Indonesian Journal of Biotechnology, June, 2012 Vol. 17, No. 1, pp.10-16

-429 T/C and -374 T/A Polymorphisms in Receptor Advanced Glycation Endproducts (RAGE) gene in Type 2 Diabetic Patients with Diabetic Retinopathy at the Dr. Sardjito General Hospital Yogyakarta

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Abstract

Receptor of advanced glycation endproduct (RAGE) plays an important role in the pathogenesis of diabetic vascular complications, such as diabetic retinopathy. The interaction between the RAGE and advanced glycation end product (AGE) leads to oxidative stress and could result in cellular activation and inflammation. The production of AGE occurs normally during aging but it increases in hyperglycemia condition. The objective of this research was to investigate the association between -429 T/C and -374 T/A polymorphisms in RAGE gene with the risk of diabetic retinopathy (DR) of type 2 diabetic patients in Javanese population. This was a case control study which consisted of 40 type 2 diabetic patients with DR as case subjects and 40 type 2 diabetic patients without DR (NDR) as control subjects. Genotyping of polymorphism was performed by PCR-RFLP. Chi-square test and odds ratio models were used to evaluate the association of both polymorphisms and DR risk and to examine 2-SNP haplotype of -429 T/C and -374 T/A polymorphisms in RAGE gene on DR. The genotype frequencies of -429 T/C polymorphism in RAGE gene in DR subjects were TT = 72.5% and TC/CC = 27.5%; while in NDR subjects were TT = 80% and TC/CC = 20%, with p = 0.431. The allele frequencies of -429 T/C polymorphism in DR subjects were T = 83.7% and C = 16.3%, while in NDR subjects were T = 87.5% and C = 12.5%, with p = 0.499. The genotype frequencies of -374 T/A polymorphism in RAGE gene in DR subjects were TT = 67.5%, TA = 32.5% while in NDR subjects were TT = 82.5%, TA = 17.5%, with p = 0.121. In DR subjects, the frequencies of T and A were 83.7% and16.3%, while in NDR subjects the frequencies of T and A were 91.2 % and 8.8%, with p = 0.151. Odds ratios of -429 T/C polymorphism were 1.52 (95% CI = 0.54 – 4.29) for TC/CC genotype and 1.358 (95% CI = 0.56 – 3.31) for C allele. Odds ratios of -374 T/A polymorphism were 2.27 (95% CI = 0.79 – 6.49) for TA genotype and 2.02 (95% CI = 0.76 – 5.37) for A allele. χ^2-value for 2-SNP haplotype was p = 0.127. The -374 T/A polymorphism in RAGE gene was a stronger risk factor of DR than -429 T/C polymorphism in RAGE gene. There were not significantly different of frequencies of genotypes, allele, and two-SNP haplotype of -429 T/C and -374 T/A polymorphisms in RAGE gene between DR subjects and NDR subjects.

Keywords : Diabetic, retinopathy, RAGE, polymorphism.

Introduction

Receptor of advanced glycation endproduct (RAGE) is the receptor for advanced glycation endproduct (AGE) which is normally expressed in low amount by endothelium, smooth muscle cells, mesangial cells, and monocytes. However, in diabetic patients, RAGE expression increaseds along with AGE accumulation (Hudson *et al*. 2001). The accumulation of AGE affects the structures and functions of cells Goldin *et al*. (2006) and induces various pathological changes. AGE is non-enzymatically reaction product between

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reducing sugar and N-terminal cluster in amino acid residues (Bierhaus *et al*. 1998). The formation of AGE occured normally during the process of aging, but it accelerates in the condition of chronic hyperglycemia (Bierhaus *et al*. 1998).

The formation of AGE is one of metabolic pathways which plays role in the development of microvascular complications of diabetes (Ciulla *et al*. 2003). The binding of AGE to RAGE activates NADPH oxidase which increases the formation free radicals (Goldin *et al*. 2006) and causes cellular oxidative stress. All of those events could damage retinal pericyte cells and leads to microaneurysm. In addition, the interaction of AGE-RAGE activates NF-κB, increases the transcription of endotelin-1, VCAM-1, tissue factors, and causes the production of pro-inflammatory cytokines (Singh *et al*. 2001). The activation of RAGE also causes the decrease of NO level and the narrowing of vascular lumen which can cause retinal vascular occlusion.

