as combination therapy was predominantly prescribed in our study sample.

We obtained different patterns of our PCR products after applying the BSuRI enzyme: wildtype homozygote (AA) indicated by one band of 369 bp, heterozygote (AC) indicated by three bands of 125, 244, and 369 bp, and mutant homozygote (CC) indicated by two bands of 125 bp and 244 bp (Figure 1). The genotype frequencies in our study sample were consistent with the Hardy-Weinberg equilibrium (HWE) equation (p=0.40). Table 2 shows clinical characteristics according to *rs857148* genotype. We found that only systolic blood pressure had a significant difference between the rs85714 genotype (p=0.03).

Table 3 displays the results of multiple regression analysis to detect any relationship between genotype model and cardiometabolic parameters. Overall, we discovered a significant relationship between SNP *rs857148* and HbA1c level in model 2. Subjects with CC genotype were detected to have significantly increased HbA1c levels (OR=7.12; 95% CI=1.05–48.26; p=0.04). It was asserted through recessive and allelic models where subjects with AA tended to have reduced HbA1c lower than those with AC+CC genotype, and subjects with C allele tended to have increased HbA1c levels (OR=0.15; 95% CI=0.02–0.97; p=0.047, OR=1.66; 95% CI=1.06–2.60; p=0.03, respectively). For the other

Table 1 Participants' Characteristics

Characteristics	Mean±SD/n (%)
Age (years)	60.58±8.16
Gender (female)	67 (62)
Blood pressure (mmHg)	137.67±20.21/77.02±10.87
HbA1c (%)	8.46±1.70
Total cholesterol (mg/dL)	196.12±39.14
HDL-c (mg/dL)	49.92±21.52
LDL-c (mg/dL)	137.57±72.39
Triglycerides (mg/dL)	158.04±102.17
Antidiabetic profiles:	
Monotherapy metformin	29 (26.9)
Metformin in combination with other OAD	79 (73.1)
Metformin dose (mg/day)	1440±364.77

Note: Numerical data were expressed as mean±standard deviation (SD), and categorical data were expressed as n (%).
Abbreviations: HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; OAD, oral antidiabetic agent(s).

cardiometabolic parameters, only total cholesterol was correlated with rs857148 calculated using model 2 (OR=1.05; 95% CI=1.01–1.10; p=0.03).

Discussion

A number of studies have explored the association between a list of genetic variants and cardiometabolic parameters.[34–36] To our knowledge, our study is the first study that observed the 3' UTR variant, especially *rs857148* related to cardiometabolic parameters, within the Yogyakarta population. This was the initial study related to personalized, patient-centered medicine, and we could only recruit a small number of participants. Cardiometabolic parameters in detail in this study include hypertension, HbA1c, and lipid profiles.

The association between hypertension and *rs857148* in our study is not well concluded yet. This is because we found significant statistical difference only in systolic blood pressure among the *rs857148* genotype, but not in diastolic blood pressure. Some studies have revealed the molecular explanation of AMPK and hypertension. AMPK activates sarcoplasmic/endoplasmic Ca2+-ATPase (SERCA), thus reducing ion Ca2+ devaluated, and the result is directly vascular smooth muscle relaxation.[37] Enhancing phosphorylated AMPK could suppress the expression of the angiotensin II type 1 receptor. Therefore, AMPK activation affects blood pressure.[38]

Our study has observed that subjects with CC genotype and C allele tended to have increased HbA1c levels after adjusted for metformin and glimepiride dose, age, gender, and considering combination therapy. Remarkably, there are limited association studies related to *rs857148*. We found a study that investigated the effect of *rs857148* variants on non-lung small cancer prognosis,[39] but no study that discussed the effect of HbA1c, previously. However, three SNPs of *PRKAA2* have been demonstrated in the previous study and it revealed no association to HbA1c.[40] These discrepancies could be due to the difference in the participants' selection. We recruited T2DM patients with less rigorous criteria than the previous study who engaged only newly diagnosed T2DM patients. Therefore, further studies are required focusing on *rs857148* with a larger sample population and applying more rigorous criteria to confirm the association that we found in our study.

A study among patients with T2DM performed by Sokolova et al in 2019 reported that phosphorylated AMPK was decreasing when the blood HbA1c level was increasing.[41] Luo et al in 2020 conducted a Mendelian randomization study

