



# The Association of Antioxidants Gene Polymorphisms (SOD2 Ala16Val, GPx1 Pro198Leu, GSTP1 Ile105Val, and Cat -21 A/T) and Risk of Type 2 Diabetes Mellitus

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## Abstract

**BACKGROUND:** Antioxidant gene polymorphism is one of the genetic risk factors associated with type 2 diabetes mellitus (T2DM) incidence.

**AIM:** This study was to analyze the association of superoxide dismutase 2 (SOD2) Ala16Val, glutathione peroxidase (GPx1) Pro198Leu, glutathione S-transferase Pi1 (GSTP1) Ile105Val, and Cat -21 A/T gene polymorphisms and risk of T2DM.

**METHODS:** We genotyped deoxyribonucleic acid of 120 T2DM patients and 80 healthy control by polymerase chain reaction and restriction fragment length polymorphism method, using a specific restriction enzyme.

**RESULTS:** This study showed that the Val/Val of SOD2 was significantly associated with an increased risk of T2DM compared to the Ala/Ala+Ala/Val ( $p = 0.011$ ; odds ratio [OR] = 2.220; confidence interval [CI] = 1.234–3.992). The TT genotype of Cat gene was also significantly associated with an increased risk of T2DM compared to the AA genotype ( $p = 0.027$ ; OR = 5.000; CI = 1.079–23.176) and TT genotype to the AA+AT genotype ( $p = 0.030$ ; OR = 4.738; CI = 1.039–21.600). However, there was no difference in all genetic models of GPx1 Pro198Leu and GSTP1 Ile105Val gene polymorphisms ( $p > 0.05$ ).

**CONCLUSION:** This study indicates that the Val/Val under the recessive model of SOD2 gene also TT genotype under the co-dominant model of Cat gene and TT genotype under the recessive model of Cat gene were associated with risk factors for T2DM occurrence.

**Edited by:** Slavica Hristomanova-Mitkovska  
**Citation:** Sari MI, Syarifah S, Daulay M, Tala ZZ. The Association of Antioxidants Gene Polymorphisms (SOD2 Ala16Val, GPx1 Pro198Leu, GSTP1 Ile105Val, and Cat -21 A/T) and Risk of Type 2 Diabetes Mellitus. Open Access Maced J Med Sci. 2020 Oct 19; 8(T1):781-786. https://doi.org/10.3889/oamjms.2020.5397  
**Keywords:** Superoxide dismutase 2 Ala16Val; Glutathione peroxidase Pro198Leu; Glutathione S-transferase Pi1 Ile105Val; Cat -21 A/T; Polymorphisms; Type 2 diabetes mellitus  
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**Received:** 29-Aug-2020  
**Revised:** 05-Oct-2020  
**Accepted:** 09-Oct-2020  
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**Funding:** This study is supported by the Ministry of Research and Technology and the Higher Education Republic of Indonesia - research grant DRPM KEMENRISTEKDIKTI of the Year 2019 (Grant No: 21/UN5.2.3.1/PPM/KP-DRPM/2019)  
**Competing Interests:** The authors have declared that no competing interests exist  
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## Introduction

Diabetes mellitus (DM) is a metabolic syndrome that occurs when the pancreas is unable to produce insulin or the insulin unable to work properly, results in hyperglycemia. Type 2 DM (T2DM) is the most common type of DM found in adults [1]. International Diabetes Federation in 2019 report the prevalence of adult population aged 20–79 years in the world suffering from T2DM reached 463 million. This number is expected to increase to 700 million by 2045. Moreover, Indonesia is the second-highest number of T2DM in the Western Pacific region, with 10.7 million [2]. In North Sumatra, the prevalence of people with T2DM was 1.7% in 2013 and rose to 1.9% in 2018 [3].

Risk factors for the emergence of T2DM are genetic, environment, or interactions of both factors [1]. Antioxidants gene polymorphisms are one of the genetic risk factors associated with T2DM incidence. Single-nucleotide polymorphisms (SNPs) are the

most common polymorphism of genetic variation in an individual. SNPs represent the difference of one nucleotide in the deoxyribonucleic acid (DNA) building block [4]. A previous study had shown the relationship of the antioxidant-superoxide dismutase 2 (SOD2) Ala16Val gene polymorphism with the incidence of T2DM [5]. SOD2 is present in the mitochondria. This enzyme gene is located on chromosome 6q25.3. Another study had shown a relationship between glutathione peroxidase (GPx1) Pro198Leu polymorphism and T2DM in populations in Poland [6]. Other antioxidant gene polymorphisms known to be associated with T2DM incidence are glutathione S-transferase Pi1 (GSTP1) Ile105Val gene polymorphism and catalase (Cat) -21A/T [7], [8].

