Study of Sex Hormone-binding Globulin Gene Polymorphism and Risk of Type 2 Diabetes Mellitus in Egyptian Men

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ABSTRACT

Objectives: *Conditions of hypoandrogenism in men have been linked to insulin resistance, suggesting that alterations in normal sex steroid physiology could play a role in the pathogenesis of Type 2 diabetes mellitus (T2DM). Sex hormone-binding globulin (SHBG) gene polymorphisms may be the cause of sex steroid alteration. The aim of this work is to study the effect of SHBG gene polymorphisms on the risk of T2DM through its impact on testosterone and oestradiol level in Egyptian men.*

Subjects and Methods: *A case control study was performed in the diabetes clinic at Zagazig University Hospital on 185 males with Type 2 diabetes and their matched healthy controls. Two polymorphisms (rs6257 and rs6259) of the gene encoding SHBG were genotyped and serum levels of SHGB testosterone and oestradiol were measured by enzyme-linked immunosorbent assay (ELISA).*

Results: *Carriers of rs6257 variant allele (CC or CT) and carriers of rs6259 wild allele (GG) appear to have a high risk of diabetes than carriers of other alleles (OR 2.241, 1.585 and 2.391, respectively). They also showed a significant decrease in plasma level of both SHBG and testosterone and a significant increase in oestradiol blood level compared with carriers of other alleles.*

Conclusions: *Sex hormone-binding globulin gene polymorphisms at position rs6257 and rs6259 are associated with higher risk of T2DM in Egyptian men, through lowering circulating levels of SHGB and consequently, lowering testosterone and elevating oestradiol level.*

Keywords: Diabetes mellitus, Egyptian men, gene polymorphism, oestradiol, sex hormone-binding globulin, testosterone

El Estudio del Polimorfismo del Gen de la Globulina Fijadora de Hormonas Sexuales y el Riesgo de Diabetes Mellitus Tipo 2 en Hombres Egipcios

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RESUMEN

Objetivos: *Las condiciones de hipoandrogenismo en los hombres se han asociado con la resistencia a la insulina, lo que sugiere que las alteraciones en la fisiología normal de los esteroides sexuales podrían desempeñar un papel en la patogénesis de la diabetes de tipo 2 (T2DM). Los polimorfismos del gen de la globulina fijadora de las hormonas sexuales (SHBG) pueden ser la causa de la alteración de los esteroides sexuales. El objetivo de este trabajo es estudiar el efecto de los polimorfismos del gen SHBG en el riesgo de la diabetes mellitus tipo 2 a través de su impacto en los niveles de testosterona y estradiol de los hombres egipcios.*

Resultados: *Los portadores del alelo variante rs6257 (CC ó CT) y los portadores del alelo salvaje rs6259 (GG) parecen tener un riesgo más alto de diabetes que los portadores de otros alelos (OR. 2.241, 1.585 y 2.391, respectivamente). También mostraron una disminución significativa en el nivel de plasma tanto de SHBG como de testosterona, así como un aumento significativo en el nivel de estradiol en sangre, en comparación con otros alelos.*

Conclusiones: *Los polimorfismos del gen de la globulina fijadora de las hormonas sexuales en las posiciones rs6257 y rs6259 se hallan asociados con un mayor riesgo de diabetes mellitus tipo 2 en los*

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hombres egipcios, mediante la reducción de los niveles circulantes de globulina fijadora de hormonas sexuales y, por consiguiente, la disminución de la testosterona y la elevación de los niveles de estradiol.

Palabras claves: Diabetes mellitus, hombres egipcios, estradiol, polimorfismo del gen, SHBG, testosterona

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INTRODUCTION

Type 2 diabetes mellitus is a chronic metabolic disorder characterized by high blood glucose level in the context of insulin resistance and impaired pancreatic β-cell function (1). It is typically a chronic disease and may lead to up to ten years shorter life expectancy (2). The expected number of patients suffering from diabetes mellitus worldwide could be 439 million by the year 2030; about 90% of those patients will have Type 2 diabetes mellitus (3).

Lifestyle and genetic factors may contribute in an additive way to the primary physiologic causes responsible for the pathophysiology of Type 2 diabetes (4). More than 36 genes have been discovered responsible for the risk of Type 2 diabetes mellitus. All of these genes comprise only about 10% of the total genetic factors of the disease (5).

