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Aldose reductase genetic polymorphism is a risk factor of diabetics retinopathy among type 2 diabetes mellitus in Yogyakarta, Indonesia

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ABSTRACT

Diabetes mellitus (DM) is a metabolic syndrome characterized by hyperglycemia and glucose intolerance, due to insulin resistance, insulin deficiency, or both. Diabetics retinopathy (DR) is a DM complication due to retinal abnormality, that causes vision reduction and even blindness. The association between DR and aldose reductase C-106T (ALR C-106T) gene polymorphism has been reported in previous studies. This genetic polymorphism increases the sorbitol level inside erythrocyte and pericyte in the retinal membrane that leads to weakness of retinal capillary vessel and microaneurism. The aim of this study was to know the presence of ALR C-106T gene polymorphism and its frequency distribution among diabetics Javanese patients in Dr. Sardjito General Hospital Yogyakarta, Indonesia. In addition, this study also aimed to analyze the difference of erythrocytes osmotic fragility (EOF) among ALR genotypes in type 2 diabetics patients with DR and without DR and to analyze whether ALR genetic polymorphism is a risk factor of DR in type 2 diabetic patients. This was a case control study that involved 40 diabetics patients with DR as case and 40 diabetics patients without DR as control groups. The C-106T ALR gene polymorphism was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) method. Erythrocytes osmotic fragility was analyzed using spectrophotometer. Genotype and allele distributions were analyzed using x^2 and other data were analyzed using independent t-test and Mann-Whitney, with $p < 0.05$ was considered as significantly different. The results showed that in type 2 diabetics patients with DR, 33 patients (82.5%) were CC homozygote individuals and 7 patients (17.5%) were CT heterozygote individuals. In type 2 diabetics patients without DR, 27 patients (67.5%) were CC homozygote individuals and 13 patients (32.5 %) were CT heterozygote individuals. The genotype and allele distributions were not significantly different between two groups ($p = 0.121$ for genotype, $p = 0.151$ for allele). Odds Ratio of genotype was 2.270 while allele was 2.023. Erythrocytes osmotic fragility of CC genotype was higher than CT genotype ($p = 0.047$). In conclusion, there was no significant difference between CC and CT genotype distribution among type 2 diabetics patients with and without DR. Erythrocyte osmotic fragility of CC genotype was higher than CT genotype. C-106T gene polymorphism was a risk factor of DR in type 2 diabetic patients.

Key words **:** ALR genes – polymorphism - type 2 DM - diabetic retinopathy - erythrocytes osmotic fragility

ABSTRAK

Diabetes Mellitus (DM) merupakan penyakit metabolik yang ditandai dengan hiperglikemia dan intoleransi glukosa, akibat resistensi insulin, kekurangan insulin atau kombinasi keduanya. Retinopati diabetika (RD) adalah komplikasi DM yang menyebabkan kelainan retina dan berakibat pada penurunan penglihatan bahkan kebutaan. Penelitian sebelumnya menunjukkan adanya hubungan antara RD dan polimorfisme C-106T gena ALR. Polimorfisme ini menyebabkan peningkatan kadar sorbitol eritrosit dan membran perisit retina yang berakibat lemahnya pembuluh kapiler retina dan mikroaneurisme. Penelitian bertujuan mengetahui adanya polimorfisme C-106T gena ALR dan distribusi frekuensi genotipe dan alel pada suku Jawa di RSUP Dr. Sardjito Yogyakarta. Selain itu juga untuk mengetahui perbedaan fragilitas osmotik eritrosit antar genotipe, antara DM tipe 2 dengan RD dan tanpa RD serta mengetahui polimorfisme C-106T gena ALR sebagai faktor risiko RD pada DM tipe 2. Penelitian ini merupakan penelitian kasus kontrol yang melibatkan 40 pasien DM tipe 2 dengan RD sebagai kasus dan 40 pasien DM tipe 2 tanpa RD sebagai

