Original article

Estrogen Receptor Alpha (Esr) Gene Polymorphism As Risk Factor For Type 2 Diabetes Mellitus (T2dm) In Javenese Menopause Women of Indonesia

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Abstract

Background: The number of menopausal women who suffer from low level estrogen-associated type-2 diabetes has been increasing recently. The role of estrogen in metabolism of glucose depends on estrogen receptor alpha expression that is regulated by estrogen receptor alpha gene (ESR ?). *PvuII* and *XbaI* polymorphisms in the ESR ? receptor may decrease the expression of ESR ? protein and receptor activity, thereby increasing the risk of developing type 2 diabetes mellitus in menopausal women. ***Purpose:*** to determine the *ESR ?* polymorphism as a risk factor for type 2 diabetes mellitus (DM) in menopause women of Javanese in Indonesia ***Methods:*** Sixty five menopausal women were recruited for the study consisted of 40 women with T2DM and 25 women as control. *PvuII and XbaI* polymorphisms were determined by polymerase chain reacton-restriction fragment length polymorphism (PCR-RFLP). The absence of PvuII and XbaI restriction sites were indicated by "P1" and "X1" and presence by "P2" and "X2", respectively. *Chai Square* test were used in statistical analyisis to measure *Hardy Weinberg Equilibrium* (HWE) and the risk of P1/P2 and X1/X2 allele for suffering T2DM. ***Results***: *PvuII* genotype was distributed as; 22.5% (P1P1), 45% (P1P2), 32.5% (P2P2) while *XbaI* genotype was distributed as 10 % (X1X1), 62.5% (X1X2) and 27.5% (X2X2) in diabetics respectively. There was no difference in distribution of P1 and P2 between diabetics and non diabetics but difference for X1 and X2 existed between groups. The frequency of P2 allele was 55 % while P1 allele frequency is 45% in diabetics. X2 allele frequency was 58.8% while X1 allele is 41.2%. X2 allele had an impact on the 3.6 times higher risk of getting type 2 diabetes in Javanese menopausal women (OR = 3.662, CI = 1.711 to 7.840) ***Conclusions:*** *PvuII* and *XbaI* polymorphism was found in Javanese menopause women of Indonesia in patients with type 2 diabetes mellitus. The allele frequency of P2 and X2 are 55% and 58.8% respectively. X2 allele was found as a risk factor for type 2 diabetes mellitus in Javanese menopause women of Indonesia.

Key Words: polymorphism, ESR, type 2 diabetes mellitus, Javanese menopause women

Introduction

Diabetes mellitus (DM) is a systemic disease characterized by an imbalance of energy metabolism, carbohydrate, lipid and protein which is mainly caused by inadequate insulin action both relative and absolute deficiency[1]. This disease affects patient lifetime and cannot be cured without the control of plasma glucose levels in the long run. In the long term DM damage organs such as kidneys, eyes, nervous system, heart, and if not treated increase the mortality rate[2].

The prevalence of DM for all age groups worldwide It is estimated that prevalence of T2DM increase from 2.8% by the year 2000 to 4.4% by the year 2030 in the world. At the age of postmenopausal, women will get suffering DM more

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than men[2]. Research has been conducted in Indonesia to prove an increase in the prevalence of diabetes mellitus. For example, research conducted in Jakarta (urban area) have showed 1.7% T2DM prevalence in 1982, and 5.7% in 1993. In 2001 the prevalence of DM in sub-urban areas in Jakarta had to 12.8%[3].

The cause of DM is combination of environmental and genetic factors impaired glucose homeostasis. In addition, insulin resistance is the main cause of metabolic syndrome characterized by hypertension, dyslipidemia, and visceral obesity which have become a worldwide health issues[4, 5]. Type 2 diabetes patients are generally older than 45 years old and have symptoms of obesity[6]. Obesity, diet, aging, lack of physical activity and urbanization is known as a major cause of increasing number of patient with DM[7].

According to Szmuilowicz *et al*. (2009) many postmenopausal women will suffer type 2 DM but still little information about how changes at menopause is typically affect the incidence of DM and may also greatly affect the DM management at the time of menopause and post menopause[8]. According to Otsuki *et al*. (2007) that non-DM menopausal women have elevated levels of fasting blood glucose than non DM women reproductive age[9]. The risk of menopause women will increase to suffer type 2 diabetes. It is estimated associated with a decrease in estrogen levels in postmenopausal women[8, 9].