SOD2, GPx1, GSTP1, and Cat are endogenous antioxidant molecules that have a significant role in the elimination of reactive oxygen species (ROS). Polymorphisms in each of these genes contribute to the emergence of phenotypic differences when gene expression occurs, which affects the antioxidant levels/

activity of eliminating ROS. Imbalance of antioxidants and ROS is thought to play a role in the occurrence of insulin resistance, which causes an increase in glucose levels [4], [9], [10].

The purpose of this study was to assess the association of SOD2 Ala16Val, GPx1 Pro198Leu, GSTP1 Ile105Val, and Cat -21A/T gene polymorphisms and risk of T2DM. To the best of our knowledge, this study is the first to be conducted on an Indonesian population in the city of Medan, North Sumatra.

## Materials and Methods

### T2DM patients and healthy control subjects

The current case-control study was including T2DM patients and healthy control subjects. T2DM was diagnosed based on the criteria established by the Indonesian Endocrinologist Association [11], recruited at Endocrinology Polyclinic in Universitas Sumatera Utara (USU) Hospital and Padang Bulan Public Health Centers. A total of 120 T2DM patients were chosen based on the inclusion and exclusion criteria. The inclusion criteria were male or female aged 20-79 years, diagnosed with T2DM since 6 months ago, willing to participate as a research subject. The informed consent that has been approved by the Faculty of Medicine, USU-Haji Adam Malik General Hospital Ethics Committee No. 447/KEPK FK USU-RSUP HAM/2019. The written informed consent for participation in this study was obtained from all subjects in accordance with the principles of the Declaration of Helsinki. The exclusion criteria were T2DM with obesity and other complications. A total of 80 subjects participated were randomly recruited among people in the Faculty of Medicine, USU staffs, and gym participants from several gyms in Medan city, North Sumatra Province. Inclusion criteria were male or female, with fasting blood glucose <126 mg/dl.

However, subjects who have a history of malignant disease were excluded from this study. Characteristics of subjects, including age, gender, duration of diabetes, and smoking, were collected through interviews using questionnaires.

### Blood glucose measurement

Blood glucose levels were measured using the Cobas 6000 analyzer with hexokinase and immunoturbidimetry method (Roche Diagnostics, Switzerland) at USU Hospital Laboratory.

### Antioxidants genotyping

Analysis of antioxidants gene polymorphism was carried out in Molecular Biology Integrated Laboratory at the Medical Faculty of USU. DNA extraction from leukocyte obtained using a commercial Wizard Genomic DNA purification kit from Promega (USA). Polymerase chain reactions (PCR) of SOD2, GPx1, GSTP1, and Cat gene were performed using the thermal cycler. The primer pairs used for the antioxidants gene, PCR condition, and the respective restriction enzyme of the restriction fragment length polymorphism (RFLP) can be seen in Table 1.

### Statistical analysis

The data were analyzed using SPSS version 19. Hardy-Weinberg equilibrium (HWE) of antioxidant genes in T2DM patients and healthy controls group were calculated using Chi-square goodness-of-fit test. A comparison of the mean values in the data between T2DM groups and control subjects was performed using non-paired t-tests. The 95% confidence intervals (CI) and odds ratios (ORs) were calculated to determine the statistical significance and risk of antioxidants gene polymorphisms to T2DM using the Chi-square test or Fisher's exact test.  $p < 0.05$  was considered as statistically significant.