Hypoandrogenism is a clinical condition characterized by decreased blood level of testosterone, together with signs and symptoms of testosterone deficiency (6, 7). It has been noticed that conditions of hypoandrogenism in men have been associated with insulin resistance, suggesting that normal sex steroid physiology alterations may play a vital role in the pathophysiology of Type 2 diabetes mellitus (8).

In human blood, circulating sex steroid hormones such as testosterone and oestradiol are primarily bound to a specific plasma transport protein, which is sex hormone-binding globulin (SHBG), a glycated homodimeric protein synthesized mainly in the liver, and which binds with a higher affinity to androgens, testosterone and dihydrotestosterone (DHT), than to oestradiol (9). Sex hormone-binding globulin has a stronger impact on androgen-bioavailability to target tissues, through differential binding and transport of sex steroids (10). Through the activation of a specific, high-affinity receptor present in the plasma membrane, SHBG could exert a direct effect on cellular uptake of sex steroid and cell proliferation in hormone-responsive tissues (11).

Sex hormone-binding globulin has been considered as one of the environmental and genetic factors that have a role in the pathophysiology of Type 2 diabetes (12). Sex hormone-binding globulin level is inversely associated with higher insulin concentration characteristic of insulin resistant states causing Type 2 diabetes. Many specific polymorphisms in the SHBG gene locus have been studied and contribute to the alteration in SHBG level and consequently affect risk of Type 2 diabetes *via* encoding a protein that regulates glucokinase activity (13).

Human SHBG has 29 amino acids encoded by the 4 kb SHBG gene at chromosome 17p12-p13 which contains eight exons separated by seven introns in-between (14). Several polymorphisms in the human (SHBG) gene were characterized; it has been found that genotype analysis of the exon 2 SHBG SNPs, rs6257 and rs6259, are associated with insulin resistance (15) and other sex hormone-dependent conditions such as reduced bone mineral density, breast cancer and prostate cancer (16). However, data examining SHBG level and polymorphisms and the risk of Type 2 diabetes in the Egyptian population are lacking.

The aim of this study was to investigate the relations of SHBG gene polymorphisms and blood levels of SHBG with the risk of Type 2 diabetes and its effect on the sex hormones blood level in a case-control study of Egyptian men with Type 2 diabetes.

SUBJECTS AND METHODS

The study included 185 Egyptian male patients (mean age 50.3 ± 6.7 years) diagnosed with Type 2 diabetes mellitus and who were randomly selected from those attending the diabetes clinic of the Zagazig University Hospital. One hundred and eighty-five age-matched [mean age 50.0 ± 6.8 years] healthy Egyptians males were recruited from Zagazig University Hospital as a control group (with normal fasting blood glucose [FBG]). The study was approved by the Ethics Committee of Zagazig University. Confidentiality and privacy of all participants were assured. Informed written consent was obtained. All participants (cases and controls) underwent routine biochemical blood analysis (which included FBG, glycated haemoglobin [HbA_{1C}], lipid profile, kidney and liver function tests). Individuals who were castrated for treatment of cancer of the testis or prostate, and those taking medications known to affect sex hormone level (*eg* antiandrogenic agents for prostate cancer) and those with impaired hepatic and renal functions were excluded from the study.

Biochemical analysis

Sampling

Peripheral venous blood (6 mL) was collected from all participants and divided into two portions, one in a tube containing EDTA and processed immediately for DNA extraction. Another portion was collected in plain tubes for serum separation by 10-minute double centrifugation at 1600 g in order to spin down any insoluble remnants in the serum. The supernatant was then stored at -80 °C for future analysis.

Enzyme-linked immunosorbent assay

Hormonal levels were assayed using specific commercially available enzyme-linked immunosorbent assay (ELISA) kits (Quantikine, R&D Systems GmbH, Germany), for SHBG and

total testosterone and BIOVENDOR Research and Diagnostic Products (Guang Zhou, China) for oestradiol. We used 50 µL of sample for SHBG and oestradiol and 100 µL for total testosterone. The instruction manual was strictly followed, and samples were assayed as duplicates.

The minimum detection limit for SHBG, total testosterone and oestradiol were 0.005 nmol/L, 0.030 ng/mL and 10 pg/ml, respectively.

DNA extraction

Genomic DNA was extracted from EDTA whole-blood sample using a spin column method according to the manufacturer's protocol (QIAamp Blood Kit; Qiagen GmbH, Hilden, Germany). The purified DNA was stored at -80 ˚C until further use.