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kelompok kontrol. Polimorfisme C-106T gena ALR ditentukan dengan metode PCR-RFLP. Fragilitas osmotik di analisa dengan metode spektrofotometri. Distribusi genotype dan alel dianalisa menggunakan uji x^2. Data lainnya dianalisa menggunakan uji t independen dan Mann-Whitney dengan nilai $p < 0,05$ untuk menentukan perbedaan secara bermakna hasil analisis. Hasil penelitian menunjukkan distribusi genotipe pasien DM tipe 2 dengan RD adalah CC 33 (82,5 %), CT 7 (17,5 %), dan pada pasien DM tipe 2 tanpa RD adalah CC 27 (67,5 %), CT 13 (32,5 %). Distribusi genotipe dan alel tidak berbeda bermakna antara ke 2 kelompok ($p = 0,121$ untuk genotipe, $p = 0,151$ untuk alel). Rasion Odds genotipe adalah 2,27, sedangkan alel adalah 2,023. Pada genotipe CC fragilitas osmotik lebih tinggi dibandingkan CT ($p = 0,047$). Dari hasil penelititan dapat disimpulkan tidak terdapat perbedaan bermakna antara distribusi genotipe CC dan CT pada pasien DM tipe 2 dengan RD dengan tanpa RD. Fragilitas osmotik eritrosit genotipe CC lebih tinggi dibandingkan CT. Polimorfisme C-106T gena ALR merupakan faktor risiko RD pada DM tipe 2.

Kata kunci **:** gena ALR – polimorfisme – DM tipe 2 – retinopati diabetika – fragilitas osmotik eritrosit

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia and glucose intolerance, as a result of insulin deficiency, impaired insulin action, or combination of both. The prevalence of DM worldwide is about 4% and is predicted to continue to increase, and by 2025 will reach 5.4%.[1] World Health Organization (WHO) has reported the increase number of people with type 2 DM in Indonesia in 2000 as many as 8.4 millions people and estimated to increase to approximately 21.3 millions in 2030.[2] The prevalence of DM in Yogyakarta, according to the national basic health research (RISKESDAS) Indonesia 2007 was about 1.6 %.

Chronic complications of DM are systemic vascular disease, heart disease, retinal degeneratur disease known as diabetic retinopathy, cataracts, kidney damage and peripheral nerve damage such as diabetic neuropathy.[3] Diabetic retinopathy is a form of DM complication that causes retinal disorder which affect vision convulsion until blindness. Hyperglycemi causes the thickening of retinal blood vessel and leakage. This complication commonly happens if its a diabetic patient has never had a treatment for at least for 15 years.[4] In Indonesia, DR prevalence was 27.2%,[5] with 8.7% was in urban area of Yogyakarta, whereas 7.73% contained in the rural.[6] Ethnic or genetic difference plays a role in the prevalence of DR.

Damage of the retina caused by hyperglycemia involves three existing pathways i.e. activation of the proteins glycation, C kinase protein, and aldose reductase (ALR).[7] Aldose reductase catalyzes the change of glucose to sorbitol through the reduction of aldehyde group of glucose. In hyperglycemia, sorbitol concentration increases and it will be converted to fructose by sorbitol dehydrogenase (SDH). In hyperglycemia, sorbitol degradation proceeds slow, as of accumulates in cells and causes an increase in osmotic pressure. Excessive accumulation of sorbitol causes pericytes on retinal capillaries weakened and causes microaneurism.[8] Hemodynamic disturbance in erythrocytes is one of the causes of blockage and leakage of retinal blood vessels which is a sign of DR.[9] Therefore, erythrocyte osmotic fragility is used as a parameter of pericytes retinal cell damage.[10]

Aldose reductase is coded by ALR gene which located in the long arm of chromosome 7 (7q35). This gene codes 316 amino acids and has 10 exon. The C-106T polymorphism in ALR gene happens in the basal promoter area and it causes changes in the expression of mRNA of ALR gene.[11] Previous study showed that C-106T polymorphisms in ALR gene is strongly associated with DR incidence in diabetic Chinese. CC genotype carrier strongly related with risk of DR.[12]

This study aimed to describe the relation of C-106T polymorphism in ALR gene with the risk for DR in diabetic Javanese in Yogyakarta, Indonesia. In addition, this study also aimed to analyze the difference of EOF among ALR genotypes in type 2 diabetics patients with DR and without DR and to analyze whether ALR genetic polymorphism is a risk factor of DR in type 2 diabetic patients.