**Table 1: Primers, PCR conditions, PCR and RFLP products, and restriction enzymes**

Genes	Primer Sequence (Macrogen, USA)	PCR condition	Restriction enzyme and temperature	PCR and RFLP product
SOD2 A1a6/Val (C/T)	(F) 5'-CAG CCC AGC CTG CGG AGA CGG-3' (R) 5'-CTT GGC CAA CGC CTC CTG GTA CTT-3'	95°C 5 min., (95°C 45 s; 54°C 30 s; 72°C 30 s) × 30, 72°C 5 min [12]	BsaW1 enzyme/30 min 65°C (Time-Saver Qualified enzyme. New England Biolabs, USA)	267 bp; Ala/Ala (CC) = 267 bp Ala/Val (CT) = 267 bp, 183 bp, 84 bp Val/Val (TT) = 183 bp, 84 bp.
GPx1 Pro198Leu (C/T)	(F) 5'- TCC AGA CCA TTG ACA TCG AG-3' (R) 5'-ACT GGG ATC AAC AGG ACC AG-3'	94°C 8 min., (94°C 30s; 59°C 30 s; 72 C 30 s) × 35, 72°C 7 min [12]	Apa1 enzyme/1 h 37°C (Promega, Madison, WI USA)	222 bp; Pro/Pro (CC) = 170bp, 52 bp Pro/Leu (CT) = 222 bp, 170 bp, 52 bp Leu/Leu (TT) = 222 bp
GSTP1 Ile105Val (A/G)	(F) 5'-GTA GTT TGC CCA AGG TCA AG-3' (R) 5'-AGC CAC CTG AGG GGT AAG-3'	95°C 5 min., (94°C 45 s; 62°C 40 s; 72°C 50 s) × 35, 72°C 7 min [13]	BsmAI enzyme/30 min 65°C (Time-Saver Qualified enzyme. New England Biolabs, USA)	433 bp Ile/Ile (AA) = 329 and 104 bp Ile/Val (AG) = 329, 222, and 104 bp Val/Val (GG) = 222 bp and 104 bp
Catalase -21 A/T	Forward primer 5'-AATCAGAAGGCAGTCCTCCC-3' Reverse primer 5'-TCGGGGAGCAGAGTGTAC-3'	95°C 4 min., (94°C 1m; 61°C 40s; 72°C 1 m) × 30, 72°C 5 min [14]	Hinf enzyme/1 h 37°C (Promega, Madison, WI USA)	250 bp; AA = 250 bp AT = 250, 177 and 73 bp TT = 177 and 73 bp

SOD2: Superoxide dismutase 2, GPx1: Glutathione peroxidase, GSTP1: Glutathione S-transferase P1, PCR: Polymerase chain reaction, RFLP: Restriction fragment length polymorphism.

## Results

The characteristics of healthy and control T2DM patients groups in this study can be seen in Table 2.

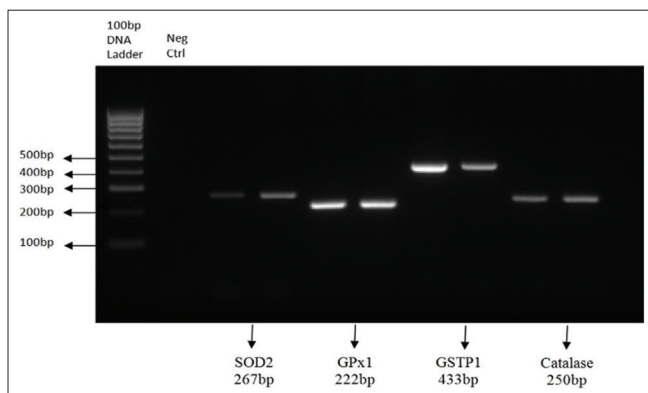
**Table 2: The characteristics of studied groups**

Characteristic	Healthy control	T2DM	p
Gender (n; %)			
Male	34 (42.5%)	53 (44.2%)	0.885
Female	46 (57.5%)	67 (55.8%)	
Age (years)	33.400 ± 15.972	57.000 ± 11.919	0.000
Duration of disease (years)	-	7.041 ± 5.837	-
Smoking			
No	64 (80.0%)	73 (60.8%)	0.005
Yes	16 (20.0%)	47 (39.2%)	
Blood glucose (mg/dl)	113.925 ± 27.772	277.500 ± 103.300	0.000

T2DM: Type 2 diabetes mellitus.

There were no sex differences between the research subjects in the T2DM group and healthy controls ( $p > 0.05$ ). The mean age of research subjects diagnosed with T2DM was higher than that of the control group ( $57.000 \pm 11.919$  vs.  $33.400 \pm 15.972$ ;  $p = 0.000$ ). There was a significant difference in smoking habits, glucose levels between the two groups ( $p < 0.05$ ).

PCR and RFLP products of antioxidants polymorphism can be seen in Figures 1 and 2.



**Figure 1: Polymerase chain reaction analysis of superoxide dismutase 2, glutathione peroxidase, glutathione S-transferase P1, and Cat gene**

The results of antioxidants genotyping for T2DM patients and healthy controls subjects are summarized in Table 3.