Detection of SHBG polymorphisms

All DNA samples were genotyped according to the method of Ding *et al* (17), based on the exonuclease activity of Taq DNA-polymerase using StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Polymerase chain reaction (PCR) amplification was carried out on 5–20 ng DNA using 1 X TaqMan universal PCR master mix, 900 nM forward and reverse primers, 200 nM of each of the allele-specific Taqman probes labelled with VIC and FAM and in 5 mL reaction volume. Amplification conditions involved the use of one cycle of 95 ˚C for 10 minutes, followed by 50 cycles of 92 ˚C for 15 seconds and 60 ˚C for one minute.

Quality control measures included blinded analyses, replicates of 10% of samples, and positive controls (blood-derived DNA from all known genotypes), and negative controls for contamination (no DNA) were run routinely with patient samples.

Statistical analysis

Statistical analysis in this study was performed using SPSS version 17.0. Percentages, frequencies, mean and standard deviation (SD) were used accordingly. Genotype frequencies for the study population were tested for Hardy-Weinberg equilibrium, and any deviation between the observed and expected frequencies was tested for significance using Chi-square test. Also, the Chi-square test was used for categorical variables and *t*-test for continuous variables. The means of the genotype groups were compared in one-way analysis of variance (ANOVA). The statistical significances of differences in frequencies of variants between the groups were tested using the odds ratios (ORs), and 95% confidence intervals (CIs) were calculated as a measure of the association of the rs6257 and rs6259 genotypes of SHBG gene polymorphism. A difference was considered significant at $p < 0.05$ level.

RESULTS

Sociodemographic characteristics, mean ± SD and median of the biochemical parameters for both groups are summarized in Table 1. We observed a significant decrease in plasma level of SHBG and testosterone and increase in oestradiol level in

Table 1: Sociodemographic characteristics and biochemical laboratory profile of the studied groups

	Diabetic patients	**Control**	
	n = 185	**n = 185**	***P-value***
Age in years			t = 0.659
Mean ± SD	50.3 ± 6.74	50.0 ± 6.80	*p* = 0.418
Family history of DM			
Negative	119 (64.3%)	168 (90.8%)	
Positive	66 (35.7%	12 (9.2%)	
Duration of DM in years			
Mean ± SD	13.0 ± 5.65		
FBS (mg/dL)			
Mean ± SD	184.6 ± 37.36	98.5 ± 12.42	t = 26.359
Median	175.0	106.0	***p* = 0.000***
HbA_{1C} % (mmol/mol)			
Mean ± SD	8.2 ± 1.23	5.0 ± 0.32	t = 34.536
Median	(66 ± 11.1 mol/mol) 8.5% (69 mmol/mol)	(31 ± 1.1 mol/mol) 5.1% (32 mmol/mol)	***p* = 0.000***
SHBG (nmol/L)			
Mean ± SD	25.1 ± 10.34	34.7 ± 12.52	t = 8.014
Median	24.0	32.8	***p* = 0.000***
Testosterone (ng/mL)			
Mean ± SD	7.8 ± 2.08	9.0 ± 2.00	t = 5.799
Median	7.5	8.6	***p* = 0.000***
Oestradiol (pg/mL)			
Mean ± SD	29.1 ± 6.53	22.5 ± 7.49	t = 8.902
Median	32.0	21.0	***p* = 0.000***

P-value is significant at < 0.05 level

DM = diabetes mellitus; FBS = fasting blood glucose; HbA_{1c} = glycated haemoglobin; SHBG = sex hormone-binding globulin

patients with Type 2 diabetes when compared to the control group.

SHBG genotype frequencies

The genotype and allele frequencies of SHBG gene rs6257 and rs6259 polymorphisms among patients with Type 2 diabetes and the control group are summarized in Table 2. Subjects with variant allele of rs6257 (CC genotype) and wild allele of rs6259 (GG genotype) were significantly more likely to develop Type 2 diabetes mellitus (Table 2).

Association between SHBG polymorphisms and SHBG hormonal levels

Among patients with diabetes and the control group, carriers of rs6257 variant allele (CC or CT) showed a significant decrease in plasma level of both SHBG and testosterone and a significant increase in oestradiol blood level compared with the wild type (TT), as described in Table 3.