Estrogen (E2) plays an important role in the pathogenesis of type 2 diabetes. Estrogen can prevent DM through different mechanisms, especially in reducing hyperglycemia and plasma insulin levels. Estrogen showed physiological effects primarily through estrogen receptor alpha (ESRα) and beta (β ESR) which are found in various tissues of the body. Estrogen interaction with its receptor will affect the metabolism of carbohydrates that participate in the pathogenesis of type 2 diabetes. Low estrogen increase vulnerability (susceptibility) a person to suffer type 2 diabetes through a variety of ways both men and women. Reference Several studies have shown that estrogen can inhibit the induction of IDDM or insulin dependent diabetes mellitus[7,10,11,12,26]. Estrogen can strengthen (modulate) the secretion of insulin, regulate K-ATP channel, and adjust the calcium signaling through the estrogen receptor. Estrogen can also stimulate fatty acid metabolism, suppress the production of glucose from the liver, protects pancreatic beta cells to continue to function and survive, increasing the expression of GLUT-4 and increased glucose uptake[1].

According to Dahlman *et al*. (2008) there is a relationship between ESR gene α polymorphism with type 2 diabetes disease and fasting glucose levels in the race of Caucasoid in Sweden and France[10]. The relationship between polymorphisms ESR α gene with type 2 diabetes have also been demonstrated in several countries such as in India[11], China[12], USA[13], and Iran[1]. From previous studies proved that the polymorphism in the ESR α gene associated with the incidence of breast cancer, postmenopausal osteoporosis, recurrent abortions, arterial hypertension, changes in serum lipid levels, cardiovasculer heart disease (CHD), and diabetes mellitus. Until now there are two types of well-known polymorphisms in the ESRα gene PvuII and XbaI of the most widely studied by researchers as a risk factor for many diseases including diabetes mellitus. PvuII polymorphism is caused by the transition C / T (P1/P2) in intron 1, located at 400 bp upstream of exon 2. *XbaI* polymorphism is caused by transition G / A (X1/X2) located at position 50 bp downstream of the *PvuII* polymorphism. In the presence of the polymorphism causes receptor expression and decreased receptor activity, thereby increasing the risk of type 2 diabetes.

Material and Methods

Subjects

Forty menopause women with type 2 diabetes mellitus as case groups and 25 non-diabetics as controls is drawn from Javanese people. Diabetic subjects were drawn from the endocrine clinic in Internal Medicine Specialist services and controls of Dr. Sardjito Hospital. The control subjects were drawn from residents in the village of Sumberharjo, Prambanan, Sleman, Yogyakarta and employees of the Faculty of Medicine of Islamic University of Indonesia, Jogjakarta. The research was conducted in 2010 with funding from the Faculty of Medicine Islamic University of Indonesia and Faculty of Medicine Gadjah Mada University.

Diagnosis of type 2 diabetes mellitus was based on

WHO 1999 criteria. Individuals with fasting blood glucose levels are equal to or greater than 126 mg/dl or blood glucose levels 2 hours post-prandial are equal to or greater than 200 mg/dl[14]. Group of individuals are selected as cases and controls with no history of taking HRT, do not suffer from liver failure and kidney failure and age over 45 years and under 60 years.

Biochemical Analysis with physical examination

Biochemical analysis or examination carried out on venous blood samples after at least 12 hours of fasting that includes an examination of total cholesterol, triglyceride, HDL and LDL levels using a commercial kit from *DiaSys* Germany. In addition to biochemical examinations are also performed a physical examination including blood pressure, the height, the weight and abdominal circumference.