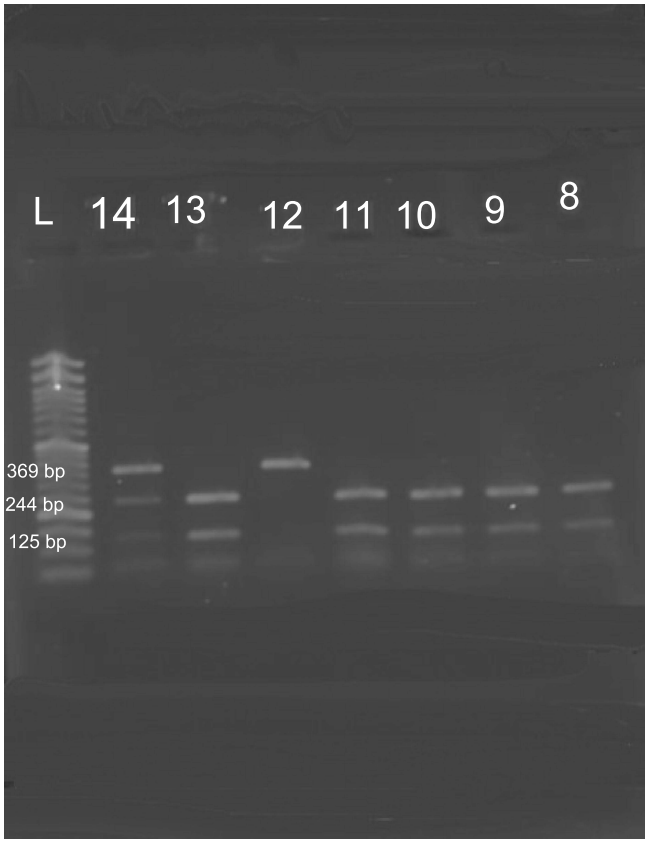

Figure 1 The PCR-RFLP results to determine *PRKAA2* rs857148 variants. L= ladder (marker 50 bp), 8, 9, 10, 11, 13 are mutant homozygote genotype, 12 is wildtype homozygote genotype, and 14 is heterozygote genotype.

and found that HbA1c reduction was instrumented by AMPK variants.[42] AMPK activation through phosphorylation induced by ATP/AMP changes and induced by metformin. Notably, AMPK controls energy metabolism in the whole body. Biologic functions of AMPK related to HbA1c include glycolysis promotion, glucose transport enhancing, and gluconeogenesis inhibition.[43,44] Therefore, it requires further investigations to confirm our findings, whether as the impact of T2DM or the effect of metformin.

Table 2 Patients' Clinical Characteristics Based on the Genotype of *rs857148*

Clinical Characteristics	AA (n=15)	AC (n=45)	CC (n=48)	*p*-value
	HWE = 0.40			
Age (years)	61.33±10.46	60.18±7.17	60.73±8.40	0.88
Systolic blood pressure (mmHg)	141.07±12.38	142.67±23.00	131.92±18.09	0.03[a]
Diastolic blood pressure (mmHg)	76.00±8.95	79.18±11.40	75.31±10.76	0.22
HbA1c (%)	8.17±1.84	8.54±1.77	8.47±1.61	0.76
Total cholesterol (mg/dL)	191.20±41.70	200.33±35.81	193.71±41.70	0.63
HDL-c (mg/dL)	51.60±15.70	48.31±13.36	50.90±28.46	0.81
LDL-c (mg/dL)	116.80±34.74	135.67±41.87	145.85±95.59	0.39
Triglycerides (mg/dL)	144.73±71.45	175.16±120.25	146.15±90.69	0.34

Notes: [a]$p<0.05$; Kruskal–Wallis test.
Abbreviations: HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol.

Table 3 Multiple Regression Analysis of *rs857148* and Cardiometabolic Parameters