The RAGE gene is identified to contribute in diabetic retinopathy (DR) development. The RAGE gene affects metabolic pathways such as the polyol pathway and the formation of AGE. The RAGE gene plays role in mediating various signals which induce pathological changes in microvascular and cause the occurrence of DR. Polymorphism in the promoter of RAGE gene has been associated with DR Hudson *et al*. (2001) reported that the -429 T/C polymorphism, -374 T/A polymorphism, and 63 bp I/D increased the transcription activity of RAGE gene as much as 2 – 4 folds. Studies performed by Hudson *et al.*(2001) hadshown that -374 A allele supporting the role of these polymorphisms in affecting RAGE transcriptional repression. Furthermore, the C allele of the -429 polymorphism was found to be related to retinopathy in subjects with type 2 diabetes.

The objective of this study was to provide description on the association between -429 T/C and -374 T/A polymorphisms in RAGE gene with DR in type 2 diabetic patients in Yogyakarta.

Materials and Methods

This study was a case control study. The total number of the research subjects was 80 type 2 diabetic patients which consisted of 40 type 2 diabetic patients with DR as the case group (DR) and 40 type 2 diabetic patients without DR as the control group (NDR). The diagnosis of type 2 diabetes was based on the criteria from American Diabetes Association (2007), while the diagnosis of retinopathy was based on the examination of fundus which indicates one of the symptoms of retinal anomalies (Proliferative Diabetic Retinopathy/PDR or Non Proliferative Diabetic Retinopathy/NPDR). Permission to perform this study was obtained from the Health and Medical Research Ethical Committee, Faculty of Medicine, Universitas Gadjah Mada. Informed consent was obtained from each individual in the studied population.

Blood chemical examination

Three ml of blood was taken from the subjects for blood chemical examination and genotyping. The blood glucose, triglyceride, cholesterol, HDL-cholesterol, and LDL-cholesterol levels were analyzed by spectrophotometry.

Genotype analysis

DNA isolation was performed using DNA Purification Kit (Promega). Primers were used for DNA amplification (forward primer 5'-GGGGGCAGTTCTCTCCTC-3' and reverse primer 5'-TCAGAGCCCCCGAT CCTATTT-3') (Hudson *et al*. 2001). The PCR mixture consisted of 2 µl DNA template, 15 µl PCR master mix, 11 ml H_2O, and 2 µl primary mix. PCR condition was as follow: early denaturation at 94^0C for 2 min, followed by 30 cycles of denaturation at 94^0 C for 1 min, annealing at 58^0 C for 1 min, extension at 72^0 C for 1 min; and final extension at 72^0 C for 5 min. For genotyping of -429 T/C

polymorphism, the PCR product (344 bp) was digested with AluI (New England Biolabs) at 37^{0}C for 16 h. While for genotyping of -374 T/A polymorphism, the PCR product was digested with Tsp509I (New England Biolabs) at 65^{0}C for 16 h. The result of DNA digestion was resolved on 2% agarose gel and was visualized using ethidium bromide.

Statistical analysis

In matching the research subjects, independent sample t-test was used for data at normal distribution, while for data without normal distribution, Mann-Whitney test was used after conducting log-transformation. Descriptive analysis was used to identify the frequency of genotype and allele of -429 T/C and -374 T/A polymorphisms in RAGE gene. The frequency difference of genotype and allele between the case and the control groups was analyzed with chi-square test. The frequency difference of 2-SNP haplotype in the case and the control groups was analyzed with chi-square test or Fisher exact test. Odds ratio test was used to measure to which extent the -429 T/C and -374 T/A polymorphisms in RAGE gene influence DR in type 2 diabetes.

Results

In this study, 80 Javanese were enrolled as research subjects. These type 2 diabetic patients with DR and 40 type 2 diabetic patients without DR (NDR). The age, blood

Table 1. Characteristics of the studied population

Characteristics	Diabetic Retinopathy (DR)	Non Diabetic Retinopathy (NDR)	p
N	40	40	
Sex: Male	15 (37.5%)	16 (40%)	
Female	25 (62.5%)	24 (60%)	
Age (years)	55.55 ± 7.96	55.40 ± 9.26	0.904$^{\#}$
BMI (kg/m^2)	22.12 ± 2.67	23.46 ± 1.85	0.032$^{\#}$
SBP (mmHg)	124.50 ± 11.31	121.25 ± 11.37	0.197$^{\#}$
DBP (mmHg)	80.25 ± 8.62	79.50 ± 7.83	0.589$^{\#}$
FBS (mg/dL)	167.78 ± 69.29	142.56 ± 63.14	0.081$^{\#}$
PPBS (mg/dL)	231.56 ± 78.03	205.28 ± 89.99	0.213^{++}
Triglycerides (mg/dL)	188.83 ± 56.26	189.29 ± 72.58	0.637$^{\#}$
Total cholesterol (mg/dL)	125.37 ± 47.46	140.92 ± 105.49	0.658$^{\#}$
HDL cholesterol (mg/dL)	76.06 ± 16.62	72.02 ± 12.95	0.073^{+}
LDL cholesterol (mg/dL)	107.68 ± 21.06	103.67 ± 16.36	0.091^{+}