MATERIALS AND METHODS

This was a case-control study. Subjects were 40 type 2 diabetic patients with DR as a case group and 40 type 2 diabetic patients without DR as a control group. The inclusion criteria of the research subject were 1). patient at Dr. Sardjito General

Hospital Yogyakarta; 2). Javanesse; 3). aged between 30-65 years old; 4). have been diagnosed for type 2 diabetes at least for 5 years. The exclusion criteria were obesity and hypertension. The patients who fulfilled the inclusion criteria were asked to sign the informed consent form as their willingness to become research subject. The protocol of the study has been approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Gadjah Mada University, Yogyakarta.

Diagnosis of type 2 DM was established based on fasting blood glucose (FBG) and 2 hours post prandial blood glucose.[2] Retinopathy criteria was according to fundusphoto which showed one of retina disorder symptoms (PDR or NPDR). The acquired data were age, sex, duration of type 2 DM, body mass index (BMI), blood pressure, and lipid profile.

Erythrocyte osmotic fragility was checked using spectrophotometer while lysis percentage and percentage of NaCl solution which cause 50% of erythrocyte lysis. Genotyping of C-106T polymorphism in ALR gene was performed using PCR-RFLP method. Sample used in this study was DNA isolated from blood. The PCR conditions were denaturation at 95^{0}C for 2 minutes, followed by 35 cycles of denaturation at 95^{o}C for 60 seconds, annealing at 67^{o}C for 60 seconds, and extension at 72^{o}C for 60 seconds. The final extension was at 72^{o}C for 5 minutes. The forward primer was: 5'-CCT TTC TGC CAC GCG GGG CGC GGG-3' (attached on -222 until - 199) and the reverse primer was: 5'-CAT GGC TGC TGC GCT CCC CAG-3' (attached on +21 until starting codon ATG).

Restriction enzyme used in this study was *BfaI* (New England Biolabs). The PCR product was incubated with the BTA for 16 hours. The digestion product the was then electrophoresed on 2% agarose gel and visualized by etidium bromide under UV light. CC genotype had 2 bands: 206 and 57 bp. TT genotype had 3 bands : 147, 59 and 57 bp, whereas genotype of CT had 4 bands : 206, 147, 59 and 57 bp.[13]

RESULTS

Subject characteristics

Subject characteristics are shown in TABLE 1. Subject characteristics such as age, blood pressure, fasting blood glucose, 2 hours post prandial blood glucose, serum triglyceride, total cholesterol, HDL and LDL cholesterol were not significantly different between type 2 diabetic patients with DR and without DR ($p>0.05$). However, the average BMI of type 2 diabetic patients with DR was lower than patients without DR ($p<0.05$).

TABLE 1. Subject characteristics of type 2 diabetic patients. Data were expressed in mean ± standard deviation except for sex in %

Variable	Type 2 DM		p*
	with DR (n=40)	without DR (n=40)	
Sex			
• Male	16 (40%	15 (37.5%)	
• Female	24 (40%)	25 (62.5%)	
Age (years)	55.68 ± 7.73	55.33 ± 8.87	0.981
BMI (kg/m^2)	21.90 ± 3.07	23.37 ± 1.83	0.028
Systolic blood pressure (mmHg)	124.00 ± 11.50	123.50 ± 12.10	0.800
Diastolic blood pressure (mmHg)	79.25 ± 8.59	80.25 ± 8.00	0.630
Fasting blood glucose (mg/dL)	168.44 ± 72.48	145.74 ± 66.19	0.128
2 hours PP blood glucose (mg/dL)	236.82 ± 75.17	214.57 ± 91.59	0.239
Triglyceride (mg/dL)	122.77 ± 46.30	153.94 ± 127.93	0.954
Total cholesterol (mg/dL)	178.35 ± 34.89	173.95 ± 28.42	0.539
HDL cholesterol (mg/dL)	75.88 ± 17.31	72.49 ± 13.25	0.207
LDL cholesterol (mg/dL)	111.68 ± 23.46	105.65 ± 20.53	0.225

* *Independent t-test and Mann Whitney test with 95% convidence interval ($p<0.05$)*

C-106T polymorphism in ALR Gene

The genotyping result of C-106T polymorphism in ALR gene using PCR-RFLP is shown in FIGURE 1. While the distribution of genotype and allele is shown in TABLE 2. Genotype distribution of ALR gene between observed value with expected value Hardy-Weinberg had no significant difference (p= 0.573). The genotype frequency between type 2 diabetic patients with DR and without DR was not different (p = 0.121), so as the allele frequency (p = 0.151). CT genotype carriers had 2.270 times increased risk for DR compared with CC genotype carries, whereas C allele carriers had 2.023 times increased risk for DR than T allele carriers. Therefore, the high risk allele for DR in type a diabetic patients was C allele.