DNA Isolation

Isolation of DNA carried out with guanidine isothiocyanate method[15]. DNA was isolated from peripheral blood leukocyte cells derived from the median cubity vein. Examination of the presence of PvuII and XbaI polymorphisms in the ESR alpha gene has been performed by PCR-RFLP. By using the forward primer 5'CTGCCACCC-TATCTGTATCTTTTCCTATTCTCC-3' and reverse primer 5'TCTTTCTCTGCCACCCTG-GCGTCGATTATCTGA3'[1]. PCR reaction was performed by using a Biometra PCR thermocycler and reagents intron. PCR reactions using the total volume of 30 μl PCR reaction consisted of 2 μl DNA, 15 μl PCR master mix containing 1 $\times$ PCR buffer, 150 mM dNTP and U Tag DNA polymerase, 2 μl primer and 1 μl of each forward and reverse, 11 μl distilled water. Temperature conditions of PCR cycles that initial denaturation for 5 min at 95^0 C, followed by 35 cycles of PCR with denaturation at 95^0 C 30 sec, annealing at 62^0 C 30 sec, extension at 72^0 C for 30 sec, 2-minute final extension and cooling at 72^0 to 4^0 C[12].

Polymorphism Analysis

PCR products have been digested by using restriction enzyme of *PvuII* dan *XbaI*. For each volume 8 μl of PCR products have incubated with 1 unit of *XbaI* for detecting polymorphism of X1/X2. Also for each 8 μl PCR product have incubated with 1 μl PvuII enzyme. Then we have added 1.5 μl of NE buffer 1x, 1.5 μl BSA10x, 3μl distilled water up to 15 μl for each reaction of PvuII and XbaI enzyme. Incubation have conducted for 8 hours at 60?C for both. To distinguish c.454-397 T > C (PvuI?) and c.454-351 A>G (XbaI) polymorphism, the amplified PCR fragment of 1372 bp was digested with restriction enzyme XbaI and PvuI? separately, followed by electrophoresis on 2% agarose gel. For PvuII, the mutated homozygous variant X2X2 (TT) produced two fragments 982 and 390 bp when heterozygote CT produced three fragments of, 1372 and 982 bp and 390. Wild-type X1X1(CC) produced one fragment of 1372 bp. For XbaI the mutated homozygous variant AA produced two fragments 936 and 436 bp when heterozygote AG produced three fragments of 1372, 936, and 436 bp and GG wild-type produced one fragment of 1372 bp[1].

Statistical analysis

Genotype distribution of polymorphism was tested for Hardy-Weinberg equilibrium by χ^2. Also analysis of risk of allele and haplotypes increasing to suffer T2 DM were conducted by χ^2 and logistic regression. A p-value less than 0.01 was considered significant. Statistical analysisi was performed with SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). and THESIAS 1.3 version[16].

Ethical Clearence

We have got ethical clearence from Bioethics Unt Faculty of Medicine Gadjah Mada University of Jogjakarta. We have done informed consent to all subjects that want to get involved in this study.

Result

Characteristics of the subjects are presented in table I. There are some variables that two groups differed significantly such as systole, diastole, height and TGA (p< 0, 01). Menopause women without DM have blood pressure higher than menopause women with DM in this research because almost all subject with DM consumed antihypertensive tablet a long with anti-diabetic treatment as consequences diabetic complication. Although the height of DM subjects is higher than non DM subjects but the BMI of both groups are normal and equal (p=0, 458).

Table I: Charaterictic of Subjects with DM and Non DM

Characteristics	Diabetic Women (n= 40)		Non diabetic Women (n=25)		P
	Mean ± SD	Median	Mean± SD	Median	
Age (years)	53.15±3.93	52.00	52.64±3.72	52.00	P= 0.605
Systole (mmHg)	113.75±5.86	110.00	127.20±20.47	120.00	P=0.001*
Diastole (mmHg)	73.75±4.90	70.00	80.60±10.64	80.00	P= 0.003*
Height (cm)	155.13±5.70	155.00	150.14±6.17	149.00	P= 0.001*
Weight (Kg)	56.53±8.57	56.00	51.50±9.69	48.00	P= 0.018*
BMI	22.85±2.97	22.00	22.81±3.87	21.49	P=0.458
Abdominal Circ. (cm)	87.43±9.88	87.00	82.68±10.35	82.00	P=0.069
Cholesterol (mg/dl)	180.18±45.69	189.00	171.64±22.91	164.10	P= 0.101*
LDL (mg/dl)	107.78±25.28	117.00	99.70±25.04	95.44	P= 0.124*
HDL (mg/dl)	64.88±26.50	59.50	52.66±16.95	51.00	P= 0.044
TGA (mg/dl)	136.88±67.24	126.00	96.39±56.94	77.00	P= 0.002*
F-GLU	163,98±64,37	160.00	83,66±13,96	80.00	P=0.000*
2 hours PP-GLU	199,78±87,23	190.00	110,94±30,48	105.00	P=0.000