This study showed that the Val/Val frequency of SOD2, Pro/Pro of GPx1, and TT genotype of Cat were the highest genotypes in the T2DM group and the control group (70.0%, 71.7%, 54.2% and 51.2%, 73.8%, 62.5%), different results, the Ile/Val of GST1 found highest in the T2DM group (49.2%), and Ile/Ile of GSTP1 Ile105Val was the highest in the control group (47.5%). The HWE analysis shows that the frequency distribution of SOD2 Ala16Val, GPx1 Pro198Leu, GSTP1 Ile105Val, and Cat -21 A/T genotypes in all groups was in agreement under the HWE law ( $p > 0.05$ ).

The Val/Val of SOD2 was significantly associated with an increased risk of T2DM compared to the Ala/Ala+Ala/Val ( $p = 0.011$ ; OR = 2.220; CI = 1.234– 3.992). The TT genotype of Cat gene was

**Table 3: Distribution of genotype of Ala16Val, GPx1 198Pro/Leu, GSTP1 Ile105Val, and Catalase -21 A/T polymorphisms in studied groups and HWE test**

Genes polymorphism	Healthy control n (%)	T2DM n (%)	Total n (%)
<b>SOD2 Ala16Val (C/T)</b>			
Ala/Ala (CC)	4 (5.0)	3 (2.5)	7 (3.5)
Ala/Val (CT)	35 (43.8)	33 (27.5)	68 (34.0)
Val/Val (TT)	41 (51.2)	84 (70.0)	125 (62.5)
C	43 (26.9)	39 (16.3)	82 (20.5)
T	117 (73.1)	201 (83.7)	318 (79.5)
HWE ; X2 (p)	0.013 (0.909)	1.023 (0.311)	0.371 (0.542)
<b>GPx1 198Pro/Leu(C/T)</b>			
Pro/Pro (CC)	59 (73.8)	86 (71.7)	145 (72.5)
Pro/Leu (CT)	19 (23.8)	32 (26.7)	51 (25.5)
Leu/Leu (TT)	2 (2.5)	2 (1.7)	4 (2.5)
C	137 (85.6)	204 (85.0)	341 (85.3)
T	23 (14.4)	36 (15.0)	59 (14.8)
HWE ; X2 (p)	0.251 (0.616)	0.099 (0.752)	0.039 (0.843)
<b>GSTP1 Ile105Val (A/G)</b>			
Ile/Ile (AA)	38 (47.5)	53 (44.2)	91 (45.5)
Ile/Val (AG)	36 (45.0)	59 (49.2)	95 (47.5)
Val/Val (GG)	6 (7.5)	8 (6.7)	14 (7.0)
A	112 (70.0)	165 (68.8)	277 (69.3)
G	48 (30.0)	75 (31.3)	123 (30.8)
HWE ; X2 (p)	0.408 (0.522)	2.496 (0.114)	2.569 (0.310)
<b>Cat -21 A/T</b>			
AA	50 (62.5)	65 (54.2)	115 (57.5)
AT	28 (35.0)	42 (35.0)	70 (35.0)
TT	2 (2.5)	13 (10.8)	15 (7.5)
A	128 (80.0)	172 (71.7)	300 (75.0)
T	32 (20.0)	68 (28.3)	100 (25.0)
HWE ; X2 (p)	2.290 (0.130)	0.703 (0.401)	0.888 (0.345)

T2DM: Type 2 diabetes mellitus, SOD2: Superoxide dismutase 2, Gpx1: Glutathione peroxidase, GSTP1: Glutathione S-transferase P1.

significantly associated also with an increased risk of T2DM compared to the AA genotype ( $p = 0.027$ ; OR = 5.000; CI = 1.079-23.176) and TT genotype to the AA+AT genotype ( $p = 0,030$ ; OR = 4.738; CI = 1.039-21.600). However, there was no difference in all genetic models of GPx1 Pro198Leu and GSTP1 Ile105Val gene polymorphisms ( $p > 0.05$ ) (Table 4).

## Discussion

Endogenous antioxidants such as SOD2, GPx1, GSTP1, and Cat act as a complex and comprehensive protection system to deal with oxidative stress on cells. Oxidative stress is defined as an imbalance between ROS and the antioxidant defense system. ROS is free radicals in the form of oxygen and derivatives that are very reactive. Antioxidants have a molecular structure that can provide electron compounds to free radical molecules and can break the chain reaction of free radicals. The presence of polymorphisms in antioxidants is associated with a decrease in antioxidant activity to eliminate ROS, so it is associated with the risk of several diseases, including the risk of suffering from T2DM [9], [15], [16].