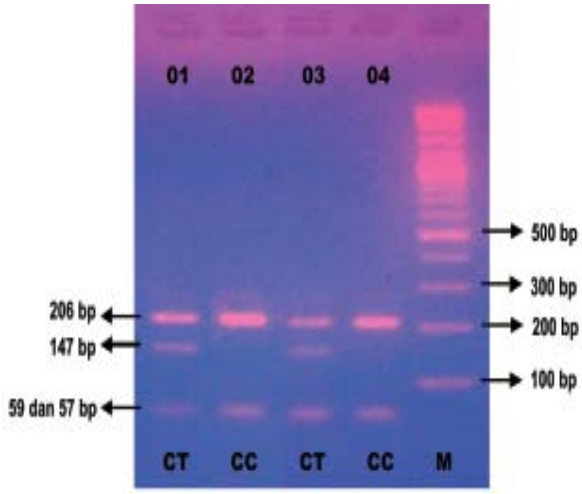

FIGURE 1. Genotyping of C-106T polymorphism in ALR gene

TABLE 2. Genotype and allele of type 2 DM risk with DR

Variable		Type 2 DM		p*	OR	CI (95%)
		with DR (n=40)	without DR (n=40)			
Genotype	CC	33 (82.5%)	27 (67.5%)	0.121	2.270	0.794 – 6.488
	CT	7 (17.5%)	13 (32.5%)			
Alelle	C	73(91.25%)	67 (83.75%)	0.151	2.023	0.762 – 5.374
	T	7 (8.75%)	13 (16.25%)			

**Statistical analysis using Chi square* (x^2) *test with a 95% confidence interval (p<0.05)*

Erythrocyte osmotic fragility

The average percentage of NaCl which caused lysis of 50% erythrocyte is shown at TABLE 3 and 4. This study showed a significant difference of erythrocyte osmotic fragility between CC and CT genotype on type 2 diabetic patients (p=0.047). The erythrocyte osmotic fragility increased in CC genotype individuals (TABLE 3). The percentage of NaCl on type 2 diabetic patients with DR was higher than to those without DR (TABLE 4). However, it was not significantly different (p=0.154).

TABLE 3. The percentage of NaCl (mean ± SD) which caused lysis of 50% erythrocyte based on the genotype of type 2 diabetic patients with and without DR

Variable	Genotype		p*
	CC	CT	
NaCl percentage which caused 50% lysis of erythrocyte	0.4135 ± 0.0129	0.4067 ± 0.0126	0.047

** Statistical analysis using Mann-Whitney test with 95% confidence interval (p<0.05)*

TABLE 4. The percentage of NaCl (mean ± SD) difference which caused lyses of 50% erythrocyte between type 2 diabetic patients with and without DR

Variable	DM type 2		p*
	with DR	without DR	
NaCl percent which caused lysis of 50% of erythrocyte	0.4139 ± 0.0110	0.4097 ± 0.0147	0.154

* *Statistical analysis using Mann-Whitney test with 95% confidence interval (p<0.05)*

DISCUSSION

The subject characteristics of type 2 diabetic patients with DR and without DR were similar except BMI. The BMI of type 2 diabetic patients without DR was higher than those with DR (p=0.028). The BMI of type 2 diabetic patients is influenced by prolonged duration of diabetes and increased insulin resistance which activated lipase-sensitive hormone, therefore the deposite of triglyceride in adipose tissue will be hydrolyzed becomes free fatty acid. This process will decrease the BMI of type 2 diabetic patients.[14]

Genotype distribution of C-106T polymorphism in ALR gene in type 2 diabetic patients in several populations has been reported by some authors from different studies (TABLE 5). The studies indicated that different population showed different genotype frequency.[7,15,16] Diabetic retinopathy development depends on environment and genetic factors. This study did not found TT genotype, whereas study in Euro-Brazil, China, and Chili populations, TT genotype was found and the number was smaller than CC and CT genotype. Frequency of CC of Javanese was higher than other populations (Euro-Brazil, China and Chili). In type 2 diabetes without DR, the number of individual with CT genotype was lower compared to Euro-Brazil and Chili, but was higher than China.