** Mann Whitney test for variable without normal distribution (α=0, 01)*

Based on clinical criteria for the result of table 1 that two groups either diabetic woman and non diabetic woman still have normal condition for all characteristic. Although diabetic women and non diabetic women have not been normal distribution statistically, both groups have been normal clinically except for diabetic criterion. Then the analysis will be conducted on genetic polymorphism between diabetic women and non diabetic women and the result is shown in Table II and III.

Table II: Genotype and Allele PvuII (P1/P2) Distribution of diabetic and non diabetic women

Subject	Genotype Frequency			Allele PvuII		CI 95%
	(P1P1))	(P1P2)	(P2P2)	(P1)	(P2)	
Diabetic Women	9 (22, 5%)	18 (45%)	13 (32,5%)	44 (55%)	36 (45%)	OR 1,688 (0,827-3,446)
Non diabetic Women	4 (16%)	14 (56%)	4 (16%)	22 (44%)	28 (56%)	
Total	13	32	17	66	64	

For PvuII X^2 =3,497, P= 0,174

There were no significant difference in prevalence of PvuII genotype in either groups (X^2=3,497, P= 0,174). The frequency of PvuII polymorphisms in patient with diabetes compared with non-diabetes group is shown in table II and also polymorphism PvuII become as risk factor for Type 2 DM in Javanese Women post menopuase but not significance (OR=1.668, CI= 0.827-3.446). This result is different from XbaI polymorphism shown in Table III.

Table III: Genotype and Allel XbaI (X1/X2) distribution of diabetic and non diabetic women

Subject	Genotype Frequency			Allel Xbal		CI 95%
	(X1X1	(X2X1)	(X2X2)	(X2)	(X1)	
Diabetic Women	4 (10 %)	25 (62,5%)	11 (27,5%)	47 (58,8%)	33 (41,2%)	OR=3,662 (1,711-7,840)
Non diabetic Women	14(56 %)	8(32 %)	3(12 %)	14(28%)	36(72%)	
Total	18	33	14	61	69	

For XbaI X^2 = 14,077 P=0,001

The frequency of X2X2,, XiX2, and X1X1 genotypes were 27,5%, 62,5%, 10 % and also 12%, 32% and 56% in women with and without diabetes, respectively. The distribution of normal allele X1 and mutated allel X2 in diabetic and non diabetic groups were 41,3% and 58,8%, and 72% and 28 % respectively. The difference between the two groups was significanct (X^2= 14,077;P=0,001). By Chai Square analysis it was found that the XbaI variant was related to type 2 diabetes mellitus so that increased the risk become 4 times to suffer type 2 DM (OR=3,662; CI=1,711-7,840) in Table III.

Table.IV: Pairs of PvuII and XbaI Genotype on diabetic and non diabetic women

Genotype Pairs	Diabetics	Non diabetics
P1P1/X2X2	3 (7,5%)	2 (8%)
P1P1/X1X2	5 (12,5%)	2 (8%)
P1P1/X1X1	1 (2,5%)	3 (12%)
P1P2X2X2	4 (10%)	1 (4%)
P1P2X1X2	13(32,5%)	6 (24%)
P1P2X1X1	2 (5%)	7 (28%)
P2P2X2X2	4 (10%)	0 (0%)
P2P2X1X2	7 (17,5%)	0 (0%)
P2P2X1X1	1 (2,5%)	4 (16%)
Total	40	25

P=0,041, no significant

We have found no association between genotype combination PvuII/ XbaI and type 2 DM in Javaneses Women (P=0,045) after combining P2P2/X2X2, P2P2/X1X2 and P2P2/X1X1 become one category P2P2/NN and then analyze with SPSS 17.0 using contigency test. The prevalence of genotype pairs in diabetic subjects in the best three groups are P1P2/X1X2 (32.5%), P2P2/NN (29.5%), and P1P1/X1X2 (12.5%) whereas in non diabetic patients are P1P2X1X1(28%) , P1P2/X1X224%), P2P2/X1X1 (16%) shown in table 4 but the difference is not significant.