On the other hand, carriers of rs6259 wild allele (GG) in our study groups demonstrated a significantly lower level in plasma SHBG and testosterone and a significant increase in

Table 2: Distribution of sex hormone-binding globulin gene polymorphisms (alleles) among the studied groups

	Diabetic patients		Control		Chi-square *(P-value)*	Odd's ratio	Confidence interval (CI) at 95%
	n = 185	**%**	**n = 185**	**%**			
rs 6257							
TT	137	74.1	157	84.9	6.624	**0.509***	**0.303, 0.856**
CT-CC		48	25.9	28	15.1	**(0.010*)**	
CT	27	14.6	18	9.7	2.049	1.585	0.840, 2.991
TT-CC	158	85.4	167	90.3	(0.152)		
CC	21	11.4	10	5.4	4.260	**2.241***	**1.025, 4.901**
TT-CT	164	88.6	175	94.6	**(0.039*)**		
rs 6259							
GG	159	85.9	133	71.9	10.982	**2.391***	**1.416, 4.036**
AG-AA	26	14.1	52	28.1	**(0.001*)**		
AG	16	8.6	32	17.3	6.128	**0.453***	**0.239, 0.857**
GG-AA	169	91.4	153	82.7	**(0.013*)**		
AA	10	5.4	20	10.8	3.627	0.471	0.214, 1.037
GG-AG	175	94.6	165	89.2	(0.057)		

P-value is significant at < 0.05 level

Table 3: Blood hormonal levels among the study participants, according to rs6257 polymorphisms in the sex hormone-binding globulin (SHBG) gene

	TT	CT	CC	*P*-value
SHBG in diabetics (*Mean ± SD)*	28.4 ± 9.57	17.8 ± 6.03	13.1 ± 3.76	F = 39.809 ***p* = 0.000***
SHBG in control (*Mean ± SD)*	37.3 ± 11.62	21.7 ± 4.18	17.9 ± 1.90	F = 31.592 ***p* = 0.000***
***t*-test**	7.136	1.818	3.811	
***p*-value**	**0.000***	0.076	**0.001***	
Testosterone in diabetics (*Mean ± SD)*	8.0 ± 2.07	7.3 ± 1.83	6.5 ± 1.92	F = 6.229 ***p* = 0.002***
Testosterone in control *(Mean ± SD)*	9.1 ± 1.99	8.4 ± 1.17	8.2 ± 2.94	
t-test	7.517	2.149	1.960	F = 1.991
***p*-value**	**0.000***	**0.037***	0.060	*p* = 0.140
Oestradiol in diabetics (*Mean ± SD)*	28.1 ± 6.82	31.0 ± 5.42	32.6 ± 3.75	F = 5.959 ***p* = 0.003***
Oestradiol in control (*Mean ± SD)*	22.0 ± 7.06	26.3 ± 9.87	24.1 ± 8.00	F = 2.892 *p* = 0.058
t-test	4.350	2.058	4.073	
***p*-value**	**0.000***	**0.046***	**0.000***	

P-value is significant at < 0.05 level

Table 4: Blood hormonal levels among the study participants, according to rs6259 polymorphism of sex hormone-binding globulin (SHBG) gene

	GG	AG	AA	*P*-value
SHBG in diabetics *(Mean ± SD)*	22.5 ± 7.27	37.0 ± 9.49	48.3 ± 11.85	F = 72.333 ***p* = 0.000***
SHBG in control (*Mean ± SD)*	26.6 ± 9.58	42.0 ± 5.69	56.6 ± 7.25	F = 94.675 ***p* = 0.000***
t-test	7.253	2.299	2.392	
***p*-value**	**0.000***	**0.026***	**0.024***	
Testosterone in diabetics (*Mean ± SD)*	7.6 ± 1.85	8.8 ± 2.65	9.3 ± 3.31	F = 6.089 ***p* = 0.003***
Testosterone in control (*Mean ± SD)*	8.5 ± 1.60	9.4 ± 2.36	11.5 ± 1.79	F = 26.237 ***p* = 0.000***
t-test	4.635	1.005	2.353	
***p*-value**	**0.000***	0.323	**0.026***	
Oestradiol in diabetics (*Mean ± SD)*	29.4 ± 6.36	26.2 ± 7.36	28.8 ± 7.61	F = 1.679 *P* = 0.189
Oestradiol in control (*Mean ± SD)*	22.7 ± 7.38	22.3 ± 7.88	21.6 ± 7.88	F = 0.224 *p* = 0.800
t-test	8.218	1.661	2.376	
***p*-value**	**0.000***	0.103	**0.025***	

***P*-value is significant at < 0.05 level**

oestradiol level when compared to carriers of the variant alleles [AA or AG] (Table 4).

