Association of Estrogen Receptor- α Gene & Metallothionein-1 Gene Polymorphisms in Type 2 Diabetic Women of Andhra Pradesh

Shilpa Reddy Ganasyam · Talluri Bhaskar Rao ·
Y. S. R. Murthy · Akka Jyothy · Madireddy Sujatha

Received: 25 May 2011 / Accepted: 10 November 2011 / Published online: 6 January 2012
© Association of Clinical Biochemists of India 2011

Abstract Type 2 diabetes mellitus (DM) is a multifactorial disease where both genetic and environmental factors contribute to its pathogenesis. Estrogen plays an important role in type 2 DM pathogenesis. A number of polymorphisms have been reported in the estrogen receptor (ESR1), including the *Xba*I and *Pvu*II restriction enzyme polymorphisms of ESR1, which may be involved in disease pathogenesis. Metallothioneins (MT) act as potent antioxidants against various oxidative damages. Very few studies have indicated the association between Estrogen Receptor- α , MT1 gene polymorphisms with type 2 DM. A total of 100 type 2 diabetic women and 100 age, sex matched controls were recruited. Using the PCR based RFLP method, the *Pvu*II and *Xba*I polymorphisms of ESR1 and in MT1A (*rs8052394* and *rs11076161*) gene polymorphisms were analysed. The genotype distribution and frequency of mutated allele showed no significant differences between diabetic and non-diabetic groups in *Pvu*II ($\chi^2 = 2.443$; $P = 0.1181$) or *Xba*I ($\chi^2 = 1.789$; $P = 0.1812$) and *rs8052394* ($\chi^2 = 1.154$; $P = 0.2840$) or *rs11076161* ($\chi^2 = 0.4141$; $P = 0.5199$), polymorphisms. This is the first Indian study to conclude that ESR1 and MT1 gene

polymorphisms are not associated with increased susceptibility to type 2 diabetes in Indian women.

Keywords Type 2 diabetes mellitus (T2DM) · Estrogen receptor1 (ESR1) · Metallothionein (MT) · Polymerase chain reaction (PCR) · Restriction fragment length polymorphism (RFLP) · Low density lipoprotein (LDL) · High density lipoprotein (HDL) · Very low density lipoprotein (VLDL) · Triglycerides (TG)

Introduction

India leads the world with largest number of diabetic subjects earning the dubious distinction of being termed the “diabetes capital of the world”. According to the Diabetes Atlas 2006 published by the International Diabetes Federation, the number of people with diabetes in India, currently around 40.9 million is expected to rise to 69.9 million by 2025 unless urgent preventive steps are taken [1, 2]. Type 2 diabetes mellitus (DM) is a multifactorial disease where both genetic and environmental factors contribute to its pathogenesis. Therefore, it is helpful to identify the population with genetic predisposition and to protect them from exposure to environmental risks.

Estrogens exert its physiological effects mainly through the estrogen receptors (ERs), ER α and ER β , and might prevent menopause syndrome, osteoporosis, arteriosclerosis, and diabetes, etc. ER is found in many tissues, including the pancreatic islets and skeletal muscle, adipose tissue, and cardiovascular system, which all participate in the pathogenesis of type 2 diabetes. Epidemiological findings have indicated that the prevalence of type 2 DM is significantly lower in patients on estrogen replacement therapy, and estrogen seems useful for the treatment of diabetes [10, 17]. Type 2 DM is a

S. R. Ganasyam (✉)
Department of Biochemistry, Institute of Genetics and Hospital
for Genetic Diseases, Osmania University, Begumpet,
Hyderabad 500016, Andhra Pradesh, India
e-mail: shilpadishi@rediffmail.com

T. B. Rao · Y. S. R. Murthy · A. Jyothy
Institute of Genetics and Hospital for Genetic Diseases,
Begumpet, Hyderabad 500016, Andhra Pradesh, India

M. Sujatha
Department of Clinical Genetics, Institute of Genetics &
Hospital for Genetic Diseases, Hyderabad, India

heterogeneous disorder caused by a combination of genetic and acquired abnormalities that affect insulin sensitivity and insulin secretion.