Data are reported as mean ± SD or %; $^{+}$t test for independent variables, $^{++}$t test for independent; $^{\#}$Mann Whitney U test after log transformed; $p < 0.05$.

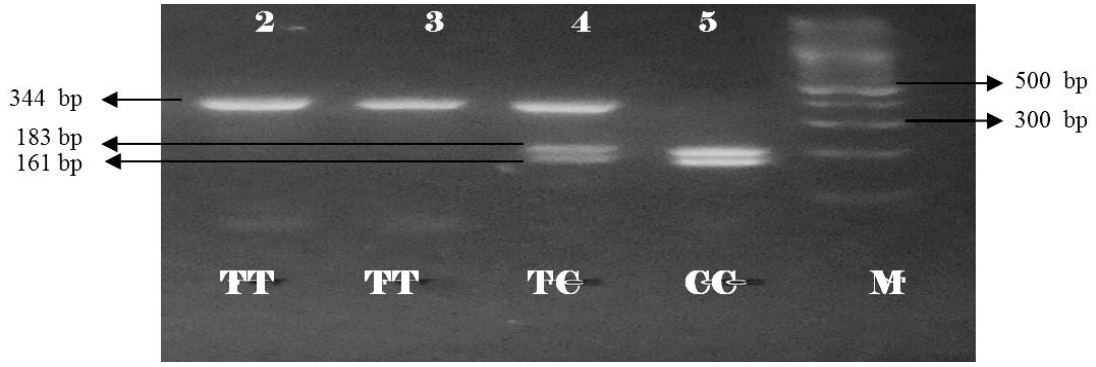

Figure 1. Genotyping of -429 T/C polymorphism in RAGE gene (TT=wild type; TC=heterozygote; CC=homozygote)

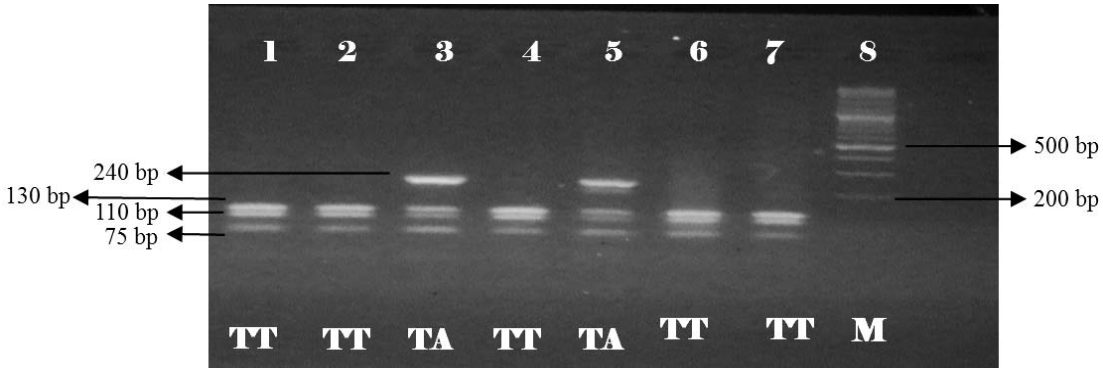

Figure 2. Genotyping of -374 T/A polymorphism in RAGE gene (TT=wild type; TA=heterozygote)

pressure (systolic blood pressure/SBP and diastolic blood pressure/DBS), fasting blood sugar (FBS), postprandial blood sugar (PPBS), triglycerides, total cholesterol, HDL cholesterol, and LDL cholesterol levels were not statistically different between type 2 diabetic patients with DR and without DR. The significant difference occurred in the body mass index (BMI) between DR and NDR subjects. However, the average BMI in both groups was still in normal range, as shown in Table 1.

The genotyping results of -429 T/C and -374 T/A polymorphisms in RAGE gene are shown in Figure 1 and 2.