Genotypes	Hypertension (Yes)		HbA1c (%)		Total Cholesterol (mg/dL)		HDL-c (mg/dL)		LDL-c (mg/dL)		Triglycerides (mg/dL)	
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Model 1												
AA	1.00 (reference)											
AC	0.31(0.06–1.66)	0.17	1.24(0.80–1.91)	0.33	1.01(0.99–1.02)	0.50	0.99(0.96–1.02)	0.62	1.01(0.99–1.02)	0.27	1.00(0.99–1.01)	0.42
CC	0.27(0.05–1.38)	0.12	1.51(0.06–2.39)	0.08	1.00(0.99–1.02)	0.74	1.00 (0.97–1.03)	0.99	1.01(0.99–1.03)	0.13	1.00(0.99–1.01)	0.98
Dominant	0.89(0.38–2.09)	0.78	1.29(0.96–1.73)	0.10	1.00(0.99–1.01)	0.80	1.01(0.99–1.02)	0.62	1.00(0.99–1.01)	0.22	1.00(0.99–1.02)	0.26
RecessiveAA	3.59(0.73–17.72)	0.12	0.74(0.49–1.13)	0.16	1.00(0.99–1.01)	0.58	1.00 (0.98–1.03)	0.81	0.99(0.98–1.00)	0.18	0.99(0.99 −1.01)	0.66
Allele A	1.00 (reference)											
Allele C	0.60(0.31–1.15)	0.12	1.22(0.99–1.50)	0.06	1.00(0.99–1.01)	0.90	1.00(1.00–1.01)	0.81	1.00(1.00–1.01)	0.12	0.99(0.99–1.02)	0.53
Model 2												
AA	1.00 (reference)											
AC	0.34 (0.03–4.39)	0.41	6.33 (0.92–43.71)	0.06	1.05 (1.01–1.10)	0.03*	1.07(0.97–1.20)	0.19	1.01(0.99–1.04)	0.34	1.01(0.99–1.02)	0.41
CC	0.47 (0.04–5.32)	0.54	7.12 (1.05–48.26)	0.04*	1.02 (0.99–1.06)	0.22	1.08 (0.97–1.21)	0.15	1.01 (0.99–1.04)	0.32	0.99 (0.98–1.01)	0.24
DominantCC	1.06 (0.27–4.23)	0.93	1.55 (0.78–3.07)	0.21	0.99 (0.97–1.01)	0.35	1.02 (0.98–1.05)	0.41	1.00 (0.99–1.01)	0.63	0.99 (0.98–1.00)	0.20
RecessiveAA	2.53 (0.24–27.12)	0.44	0.15 (0.02–0.97)	0.047*	0.97 (0.94–1.01)	0.10	0.93 (0.83–1.03)	0.16	0.99 (0.96–1.01)	0.30	1.00 (0.99–1.01)	0.88
Allele A	1.00 (reference)											
Allele C	0.85 (0.30–2.42)	0.76	1.66 (1.06–2.60)	0.03*	1.00 (0.99–1.02)	0.80	1.02 (0.99–1.06)	0.26	1.00 (0.99–1.01)	0.44	0.99 (0.99–1.00)	0.15

Note: *$p<0.05$.

Abbreviations: CI, confidence interval; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; OR, odds ratio.

Concerning the lipid profiles, no significant associations were found between *rs857148* and total cholesterol, HDL-c, LDL-c, and triglyceride in model 1. Nonetheless, model 2 showed that the AC genotype had a significant correlation to total cholesterol level compared to the AA genotype. Nevertheless, only a little effect of AC was detected to total cholesterol level where the odds ratio was only 1.05. This agrees with the study conducted by Jones et al in 2006, 5 SNPs of *PRKAA2* (rs1124900, rs2796516, rs2746342, rs2796498 and rs1418442) were significantly correlated to total cholesterol in a Caucasian population.[45]

The synthesis and disposal of cholesterol are coregulated through phosphorylation of key enzymes activated by AMPK. Those activated via sterol-regulatory element-binding protein (SREBP) and acetyl-CoA carboxylase (ACC) as the AMPK downstream.[46–48] An in vivo study revealed that AMPK influence the signalling of the mevalonate pathway 3-hydroxy-3-methylglutaryl (HMG) coenzyme A (CoA) reductase (HMGCR), thus regulating the cholesterol biosynthesis.[49]

As mentioned above, *rs857148* have not been observed related to cardiometabolic parameters, especially among T2DM patients, yet. We found one study that reported the frequency of the genotypes among health participants (as control of pancreatic cancer). The percentages of AA vs AC vs CC were 15.5%, 25.6%, and 11.3% respectively, among non-Hispanic whites as a control group.[50]

The clinical importance explained to us that the homozygote mutant of rs857148 could increase HbA1c than wildtype, even heterozygotes tend to increase total cholesterol. Since it is a pilot study among Indonesian subjects, it requires a larger sample to clarify our findings. However, these findings could indicate that rs857148 is worth considering as one of SNP contributing to cardiometabolic parameters, especially among patients with T2DM.

Notably, several limitations have been detected in our study. First, we could not explore whether the effect of SNP is influenced by pathologic conditions or metformin therapy because we did not collect HbA1c baseline and the information related to metformin as monotherapy or as combination therapy. Second, we did not measure physical activities, diet habits, and coexisting diseases that contribute to cardiometabolic physiologic. We have tried to minimalize these effects through multinomial logistical regression analyses. However, it would be better to apply more rigorous criteria during data collection.

Conclusions

In summary, CC genotype and C allele of rs857148 had a significant correlation to HbA1c level. Our study gives evidence that AC genotype could significantly increase total cholesterol compared to AA genotype. Since our results as a preliminary study showed significant findings, it requires further investigation with a larger sample and more rigorous criteria to determine the effect of metformin on *PRKAA2* variants accurately.

Abbreviations

T2DM, type 2 diabetes mellitus; AMPK, adenosine monophosphate protein; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; SNP, single nucleotide polymorphism; UTR, untranslated region; SERCA, sarcoplasmic/endoplasmic Ca2+-ATPase; SREBP, sterol-regulatory element-binding protein; ACC, acetyl-CoA carboxylase; HMGCR, mevalonate pathway 3-hydroxy-3-methylglutaryl (HMG) coenzyme A (CoA) reductase.

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Disclosure

The authors report no conflicts of interest in this work.

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