Estrogen has been implicated in adipose tissue biology [18], stimulation of liver fatty acid metabolism, suppression of hepatic glucose production, reduction of both hyperglycemia and plasma insulin levels. It also helps in protection of pancreatic β -cell function/survival and insulin secretion in conditions of oxidative stress, and the blunting of neuroendocrine and metabolic responses to hypoglycemia. Estrogen modulates insulin secretion, regulates K-ATP channel activity, and regulates calcium signals via plasma membrane receptors [3, 4]. Thus, the gene encoding ER α gene (*ESR1*, gene map locus 6q25.1) is a potential candidate gene for susceptibility to type 2 diabetes. A number of polymorphisms in *ESR1* have been reported. The *PvuII* and *XbaI* restriction fragment length polymorphisms (RFLPs) are two commonly employed markers for genetic analyses of the *ESR1*; both are located in intron 1 of the *ESR1*. Association between *ESR1* and disease has been demonstrated in several cases. An association has been found between the T allele (an allele with a T/C transition) and the absent expressed *ESR1* gene in breast.

Cancer cell and breast tumor tissue. *PvuII* polymorphism may affect the splicing of ER mRNA, resulting in the alteration of protein expression. *XbaI* and *PvuII* RFLPs of *ESR1* may play a role in the pathogenesis of such diseases as breast and prostate cancer, osteoporosis, Alzheimer's disease, cardiovascular diseases, and stroke.

MTs are a family of proteins with low molecular mass and high affinity to certain metal ions. MTs exist in multiple organs with several molecular forms, including isoforms of MT1, MT2, MT3, and MT4 [19, 20]. A multigene family with at least 14 closely related and pseudo genes encodes MT proteins, and the most MT genes, including the functional MT genes (*MT1A*, *MT1B*, *MT2A*), lie on human chromosome 16 [7]. MTs can function on the regulation of zinc and copper homeostasis and also act as potent antioxidants against various oxidative damages. Major cause of diabetes and diabetic complications is related to the induction of oxidative stress, and others have demonstrated protection by increased MT expression in the pancreas [21, 22] and in the heart against initiation of diabetes and diabetic cardiomyopathy, respectively. Furthermore, MT was found also to play a critical role in energy metabolism [23, 24].

Genomic scanning has suggested chromosomal regions possibly containing T2DM susceptibility genes. The chromosomal regions in *ESR1* is 6q 25.1 [3–5] and *MT1* is located on chromosome 16q22 [6–8]. Therefore, any changes in *ESR1* or *MT1* genes may be associated with T2DM predisposition. We investigated whether type 2 DM is associated with *ESR1* or *MT1* gene polymorphisms in Andhra Pradesh women.

The prevalence of type 2 DM and associated traits such as obesity, dyslipidemias, and hypertension in the overall population has become a worldwide challenge for health care systems. Estrogen has beneficial effects on lipids and lipoproteins metabolism [9–11]. In order to find if the *ESR1* variant relates to serum lipid level, which may contribute to the mechanism causing type 2 diabetes, the relationship between the gene polymorphism and serum lipid level was also investigated.

Materials and Methods

Study Design

The participants were selected who were Hyderabad residents, aged 30–70 years and had no significant liver damage or renal dysfunction. Patients and control subjects had no history of a sex-hormone-dependent disease and never received hormone replacement therapy. Informed written consent was obtained from each individual.

A total of 100 T2DM female patients and 100 age and sex matched controls of Indian population were recruited for the genetic analysis at the Institute of Genetics and Hospital for Genetic Diseases (Hyderabad, Andhra Pradesh, India). They were diagnosed as T2DM based on the World Health Organization 1999 guideline that was adopted from the American Diabetes Association 1997 standard.

Biochemical Analyses

Blood samples were collected from the patients after a 12-h fasting. Total cholesterol, HDL cholesterol, and triglyceride levels were determined by standard methods using commercial kits from ERBA (Transasia Biomedical LTD, India).

Genotyping

PCR–RFLP assays were used to determine the *ESR1* and *MT1* genotypes. The oligonucleotide primers used to determine the *PvuII* and *XbaI* polymorphisms within the *ESR1* gene were the same as those used by Yaich et al (forward, 5'-TGCCACCCTATCTGTATCTTTTCCTATTCTCC-3'; reverse, 5'-TCTTTCTCTGCCACCCTGGCGTC-GATTATCTGA-3'). Genomic DNA used for PCR amplification was extracted from the whole blood sample using a DNA extraction kit (Hi media, India). The PCR product of approximately 1.3 kb was completely digested with restriction endonuclease *PvuII* or *XbaI* for 8 h, and electrophoresed in a 1.5% agarose gel. The *PvuII* polymorphism, which is caused by a T/C transition in intron 1, is located approximately 0.4 kb upstream of exon 2. The *XbaI* polymorphism, which is caused by an A/G transition in

intron 1, is approximately 50 bp away from the *PvuII* polymorphism site [12].