The distribution of genotype and allele of -429 T/C and -374 T/A polymorphisms in RAGE gene is shown in Table 2 and 3.

Table 2. Distribution of genotype and allele of -429 T/C polymorphism in RAGE gene

	All subject	DR n = 40	NDR n = 40	p (χ^2)	H-W
Genotype					
TT (%)	61	29 (72.5)	32 (80)	0.431	0.483
TC (%)	15	9 (22.5)	6 (15)		
CC(%)	4	2 (5)	2 (5)		
Allele					
T	137	67 (83.7)	70 (87.5)	0.499	
C	23	13 (16.3)	10 (12.5)		

$p < 0.05$; χ^2 = chi-square test; H-W = Hardy-Weinberg equilibrium.

Table 3. Distribution of genotype and allele of -374 T/A polymorphism in RAGE gene

	All subject	DR n = 40	NDR n = 40	p (χ^2)	H-W
Genotype					
TT (%)	60	27 (67.5)	33 (82.5)	0.121	0.573
TA (%)	20	13 (32.5)	7 (17.5)		
AA (%)	0	0 (0)	0 (0)		
Allele					
T	140	67 (83.7)	73 (91.2)	0.151	
A	20	13 (16.3)	7 (8.8)		

$p < 0.05$; χ^2 = chi-square test; H-W = Hardy-Weinberg equilibrium.

The odds ratio of genotype and allele of -492 T/C and -374 T/A polymorphisms in RAGE gene is shown in Table 4. The distribution and odds ratio of 2-SNP haplotype of -429 T/C and -374 T/A polymorphisms in RAGE gene are shown in Table 5.

Table 4. The odds ratio of -429 T/C and -374 T/A polymorphisms in RAGE gene

Value	-374 T/A polymorphism		-429 T/C polymorphism	
	Genotype	Allele	Genotype	Allele
	TA	A	TC/CC	C
Odds ratio	2.27	2.023	1.517	1.358
95% CI	0.79–6.49	0.76–5.37	0.54–4.29	0.56–3.31

95% CI: 95% Confidence Interval.

Table 5. Haplotype of -429 T/C and -374 T/A polymorphisms in RAGE gene

Haplotype -374/-429	DR	NDR	OR (95% CI)	p
TT-TT	21 (52.5%)	25 (62.5%)	-	
TT-TC/CC	6 (15%)	8 (20%)	0.706 (0.22 – 2.26)	0.556[+]
TA-TT	8 (20%)	7 (17.5%)	1.179 (0.38 – 3.63)	0.775[+]
TA-TC	5 (2.5%)	0 (0%)	-	0.055[#]
				p = 0.127[++]

Data are reported as number (%); p < 0.05; OR = odds ratio; 95% CI = 95% Confidence Interval; [+,#]p-value for each haplotype vs other combination; [*]chi-square test; [#]Fisher exact test; [++]p-value for the overall test of association.

Discussion

Genotyping of -429 T/C polymorphism in RAGE gene found all of the possible genotypes, which were TT, TC, and CC. Similar result was reported by Ramprasad *et al* (2007) and Santos et al (2010). On the other hand, genotyping of -374 T/A polymorphism in RAGE gene only found 2 genotypes, which were TT and TA. This result was different with the report by Ramprasad *et al.*(2007) and Lindholm *et al*.(2006) in which they found that all of the possible genotypes of -374 T/A polymorphism, which were TT, TA, and AA genotypes.

The chi-square test result indicated that the frequency distribution of genotype and allele of -429 T/C polymorphism in RAGE gene was not statistically different between DR and NDR subjects. This study found that the carrier of TC or CC genotype had 1.52 times higher risk of DR than the carrier of TT genotype, while the carrier of C allele had a possibility of 1.36 times to have DR.

Previously, Santos *et al*. (2010) reported that in gestational diabetic patients from Euro-Brazilians ethnic, there was no significant difference on -429 T/C polymorphism in RAGE gene between case and control subjects (p = 0.054). Ramprasad *et al*. (2007) also reported that there was no association between -429 T/C polymorphism in RAGE gene with DR (p = 0.884; OR = 0.968; 95% CI = 0.63 – 1.49).

The chi-square analysis result of this study indicated that the frequency distribution of genotype and allele of -374 T/A polymorphism in RAGE gene was not statistically different between DR and NDR subjects. The carriers of TA genotype had 2.27 times higher risk of DR than the carriers of TT genotype, while the carriers of A allele had a possibility of 2.02 times to have DR.