In this study, genotyping of SOD2 Ala16Val, GPx1 Pro198Leu, GSTP1 Ile105Val, and Cat -21 A/T gene variants have been carried out. The results of the Chi-square goodness-of-fit test analysis, which examined the distribution of genotype frequencies of all groups, were in agreement under the HWE law in accordance with the HWE law ( $p > 0.05$ ). HWE is a mathematical relation model which states that a

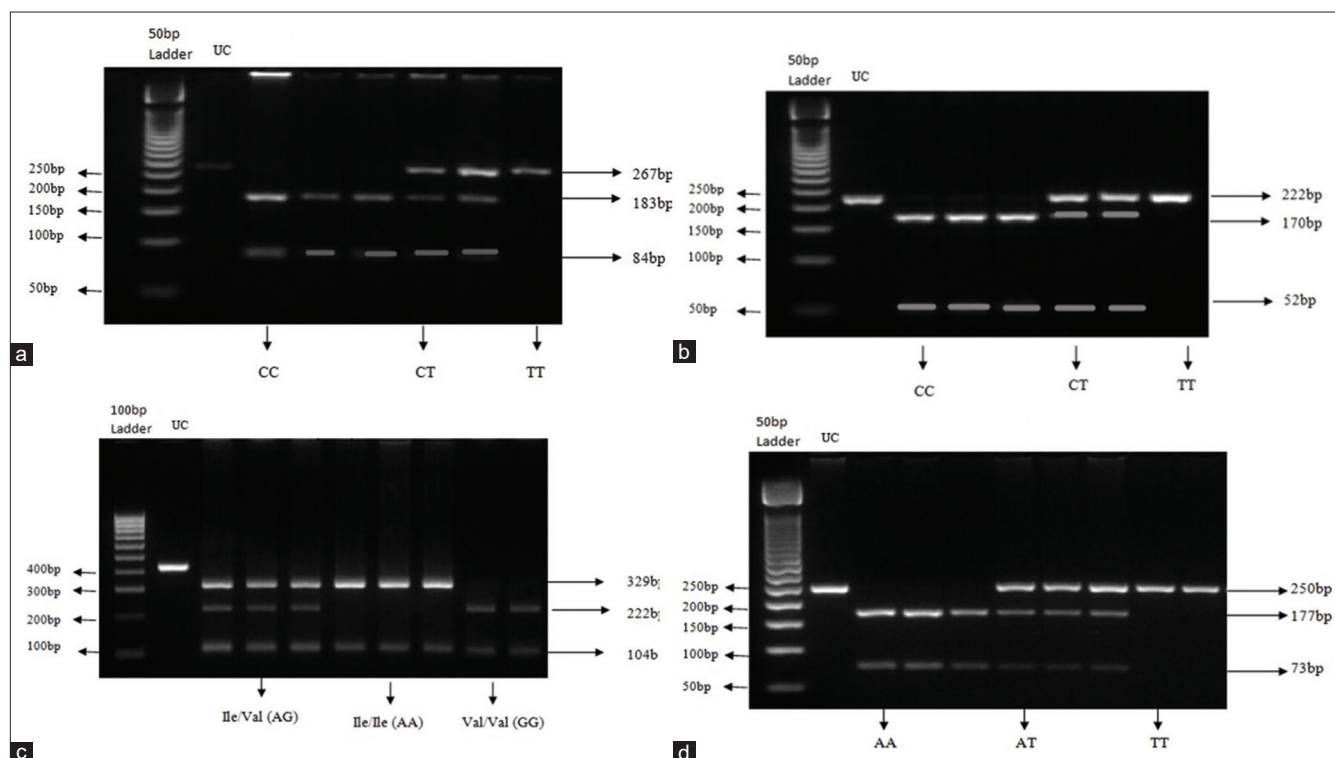


Figure 2: Restriction fragment length polymorphism analysis of (A) Ala16Val; (B) glutathione peroxidase 198Pro/Leu; (C) glutathione S-transferase Pi1 Ile105Val; and (D) Catalase -21 A/T polymorphisms

genotype or allele frequency in a population will be constant from one generation to another, without the influence of evolution [17].