The oligonucleotide primers used to determine the *rs8052394* and *rs11076161* polymorphisms within the MT1 gene were *rs8052394* (forward: 5-ACTAAGTGTCC TCTGGGGCTG, reverse: 5-AATGGGTCACGGTTGTAT GG 3) and *rs11076161* (Forward: 5-TTCGGGATTAAGG ACATAAAGC-3, reverse: 5-GAAATGGATCATTGGC CTAATC-3). The PCR amplification was performed in a 25- μ l reaction mixture with each primer, for which the conditions included an initial denaturation at 94°C for 5 min, followed by 35–40 cycles of 94°C for 45 s, 55–60°C for 1 min and 72°C for 1 min, and a final elongation at 72°C for 10 min. A 15- μ l aliquot of the PCR products was completely digested with 7–10 units of restriction enzymes (Table 1).

Statistical Analysis

All quantitative variables were presented as mean $\pm$ standard deviation while categorical variables were summarized by absolute frequencies and percentages. Allele frequencies were calculated for each genotype by allele counting. Continuous variables were compared using independent two-sample *t* test, and categorical variables were compared using Chi-square test.

Results

A total of one hundred patients with diabetes and 100 controls without diabetes were genotyped for the four common polymorphisms *PvuII*, *XbaI* of ESR1 gene and *rs8052394*, *rs11076161* of MT1 gene. All participants were aged 35–86 years. The mean age of the patients with

diabetes and controls were 44.6 ± 9.89 years and 40.4 ± 6.19 years ($P = 0.0798$), respectively.

Genotyping Analysis for the Association with T2DM

All four polymorphisms were detected in the study. The frequencies of polymorphisms in patients with diabetes compared with non-diabetic group is shown in Table 2. The blood LDL, HDL, total cholesterol, and triglyceride levels of the participants were measured to determine their relations with type 2 diabetes. Lipid profile of the participants are summarized in Table 3.

Discussion

Type 2 DM is thought to be a multifactorial disease and both genetic and acquired factors contribute to its pathogenesis. Identification of the susceptibility genes for type 2 DM thus may lead to primary prevention of the disease. In most patients, type 2 DM results from genetic changes, each has a partial and additive effect. The inheritance pattern is thus complex, and environmental factors play an important role in favoring or delaying the expression of the disease.

Sex steroids clearly have an impact on insulin resistance risk. Recent data have revealed a surprising role of estradiol in regulating energy metabolism, which opened new insights into the role of the two estrogen receptors. Therefore, estrogen receptors seem to play a role in the prevention or in the occurrence of diabetes type 2 [25]. *ER α* polymorphisms have attracted great interest in the last few years and the *PvuII* and *XbaI* are the most extensively investigated issues. The abnormal expression of MT may cause uncontrolled oxidative stress and inflammatory responses and may accelerate the development of diabetic complications.

We report for the first time no significant difference in the ESR1 and MT1 gene polymorphisms in Indian population. There are few studies showing the significant association of ESR1 gene (*PvuII*) polymorphisms in the Chinese [13], African-Americans and European-Americans [14]. We did not observe any association between *PvuII* polymorphism and diabetes or significant relation between *XbaI* polymorphism and diabetes. Our findings correlated with the Iranian study [15].

Study from Chinese people of Han (397 T2DM and 454 controls) supports that there was a significant Association of MT1A gene (*rs8052394*) polymorphism with T2DM patients [16] but no association with MT1B gene *rs11076161* [15]. In our study no significant association of the MT1A (*rs8052394*) gene and MT1B (*rs11076161*) gene

Table 1 The primer sequence for PCR–RFLP in a Indian population

Gene	RFLP	Primer sequence
ESR1	<i>PvuII</i> , <i>XbaI</i>	Forward, 5'-CTG CCACCC TAT CTG TAT CTT TTC CTA TTC TCC-3
		Reverse, 5'-TCT TTC TCT GCC ACC CTGGCG TCG ATT ATC TGA-3'
MT1 A	<i>rs8052394</i>	Forward: 5-ACTAAGTGTCTCTGGG GCTG 3
		Reverse: 5-AATGGGTCACGGTT GTATGG 3
MT1 B	<i>rs11076161</i>	Forward: 5-TTCGGGATTAAGGACATAAAGC-3
		Reverse: 5-GAAATGGATCATTGGCCTACTC-3

Table 2 Genotype distribution and allele frequencies of *PvuII*, *XbaI* and *rs8052394*, *rs11076161* polymorphisms in type 2 diabetic patients ($n = 100$) and control subjects (100)