We observed the four possible PvuII-XbaI haplotype alleles in the following frequencies in diabetic subjects: haplotype 1 (P1-X1) 19,37%, haplotype 2 (P1-X2) 25.63%, haplotype 3 (P2-X1) 19.37% and haplotype 4 (P2-X2) 35.63% in 40 diabetic subjects. We have found distribution difference from 25 control subjects in following frequencies 38%, 20%, 34% and 8% respectively (Table 5). Genotype distributions were in Hardy–Weinberg equilibrium both control subjects and diabetic subjects after testing by THESIAS program.

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P1P1/X1X2	5 (12,5%)	2 (8%)
P1P1/X1X1	1 (2,5%)	3 (12%)
P1P2X2X2	4 (10%)	1 (4%)
P1P2X1X2	13(32,5%)	6 (24%)
P1P2X1X1	2 (5%)	7 (28%)
P2P2X2X2	4 (10%)	0 (0%)
P2P2X1X2	7 (17,5%)	0 (0%)
P2P2X1X1	1 (2,5%)	4 (16%)
Total	40	25

P=0,041, no significant

*Chi Square *haploptype P1X2, **haploptype P2X1 not significance, ***haploptype P2X2*
Subject with P2X2 and P1X2 haploptype will increase the risk to suffer DM type 2 more than P1X1 as normal haploptype (OR 8.7.4 & 2.51).

Discussion

Diabetes mellitus is a group of metabolic diseases of multiple etiologies characterized by hyperglycemia together with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both[17]. Type 2 diabetes mellitus results from the interaction of environmental factors with a combination of genetic variants, most of which were hitherto unknown[18]. A systematic search for these variants was recently developed by some researcher to ascertain the genetic factor. Several risk factors that related to type 2 diabetes are a family history of diabetes, history of gestasional diabetes, obesity, impaired glucose tolerance, sedentary life or physical inactivity, age more than 45 years old, moderate alcohol consumption, smoking, low fiber diet, high LDL, low HDL, menopause for woman, hypertension, eating habits, ethnic, and polycistic ovary syndrome[19,20,21,22].

Identification of the susceptibility genes for type 2 DM thus may lead to primary prevention of the disease1. The most patients type 2 DM have affected by genetic factor contributing a partial and additive effect. The inheritance pattern is thus complex, and environmental factor play an important role in favoring or delaying the expression of the disease[11].

Sex steroid clearly have an impact on insulin resistance risk. Recent data have revealed a surprising role of estrogen in regulating energy metobolis, which opened new insight into the role of the two estrogen receptors[23]. Therefore, estrogen receptors seem to play a role in the prevention or in the occurence of diabetes type 2 [24]. ESR ? polymorphism have attracted great interest in the last few years and the PvuII and XbaI are the most extensively investigated issue[11].

We report for the first time no significant difference in the PvuII polymorphism in Javanese population menopausal women. There are few studies showing the significant association of ESR ? gene PvuII polymorphism in the Chinese[12], African-American and European-American[25]. We have observed association between XbaI polymorphism and diabetes significantly. Our findings become the new discovery for association between XbaI polymorphism and diabetes in Javanese population menopausal women. The risk XbaI polymorphism to get diabetes will increase 3.6 times and haplotype P2X2 had greater risk compared to others. Our finding for XbaI polymorphism correlated with the African-American study related to metabolic syndrome[26]. The crucial difference of our finding with other studies are A-allele (X2) become mayor allel whereas in India population, Iran population, African-American and Chinese population become minor allele. A-allele as mayor allele in the first our study correlated with European-American population[13, 26].