One-way analysis of variance for plasma level of SHBG, testosterone and oestradiol in patients with Type 2 diabetes and the control group in relation to different alleles (TT, CT and CC) of rs6257 and alleles of rs6259 (GG, AG and AA) are expressed in Tables 3 and 4, respectively.

DISCUSSION

Increased risk of Type 2 diabetes with low SHBG levels may predict the stronger effects of more bioavailable testosterone, and thus, the role of SHBG in the pathogenesis of Type 2 diabetes can be explained by its alteration in the sex hormones levels (18). Studies suggest that sex hormones bound to SHBG may also be biologically active, increasing their signalling, endocytosis, or overall biologic actions (18).

In the present study, there was a significant reduction in SHBGs, resulting in significant decrease in total testosterone levels and increased oestradiol level in patients with Type 2 diabetes compared to the control group. These findings are in accordance with the results reported by Ding *et al* and Perry *et al* (17, 19).

Presumed mechanisms of insulin resistance due to SHBG alteration include modulating the biologic effects of sex hormones (oestrogen and testosterone) on peripheral tissues (*ie* liver, muscle and fat). Low testosterone levels may increase insulin resistance through mechanisms involving muscle (20), liver (21), and bone (22). *In vitro*, testosterone promotes commitment of pluripotent stem cells to the myogenic lineage but inhibits their differentiation into adipocytes *via* an androgen receptor (AR)-mediated pathway. These findings provide mechanistic insights into the well-documented effects of testosterone therapy on body composition in men, namely a reduction in fat mass and an increase in muscle mass, changes expected to decrease insulin resistance. Testosterone decreases insulin resistance by regulating mature adipocytes and myocytes, enhances catecholamine-induced lipolysis *in vitro* (23), and reduces lipoprotein lipase activity and triglyceride uptake in human abdominal adipose tissue *in vivo* (24).

Moreover, another study suggested that blood levels of testosterone have a positive correlation with genetic and functional mitochondrial indices caused by increased insulin sensitivity in human skeletal muscle (20). In another study, castration of male rats caused a decrease in muscular glucose uptake which increased insulin resistance. Low testosterone level may enhance more accumulation of visceral fat, which increases insulin resistance and diabetes *via* increased inflammatory cytokines (25).

In the present study, decreased SHBGs in Type 2 diabetes led to increased level of free oestradiol in the blood which has direct effects on glucose transport or metabolism, as proven by different studies (26).

Two single-nucleotide polymorphisms at the SHBG locus (17p12-p13) (rs6257, rs6259) were identified as independently associated with serum testosterone concentration (17).

The present study revealed that carriage of variant alleles of rs6257 was associated with lower levels of SHBG, and variant alleles of rs6259 was associated with higher levels of SHBG. For rs6257, a SNP that flanks, and is located 17 bp upstream of exon 2 suggests the presence of potential key splicing or regulatory elements in that region (17). This SNP results in the production of a SHBG variant with reduced affinity for testosterone that provides an explanation for the asso-

ciation between rs6257 and low serum testosterone concentrations. Rs6257 is likely to be a functional polymorphism with impact on testosterone binding to SHBG as well as testosterone bioavailability and action at target tissue level (27).

Elevated levels of circulating SHBG among carriers of an rs6259 variant allele may be explained by substitution of asparagine amino acid for aspartic acid (D356N) within exon 8 and introduction of additional N-glycosylation consensus site that alters the binding of SHBG to membrane receptors and other proteins and consequently reduces its clearance rate from the circulation, resulting in higher plasma levels of the globulin (28).

A higher risk of diabetes mellitus associated with low serum testosterone concentrations is complicated by the fact that free testosterone levels are maintained in men by a strong feedback loop whereby luteinizing hormone (LH) production from the pituitary gland in the presence of low androgen levels stimulates testosterone production in the testes. This should mean that genetically lower SHBG levels will not greatly affect free testosterone levels in men. However, there is recent evidence that bound testosterone may be biologically active (18).

Linking plasma levels of SHBG to the risk of Type 2 diabetes obtained from our genotyping analysis may represent the average lifetime risk attributable to SHBG alone, independent of traditional risk factors.

CONCLUSION

Sex hormone-binding globulin may play an important role in the development of Type 2 diabetes at both the genomic and phenotypic levels. Sex hormone-binding globulin could be an important target in stratification for the risk of Type 2 diabetes and early intervention.

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