The results of the current study found that individuals with the Val/Val of SOD2 under the recessive model had 2.220 fold to the risk of T2DM compared to the Ala/Ala+Ala/Val ( $p = 0.011$ ; OR = 2.220; CI = 1.234-3.992). These study results are in line with previous studies, which also found an association between SOD2 Ala16Val polymorphism and T2DM in Lucknow, India, and Lebanese populations [5], [18]. In contrast, different results were found in the Western Indian population [19].

SOD2 is present in the mitochondria. This enzyme gene is located on chromosome 6q25.3. SOD2 Ala16Val (+ 47C/T) is the most common dimorphism found. The dimorphism of this gene is the substitution of nucleotide C to T at nucleotide 47 (GCT to GTT), in which the amino acid alanine changes to valine. The amino acid alanine changes to valine cause conformational changes of MTS from  $\beta$ -sheet to  $\alpha$ -Helix. Val/Val or T allele of the SOD2 gene would encode a  $\beta$ -sheet conformation, arrested in the inner mitochondrial space, and degraded as proteasome leading to low mitochondrial-SOD activity [5]. This underpins the SOD2 Ala16Val polymorphism associated with the pathogenesis of several diseases. Increased Val/Val of the SOD2 gene or T allele causes less SOD ability to fight oxidative stress [20], [21].

In the present study, no significant relationship was found between GPx1 Pro198Leu and GSTP1 Ile105Val in all genetic models (co-dominant, dominant,

and recessive) with T2DM risk ( $p > 0.05$ ). The GPx1 polymorphism that has been identified is the substitution of cytosine to thymine (C > T), it causes the substitution of proline with leucine occurring in codon 198 in exon 2 (Pro198Leu, rs1050450). The GSTP1 gene is one of the antioxidant gene located on the long arm of chromosome 11. In the GSTP1 Ile105Val (rs1695), the substitution of adenine to guanine (A/G) is found in the DNA coding sequence. This results in a substitution of isoleucine residue to valine (Ile/Val). Previous studies have shown that GPx1 Pro198Leu and GSTP1 Ile105Va are not related to the risk of T2DM, but these results are inconsistent [5], [7], [22], [23].

Analysis of the Cat -21 A/T gene shows a significant relationship between the TT genotype of Cat -21 A/T under the co-dominant model and the TT Cat -21 A/T under the recessive model with the risk of suffering from T2DM (OR = 5.000, 95% CI: 1.079-23.176,  $p = 0.027$  and OR = 4.738, 95% CI: 1.039-21.600,  $p = 0.030$ ). CAT is a homotetrameric protein with a mass of 224 kDa containing 527 amino acid residues, which have four heme groups in their structure. The gene is localized on chromosome 11p13.31, which consists of 13 exons and 12 introns. The TT genotype in Cat -21 A/T gene polymorphism can affect CAT production or activity because of its ability to change binding affinity at the transcription factor area. The decreased activity of CAT can increase oxidative stress that causes damage to a certain gene, trigger many diseases [8], [24].

Several previous researchers have investigated the relationship between the -21 A/T polymorphism and

**Table 4: The relationship between SOD2 Ala16Val, GPx1 Pro198Leu, GSTP1 Ile105Val, and Cat -21 A/T polymorphism with T2DM risk**