ESR1						
<i>PvuII</i>						
Subjects	TT (%)	CT (%)	CC (%)	T	C	OR (95% CI)
Patients	39	43	18	121 (63.6%)	79 (41.5%)	1.387
Controls	52	32	16	136 (68%)	64 (32%)	
For <i>PvuII</i> $\chi^2 = 2.443$; $P = 0.1181$, heterozygous T/C, homozygous mutant C/C, normal T/T						
<i>XbaI</i>						
Subjects	GG (%)	AG (%)	AA (%)	G	A	OR (95% CI)
Patients	75	20	5	170 (85%)	30 (15%)	1.492
Controls	86	10	4	186 (89.4%)	22 (10.5%)	
For <i>XbaI</i> $\chi^2 = 1.789$; $P = 0.1812$, heterozygous G/A, homozygous mutant A/A, normal G/G						
MT1A						
<i>rs8052394</i>						
Subjects (MT1A)	GG (%)	GA (%)	AA (%)	A	G	OR (95% CI)
Patients	86	8	6	20 (10%)	180 (90%)	1.476
Controls	90	6	4	14 (7%)	186 (93%)	
For MT1A (Pst1) $\chi^2 = 1.154$; $P = 0.2840$, heterozygous G/A, homozygous, mutant A/A, normal G/G						
<i>rs11076161</i>						
Subjects (MT1B)	AA (%)	AG (%)	GG (%)	G	A	OR (95% CI)
Patients	90	7	3	13 (6.5%)	187 (93.5%)	1.321
Controls	92	6	2	10 (5%)	190 (95%)	
For MT1B (Tsp5091) $\chi^2 = 0.4141$; $P = 0.5199$, heterozygous A/G, homozygous, mutant G/G, normal A/A						

Table 3 Lipid profile of the study subjects

Characteristics	Women with diabetes ($n = 100$)		Women without diabetes ($n = 100$)		P value
	Mean $\pm$ SD	Median	Mean $\pm$ SD	Median	
Age	44.6 $\pm$ 9.89	41	40.4 $\pm$ 6.19	40	0.0798
GLU	127.7 $\pm$ 61.74	113.65	110.85 $\pm$ 71.83	82	0.5178
CH	165 $\pm$ 29.42	162.9	174 $\pm$ 20.21	171	0.6824
TG	259.7 $\pm$ 2 82.6	185.22	186.5 $\pm$ 108.73	150	0.7211
HDL	37.35 $\pm$ 20.47	31.92	30 $\pm$ 1 2.21	25	0.7164
LDL	82.79 $\pm$ 33.5	96.8	106 $\pm$ 25.65	113	0.8825
VLDL	59.95 $\pm$ 56.55	37	37.05 $\pm$ 21.72	30	0.5318

TC total cholesterol; TG triglycerides; LDL-c low-density lipoprotein cholesterol; HDL-c high-density lipoprotein cholesterol

polymorphisms was observed in Indian women of type 2 DM.

We also demonstrated that ESR1 and MT1 gene polymorphisms are not significantly associated with the lipid levels, since the patients were on treatment. The present findings are limited in the way that they were obtained

from a relatively small study population and our non-significant findings may be caused by the poor statistical power. The results of our study should be considered exploratory and confirmed by additional studies, which include larger sample size and other polymorphisms in estrogen receptor and MT genes. This will help to identify

the population with genetic predisposition and to protect them from exposure to environmental risks.

This is the first Indian study to show that ESR1 and MT1 gene polymorphisms are not associated with increased susceptibility of Indian women to type 2 DM.