Significant difference between nonproliferative diabetic retinopathy (NPDR) subjects with non DR subjects was reported in Indian population (p = 0.048; OR = 1.537; 95%CI = 0.885 – 2.672) (Ramprasad *et al*. 2007). However, a conflicting result was reported in Scandinavian population, in which there was no significant difference between DR and NDR subjects (OR = 1.16; 95% CI = 0.86 – 1.55) (Lindholm *et al*. 2006).

Ethnic difference is one of the factors affecting the difference of genotype frequency. The Multi-Ethnic Study of Atherosclerosis (MESA) reported that ethnic affected the risk of DR (Liew *et al*. 2009). In addition, the development of DR is also affected by different environmental factors in each population and also by other genetic factors (Rema *et al*. 2002).

This study suggested that -374 T/A polymorphism had more influence on the occurrence of DR than -429 T/C polymorphism. The result was supported by the fact that TA genotype of -374 T/A polymorphism had higher risks toward the occurrence of diabetic retinopathy than TC genotype of 429 T/C polymorphism (OR = 1.35; 95% CI = 0.37 – 4.93). Hudson *et al*. (2001) reported that the substitution of thymine by adenine in the position of -374

in the promoter of RAGE gene disturbed the binding of transcription factors (CTF/NF1) to the promoter, therefore the transcription of RAGE gene was affected. The disturbance of that binding was the increase of RAGE gene transcription to 2 – 4 folds, which results in the increase of RAGE expression.

The binding of AGE on its receptor causes the formation of free radical that increases the cellular oxidative stress and causes the damage of the retinal pericyte cells which could lead to the formation of microaneurysm (Ilyas, 2006). The increase of free radical caused by the interaction of AGE-RAGE also causes the increasing of NF-κB activity. NF-κB is a transcription factor which sensitive to free radical (Schmidt *et al*. 1994). One of proteins that undergoes increase transcription due to the AGE-RAGE interaction is endothelin-1 (Singh *et al*. 2001). Endothelin-1 which is produced by endothelial cells, is a vasoactive substance that plays role in vasoconstriction (Sherwood, 2001). The activation of RAGE also causes the decrease of NO level which plays role in the vasodilatation of local artery (Sherwood, 2001). This event causes the narrowing of vascular lumen which can cause retinal vascular occlusion.

The result of Hardy-Weinberg (H-W) equilibrium and statistical test on genotype of -429 T/C and -374 T/A polymorphisms in RAGE gene in Javanese population indicated that there was no significant difference between the genotype distributions in the studied population with H-W equilibrium. It can be interpreted that TT, TA, and AA genotypes of -374 T/A polymorphism as well as TT, TC, and CC genotypes of -429 T/C polymorphism in RAGE gene were at equilibrium or well distributed in Javanese population.

The chi-square analysis result indicated that the difference on the frequency of 2-SNP haplotype between DR and NDR subjects were statistically insignificant. This study found that all of the carriers of double heterozygote haplotype (TA-TC) were DR subjects. It is interesting to study the association between double heterozygote haplotype with the other risk factors, such as lipid profile and the duration of diabetes. This study reported that the lipid profile of DR subjects whom carried double heterozygote was normal. It can be assumed that this genetic variant did not affect triglyceride, total cholesterol, LDL cholesterol, and HDL cholesterol levels. However, this study found that 3 of 5 DR subjects whom carried double heterozygote had diabetes for only less than 6 years. Therefore, it indicated that DR occurred earlier in type 2 diabetic patients who carry double heterozygote. Further study on this matter is required, with a larger number of research subjects. Therefore, the risk factors that contribute to the development of DR in type 2 diabetic patients who carry double heterozygote could be identified.

Nowadays, DR treatment requires early detection and good blood glucose control to decelerate its development. However, those efforts are often failed due to the absence of symptoms in the early stage of DR. The treatment for DR, such as photocoagulation also can prevent the development of the disease (Ciula *et al*. 2003). In addition, the use of AGE inhibitor such as aminoguanidine and antioxidant compounds, for example vitamin C, can be the alternative treatment to decelerate the development of DR since those compounds can decrease the formation of AGE through its role in inhibiting polyol sorbitol pathway.

In Javanese ethnic, TA genotype of -374 T/A polymorphism was the stronger risk factor of DR than TC and CC genotypes of -429 T/C polymorphism. Type 2 diabetic patients whom carried double heterozygote of -439 T/C and -374 T/A polymorphisms in RAGE gene could developed DR earlier than type 2 diabetic patients whom did not carry double heterozygote of -439 T/C and -374 T/A polymorphisms.