TABLE 5. Genotype distribution of C106T polymorphism in ALR gene in type 2 diabetic patients in several populations

Population	Subject	Genotype*			n
		CC	CT	TT	
Euro-Brazil (Santos *et al.*[16])	DR	32 (32.32 %)	46 (46.47 %)	21 (21.21 %)	99
	without DR	47 (42.73 %)	39 (35.45 %)	24 (21.82 %)	110
China (Wang *et al.*[17])	DR	111 (59.36 %)	63 (33.69 %)	13 (6.95 %)	187
	without DR	360 (65.34 %)	163 (29.58 %)	28 (5.08 %)	551
Chili (Olmos *et al.*[7])	DR	24 (45.28 %)	26 (49.06 %)	3 (5.66 %)	53
	without DR	57 (59.38%)	32 (33.33 %)	7 (7.29 %)	96
Jawa (this study)	DR	33 (82.5 %)	7 (17.5 %)	0 (0 %)	40
	without DR	27 (67.5 %)	13 (32.5 %)	0 (0 %)	40

* The data were expressed in sum (%); n=total sample

Studies on C-106T polymorphism has showed a strong association of the polymorphism with DR complication, although the case and control subjects had no significant difference.[12] For Euro-Brazil population there was no significant difference between type 2 diabetic patients with DR and without DR. CC genotype and C allele were the high risk genotype and allele for DR complication The same result also found in Chili.[7]

There was correlation between C-106T polymorphism with (CA) dinucleotide repetition on ALR gene promoter region,[17] this might happen because the location of polymorphism was contiguous with the sequence of ALR gene which causes DR

sensitivity. From the other research which showed that CC was a high risk genotype for DR.[13]

C-106T polymorphism on basal promoter area of ALR gene will cover the CCAAT promoter element which located between -98 and -94, in cases where that region performs as active functional element. Research showed that CCAAT element was recognized by nuclear factor of Hep G2 cell.[11] Other study showed that different allele on location -106 would affect the CCAAT element function by changing the factor binding activity and finally change the basal transcription level of ALR gene.[18] The increase of ALR gene expression was identified on retinal epithelial cell of CC homozygote carrier compared with heterozygote genotype CT-106.[19]

A significant difference of erythrocyte osmotic fragility between CC and CT genotype on type 2 diabetic patient was observed in this study. The erythrocyte osmotic fragility significantly increased in CC genotype individuals (TABLE 3). Previous study showed that higher ALR expression occured in CC genotype individuals compared with CT genotype individuals.[7] The increase also happens on blood and sorbitol level of erythrocyte on type 2 diabetic patients with DR than without DR.[20] Sorbitol accumulation will affect osmolality of erythrocyte and causes its fragility to increase.

Higher percentage of NaCl on type 2 diabetic patients with DR indicated a higher erythrocyte fragility compared to those without DR. High level of plasma glucose can affect osmotic pressure and will cause cellular dehydration.[21] Sorbitol level in erythrocyte and ALR in the blood correlates with level of plasma glucose.[22] The increase of plasma glucose level in hyperglycemia will increase the sorbitol level.

Sorbitol is not easy to diffuse through cell membrane, as a consequence sorbitol will accumulate and disturb water entry inside the cell and impair the osmotic.[14] Sorbitol concentration raise significantly in type 2 diabetic patients with DR, compared to those type 2 diabetic patients without complication. The sorbitol accumulation decreases the number of pericyte of retina.[23]

The beneficial impact of strict glycemic control to prevent diabetic complications has been reported. However, most diabetic patients rarely achieve consistent glucose level. Hence, agents that can prevent diabetic complications, irrespective of glycemic control, would be an alternative.[20] Aldose reductase inhibitor and protein kinase inhibitor can be included as an alternative treatment to prevent diabetic complications. However, further study is needed in order to obtain optimal benefit of these agents.

CONCLUSION

C-106T polymorphism in ALR gene occured in type 2 diabetic patients with DR on Dr. Sardjito General Hospital Yogyakarta. There was no significant difference on genotype frequency between CC and CT as well as C and T allele between type 2 diabetic patients with or without DR. Significant difference in osmotic erythrocyte fragility was observed between CC and CT genotype of type 2 diabetic patients, but there was no significant difference between type 2 diabetic patients with and without DR. C-106T polymorphism in ALR gene was a risk factor of DR on type 2 diabetic patients.

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