References

1. Mohan V, Sandeep S, Deepa R, Shah B, Varghese C. Epidemiology of type 2 diabetes: Indian scenario. *Indian J Med Res.* 2007;125:217–30.
2. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care.* 2004;27:1047–53.
3. Nadal A, Rovira JM, Laribi O, Leon-quinto T, Andreu E, Ripoll C, et al. Rapid insulinotropic effect of 17beta-estradiol via a plasma membrane receptor. *FASEB J.* 1998;12:1341–8.
4. Song RX, Barnes CJ, Zhang Z, Bao Y, Kumar R, Santen RJ. The role of Shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor alpha to the plasma membrane. *Proc Natl Acad Sci USA.* 2004;101:2076–81.
5. Lu H, Higashikata T, Inazu A, et al. Association of estrogen receptor-alpha gene polymorphisms with coronary artery disease in patients with familial hypercholesterolemia. *Arterioscler Thromb Vasc Biol.* 2002;22:817–23.
6. Doria A, Caldwell JS, Ji L, Reynet C, Rich SS, Weremowicz S, Morton CC, Warram JH, Kahn CR, Krolewski AS. Trinucleotide repeats at the rad locus. Allele distributions in NIDDM and mapping to a 3-cM region on chromosome 16q. *Diabetes.* 1995;44:243–7.
7. Le Beau MM, Diaz MO, Karin M, Rowley JD. Metallothionein gene cluster is split by chromosome 16 rearrangements in myelomonocytic leukaemia. *Nature.* 1985;313:709–11.
8. Reynet C, Kahn CR. Rad: a member of the Ras family overexpressed in muscle of type II diabetic humans. *Science.* 1993;262:1441–4.
9. Lundholm L, Moverare S, Steffensen KR, Nilsson M, Otsuki M, Ohlsson C, et al. Gene expression profiling identifies liver X receptor alpha as an estrogen-regulated gene in mouse adipose tissue. *J Mol Endocrinol.* 2004;32:879–92.
10. Knopp RH, Zhu X. Multiple beneficial effects of estrogen on lipoprotein metabolism. *J Clin Endocrinol Metab.* 1997;82:3952–4.
11. Di Croce L, Bruscalupi G, Trentalance A. Independent behavior of rat liver LDL receptor and HMGCoA reductase under estrogen treatment. *Biochem Biophys Res Commun.* 1996;224:345–50.
12. Yaich L, Dupont WD, Cavener DR, Parl FF. Analysis of the PvuII restriction fragment-length polymorphism and exon structure of the estrogen receptor gene in breast cancer and peripheral blood. *Cancer Res.* 1992;52:77–83.
13. Huang Q, Wang TH, Lu WS, et al. Estrogen receptor alpha gene polymorphism associated with type 2 diabetes mellitus and the serum lipid concentration in Chinese women in Guangzhou. *Chin Med J (Engl).* 2006;119:1794–801.
14. Sale MM, Freedman BI, Langefeld CD, Williams AH, Hicks PJ, Colicigno CJ, et al. A genome-wide scan for type 2 diabetes in African-American families reveals evidence for a locus on chromosome 6q. *Diabetes.* 2004;53:830–7.
15. Golkhu Sh, Ghaedi M, Mohammad Taghvaei N, Boroumand MA, Davoodi GhR, Aminzadegan AR, Poorgoli L, Sheikh Fathollahi M. Genetic polymorphisms of estrogen receptors in Iranian women with diabetes and coronary artery disease. *Iran J Med Sci.* 2009;34(3):208–12.
16. Yang L, Hongyan L, Ting Y, Zhao H, George Cherian M, Cai L, Liu Y. Polymorphisms in metallothionein-1 and -2 genes associated with the risk of type 2 diabetes mellitus and its complications. *Am J Physiol Endocrinol Metab.* 2008;294:E987–92.
17. Brussaard HE, Gevers Leuven JA, Frolich M, Kluft C, Krans HM. Short-term oestrogen replacement therapy improves insulin resistance, lipids and fibrinolysis in postmenopausal women with NIDDM. *Diabetologia.* 1997;40:843–9.
18. Speer G, Cseh K, Winkler G, Vargha P, Braun E, Takacs I, et al. Vitamin D and estrogen receptor gene polymorphisms in type 2 diabetes mellitus and in android type obesity. *Eur J Endocrinol.* 2001;144:385–9.
19. Cai L. Diabetic cardiomyopathy and its prevention by metallothionein: experimental evidence, possible mechanisms and clinical implications. *Curr Med Chem.* 2007;14:2193–203.
20. Li X, Cai L, Feng W. Diabetes and metallothionein. *Mini Rev Med Chem.* 2007;7:761–8.
21. Chen H, Carlson EC, Pellet L, Moritz JT, Epstein PN. Overexpression of metallothionein in pancreatic beta-cells reduces streptozotocin-induced DNA damage and diabetes. *Diabetes.* 2001;50:2040–6.
22. Yang J, Cherian MG. Protective effects of metallothionein on streptozotocin-induced diabetes in rats. *Life Sci.* 1994;55:43–51.
23. Beattie JH, Wood AM, Newman AM, Bremner I, Choo KH, Michalska AE, Duncan JS, Trayhurn P. Obesity and hyperleptinemia in metallothionein (-I and -II) null mice. *Proc Natl Acad Sci USA.* 1998;95:358–63.
24. Miura N, Koizumi S. Gene expression profiles in the liver and kidney of metallothionein-null mice. *Biochem Biophys Res Commun.* 2005;332:949–55.
25. Xiang K, Wang Y, Zheng T, Jia W, Li J, Chen L, et al. Genome-wide search for type 2 diabetes/Impaired glucose homeostasis susceptibility genes in the Chinese (significant linkage to chromosome 6q21–q23 and chromosome 1q21–q24). *Diabetes.* 2004;53:228–34.