How do these specific polymorphisms influence ESR1 gene expression and consequently suffer diabetes mellitus type 2 ?. The PvuII and XbaI polymorphisms have been an important area of research in diseases such as osteoporosis, cardiovascular disease, cancer, demensia and diabetes mellitus[1,11,12,27] because of estrogen receptor? pleiotrophic effect . A number of hypotheses for the functional significance of these polymorphisms have been reported in the literature. Given their location, 397 and 351 bp upstream from the start of exon 2, possible functional mechanisms include changed ESR1 expression via altered binding of transcription factors and influence on alternative splicing of the ESR1 gene. Both these mechanisms can be a direct result of either of these polymorphisms or through linkage disequilibrium with a truly functional, but so far unknown, sequence variation elsewhere in the ESR α gene[27]. The first mechanism was recently supported by findings of Herrington et al.[28] and was confirmed by Schuit et al. Herrington *et al*. showed that the T-allele of the *PvuII* Restriction Fragment Length Polymorphism (RFLP) eliminates a functional binding site for the transcription factor B-myb. This implies that the presence of this allele may result in lower ESR α transcription. The present study reports that the XbaI A-allele, represented in haplotype P2X2, is associated with increased risk of diabetes mellitus in Javanese menopausal women. This suggests that the potentially lower ESR1 expression caused by the *PvuII* P2-allele leads to a lower expression of an enzyme in the estrogen synthesis pathway, such as 17b-HSD, and, subsequently, reduced E2 synthesis. These findings are further supported by the observation in our study population, as well as in

others, that this T-allele of the PvuII polymorphism is associated with increased risk of osteoporosis and myocardial infarction, decreased risk of osteoarthritis and hysterectomy, lower BMI, shorter stature, and later age at menopause[27]. These phenotypes are known to be related to decreased E2 effects. The fact that the XbaI polymorphism A-allele is also associated with E2 levels may be due to linkage disequilibrium with the PvuII SNP or another functional polymorphism, or to functional significance of the *XbaI* polymorphism itself[24]. In addition, Estrogen receptor? has pleiotropic influences, including effects on reproductive fitness, so these uncommon alleles may have been retained in the population because of positive selective pressure unrelated to diabetes[11].

Although rs2234693 (PvuII) allele C (P1) has been associated with lower BMI, waist circumference and lower small LDL concentration, this allele in our finding was associated with no increased risk for suffering DM type 2. According to Gallagher et al. C-allele has been associated with reduced insulin sensitivity index or increased insulin resistency[24] but in this present study no association with type 2 DM.homozygote P2P2 have increased insulin sensitivity (Si). An influence of ESR α on Si is consistent with the finding that mice with a non functional ESR α gene and the only human male identified without a functional copy of ESRα exhibit insulin resistency[26]. The rs9340799 (*Xba*I) minor allele G has been associated with reduced waist circumference, LDL cholesterol, and apolippoprotein B but showed an additive risk for metabolic syndrome in the IRAS Family Study families in England. The modest associations observed could be the result of type I error. Alternatively, these alleles may have differential effects on these traits or be in LD with a functional SNP present on different haplotypic backgrounds[29]. This first finding in Javanese population with A allele as mayor allele is consistent with Gallagher et al. studies in African-American population as additive risk factor of metabolic syndrome (1,5 odds ratio 95% CI 1.05-2.27)[26].

The present findings are limited in the way that they were obtained from a relatively small study population and our significant findings of *XbaI* polymorphism may be caused by the poor statistical power. The result of our study should be considered exploratory and confirmed by additional studies, which include larger sample size and other polymorphism in estrogen receptor gene. This will help to identify the population with genetic predisposition and to protect the from exposure to enviromental risk.

This is the first Indonesian study in Javanese population to show that ESR ? polymorphism especially *XbaI* polymorphism is associated with increased suspectibility of Javanese menopausal women to type 2 DM. Investigation of these polymorphisms in other ethnic groups and comparing premenopausal with postmenopausal women are recommended. The molecular mechanism of type 2 diabetes pathogenesis mediated by PvuII and XbaI polymorphism should also be elucidated in experimental animals[1].

Conclusion

In conclusion, we have able to find association between variations in the ESRα gene and risk type 2 diabetes mellitus especially for XbaI polymorphism. A-allele in the first our study become mayor allele and increased risk 4 time to suffer type 2 DM whereas PvuII polymorphism did not associate with type 2 DM in Javanese menopausal women.

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