Genotyping of -374 T/A polymorphism in RAGE gene is required as a complementary examination for type 2 diabetic patients. The carriers of high risk genotype and allele can be treated earlier by using compounds that

can decelerate the formation of AGE or by other alternative treatments. Consequently, the retinopathy can be controlled.

Acknowledgement

The authors would like to express deepest gratitude to Politeknik Kesehatan Kementerian Kesehatan Kupang as the sponsor, dr. Ahmad Hamim Sadewa, Ph.D., and Prof. Dr. Mustofa, M.Kes., Apt., for their suggestions, Dra. Tasmini, M.Kes. and dr. Maliyah, dr. Haryo Yudono, Sp.M., M.Sc., and dr. Elly, the technicians of Laboratory of Biochemistry, Faculty of Medicine, Universitas Gadjah Mada for their help, Jujuk A. Cahyono, Jems K.R. Maay, and Putri W. Welkriana for the assistance and cooperation during the research.

References

American Diabetes Association. 2007. Diagnosis and classification of diabetes mellitus. *Diabetes Care*, **30**(1), S42-S47.

Bierhaus, A., Hofmann, M.A., Ziegler, R., and Nawroth, P.P., 1998. AGEs and their interaction with AGE-receptors in vascular disease and diabetes mellitus. I. The AGE concept. *Cardiovas. Res.*, **37**(3), 586-600.

Ciulla, T.A., Armando, G.A., and Bernard, Z., 2003. Diabetic retinopathy and diabetic macular edema. *Diabetes Care*, **26**(9), 2653–2664.

Goldin, A., Beckman, J.A., Schmidt, A.M., and Creager, M.A., 2006. Advanced glycation endproducts: sparking the development of diabetic vascular injury. *Circulation*, **114**, 597-605.

Hudson, B.I., Stickland, M.H., Futers, T.S., and Grant, P.J., 2001. Effects of novel polymorphisms in the RAGE gene on transcriptional regulation and their association with diabetic retinopathy. *Diabetes*, **50**,1505–1511.

Ilyas, S. 2006. *Ikhtisar Ilmu Penyakit Mata*. Jakarta: Fakultas Kedokteran Universitas Indonesia.

Liew, G., Klein, R., Wong, T.Y., 2009. The role of genetics in susceptibility to diabetic retinopathy. *Int. Ophthalmol. Clin.*, **49**(2), 35–52.

Lindholm, E., Bakhtadze, E., Sjogren, M., Cilio, C.M., Agardh, E., Groop, C., and Agardh, D., 2006. The -374 T/A polymorphism in the gene encoding RAGE is associated with diabetic nephropathy and retinopathy in type 1 diabetic patients. *Diabetologia*, **49**, 2745-2755.

Ramprasad, S., Radha, V., Mathias, R.A., Majumder, P.P., Rao, M.R.S., and Rema, M., 2007. Rage gene promoter polymorphisms and diabetic retinopathy in a clinic-based population from South India. *Eye,* **21**, 395-401.

Rema, M., Saravanan, G., Deepa, R., and Mohan, V., 2002. Familial clustering of diabetic retinopathy in South Indian Type 2 diabetic patients. *Diabet. Med.*, **19**(11), 910-916.

Santos, I.C.R., Daga, D.R., Frigeri, H.R., Rea, R.R., Almeida, A.C.R., Souza, E.M., Pedrosa, FO., Fadel-Picheth, CMT., and Picheth, G., 2010. The functional polymorphisms -429 T>C and -374 T>A of the *RAGE* gene promoter are not associated with gestasional diabetes in Euro-Brazilians. *GMR,* **9**(2), 1130-1135

Schmidt, A.M., Hori, O., Brett, J., Yan, S.D., Wautier, J.L., and Stern, D., 1994. Cellular receptors for advanced glycation end products. Implications for induction of oxidant stress and cellular dysfunction in the pathogenesis of vascular lesions. *Arterioscler. Thromb.*, **14**, 1521-1528.

Sherwood, L. 2001. *Fisiologi Manusia dari Sel ke Sistem*. Alih bahasa Pendit, B.U., Ed. 2. Jakarta : EGC.

Singh, R., Barden, A., Mori, T., and Beilin, L., 2001. Advanced glycation end products: a review. *Diabetologia*, **44**,129–146.