Genes polymorphism	Healthy control n (%)	T2DM n (%)	p	OR (95% CI)
<b>SOD2 Ala16Val (C/T)</b>				
Co-dominant				
Ala/Ala (CC)	4 (5.0)	3 (2.5)	1	Reference
Ala/Val (CT)	35 (43.8)	33 (27.5)	1	1.257 (0.261–6.047)
Val/Val (TT)	41 (51.2)	84 (70.0)	0.229	2.732 (0.584–12.778)
Dominant				
Ala/Ala (CC)	4 (5)	3 (2.5)	1	Reference
Ala/Val (CT)+Val/Val (TT)	76 (95.0)	117 (97.5)	0.441	2.053 (0.447–9.428)
Recessive				
Ala/Ala (CC)+Ala/Val (CT)	39 (48.8)	36 (30.0)	1	Reference
Val/Val (TT)	41 (51.3)	84 (70.0)	0.011	2.220 (1.234–3.992)
<b>GPx1 198Pro/Leu(C/T)</b>				
Co-dominant				
Pro/Pro (CC)	59 (73.8)	86 (71.7)	1	Reference
Pro/Leu (CT)	19 (23.8)	32 (26.7)	0.741	1.155 (0.599–2.229)
Leu/Leu (TT)	2 (2.5)	2 (1.7)	1	0.868 (0.094–5.008)
Dominant				
Pro/Pro (CC)	59 (73.8)	86 (71.7)	1	Reference
Pro/Leu (CT)+Leu/Leu (TT)	21 (26.3)	34 (28.3)	0.872	1.111 (0.587–2.1000)
Recessive				
Pro/Pro (CC)+Pro/Leu (CT)	78 (97.5)	118 (98.3)	1	Reference
Leu/Leu (TT)	2 (2.5)	2 (1.7)	1	0.661 (0.091–4.791)
<b>GSTP1 Ile105Val (A/G)</b>				
Co-dominant				
Ile/Ile (AA)	38 (47.5)	53 (44.2)	1	Reference
Ile/Val (AG)	36 (45.0)	59 (49.2)	0.590	1.175 (0.653–2.115)
Val/Val (GG)	6 (7.5)	8 (6.7)	1	0.956 (0.306–2.982)
Dominant				
Ile/Ile (AA)	38 (47.5)	53 (44.2)	1	Reference
Ile/Val (AG)+Val/Val (GG)	42 (52.5)	67 (55.8)	0.666	1.144 (0.648–2.018)
Recessive				
Ile/Ile (AA)+Ile/Val (AG)	74 (92.5)	112 (93.3)	1	Reference
Val/Val (GG)	6 (7.5)	8 (6.7)	1	0.881 (0.294–2.642)
<b>Cat -21 A/T</b>				
Co-dominant				
AA	50 (62.5)	65 (54.2)	1	Reference
AT	28 (35.0)	42 (35.0)	0.759	1.154 (0.631–2.110)
TT	2 (2.5)	13 (10.8)	0.027	5.000 (1.079–23.176)
Dominant				
AA	50 (62.5)	65 (54.2)	1	Reference
AT+TT	30 (37.5)	55 (45.8)	0.307	1.410 (0.791–2.513)
Recessive				
AA+AT	78 (97.5)	107 (89.2)	1	Reference
TT	2 (2.5)	13 (10.8)	0.030	4.738 (1.039–21.600)

T2DM: Type 2 diabetes mellitus, OR: Odds ratio, CI: Confidence interval, SOD2: Superoxide dismutase 2, GPx1: Glutathione peroxidase, GSTP1: Glutathione S-transferase P1.

the risk of T2DM. Research by Saravani *et al.* (2015) on T2DM populations in Iran found that TT genotype of Cat -21 was associated with a risk of T2DM occurrence of 1797 times. Other studies by Vat *et al.* (2013) in the Lucknow population, India, found that Cat -21 A/T genotypes are associated with T2DM but not Cat -21 A/T alleles [8], [25]. A different result that Cat -21 A/T polymorphism is not related to T2DM in populations in the Czech Republic [26].

In humans, genes that play a role in coding antioxidants are known to be highly polymorphic cause gene variation. Race and ethnicity are known to affect individual gene variations. The association between genotype or allele of single-nucleotide polymorphisms and several diseases is also known to vary according to race or ethnicity. The association between ethnicity and diseases or susceptibility of the disease has been reported in several previous studies. The existence of racial and ethnic differences is a factor that allows differences in the results of this study with previous research in various populations [27], [28].

## Conclusion

This study indicates that the Val/Val in the recessive model of SOD2 gene also TT genotype in the co-dominant model of Cat gene and TT genotype in the recessive model of Cat gene are associated with risk factors for T2DM occurrence. However, this was not found in GPx1 Pro198Leu and GSTP1 Ile105Val. The limitations of this study were the small number of samples and examination of antioxidant serum's activity/levels of subjects was not performed. Further research is needed on larger samples and analyzing the relationship of antioxidant variants with antioxidant activity/levels of subjects.

## Acknowledgments

This study is supported by the Ministry of Research and Technology and the Higher Education Republic of Indonesia. The support is under the research grant DRPM KEMENRISTEKDIKTI of the Year 2019 (Grant No: 21/UN5.2.3.1/PPM/KP-DRPM/2019). The authors are grateful to all participants in this study, Director of USU Hospital, Dean of Faculty of Medicine USU, and Heads of the Padang Bulan Public Health Centers of Teladan.

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