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Association of the (TAAAA)*n* repeat and Asp327Asn polymorphisms in the sex hormone-binding globulin (SHBG) gene with idiopathic male infertility and relation to serum SHBG concentrations

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ABSTRACT

Sex hormone binding globulin (SHBG) is involved in delivering sex hormones to target tissues. We investigated the association between the (TAAAA)*n* repeat polymorphism, and Asp327Asn polymorphism in the SHBG gene with semen quality and idiopathic male infertility. We studied 168 men with idiopathic infertility [oligoasthenoteratozoospermia (OAT)] and equal number of age-matched normal controls. The serum levels of SHBG, reproductive and thyroid hormones, and Inhibin B were measured. Semen parameters were also assessed. The genotype assays for the SHBG polymorphism were done using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Baseline SHBG levels tended to be lower in infertile men (21.1 ± 7.2 nmol/l) compared to normal fertile men (24.7 ± 7.9 nmol/l). SHBG levels tended to be higher among the subjects with the Asn/Asn (25.84 ± 3.6 nmol/l) and S/S (24.50 ± 5.4 nmol/l) genotypes compared to subjects with the Asp/Asn (24.38 ± 3.2 nmol/l) and L/L (18.44 ± 4.2 nmol/l) genotypes of the SHBG gene. The genotype frequencies of Asp/Asp were 80.9% in cases and 71.4% in controls ($P = 0.001$). The variant Asp/Asn genotype was associated with a more than 50% reduced risk of infertility (OR: 0.46, 95% CI: 0.25–0.80, $P = 0.001$). Genotype analysis demonstrated six SHBG (TAAAA)*n* alleles with 6–11 repeats. Long SHBG (TAAAA)*n* alleles (>8 repeats) were at greater frequency in infertile men than fertile subjects ($P = 0.001$), whereas short SHBG (TAAAA)*n* alleles ($\leq$8 repeats) tended to be more frequent in fertile men than cases ($P = 0.001$). Men with the 9/X TAAAA repeat genotype displayed a 2.82-fold increased risk of infertility (95% CI: 1.27–4.79, $P = 0.01$). There were strong and significant positive correlations between plasma SHBG and sperm count ($r = 0.672$, $P = 0.01$), sperm motility ($r = 0.721$, $P = 0.01$) and sperm morphology ($r = 0.574$, $P = 0.02$). We concluded that the SHBG Asp237Asn and (TAAAA)*n* polymorphisms may influence SHBG levels and as a result, male infertility. Multicenter large scale studies are warranted to better elucidate the role of SHBG gene polymorphism in male infertility.

1. Introduction

Androgens play a central role in the maintenance of normal spermatogenesis, if androgen levels are decreased, infertility could ensue. Estrogen reduces gonadotropins [luteinizing hormone (LH) and follicle stimulating hormone (FSH)] secretion at pituitary level [1] resulting in decreased testicular function and reduction in testosterone production and intratesticular and serum testosterone levels. The balance between serum androgens and estrogens is essential for normal semen parameters [2]. A decrease in testosterone/estrogen ratio is associated with infertility [2]. Also, estrogen has a direct deleterious effect on spermatogenesis [3].

Significant number of infertile men is considered idiopathic due to lack of obvious medical or surgical causes for their infertility. While many such cases are attributed to genetic factors, few genetic causes have been confirmed in humans to date [4]. Plasma sex hormone binding globulin (SHBG) binds testosterone and estradiol with high affinity, and selectively transports sex hormones in plasma [5]. SHBG affects the bioavailability of androgens and estrogens and thus their access to target tissues [6,7]. The SHBG gene is located on chromosome 17p12–p13 and encodes a 402-amino-acid polypeptide [8]. Substantial variations in serum SHBG concentration exist between individuals, and can therefore influence sex hormones actions. A genetic influence on SHBG levels has been well documented [9]. In the last decade, several genetic polymorphisms have been determined in the human SHBG gene. There are 11 detected single-nucleotide polymorphisms (SNPs) in

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the SHBG gene and seven SNPs and a (TAAAA)*n* microsatellite in the genomic region [10]. Polymorphisms in the SHBG gene may change either the production or the metabolism of the protein, thus resulting to individual variability in SHBG concentration [9].

A single-nucleotide polymorphism (G to A) at nucleotide 5790 (M31651, GenBank) in exon 8 of this gene results in an amino acid substitution of asparagine for aspartic acid at residue 327 (Asp327Asn) (reference sequence [*rs*] *rs*6259) in the SHBG polypeptide [11]. This amino acid substitution generates an additional N-linked carbohydrate chain and adds an extra consensus site for N-glycosylation (Asn-X-Ser or Thr) within the carboxyl-terminal globular domain [12,13]. The presence of the additional carbohydrate chain in the variant SHBG decreases its metabolic clearance rate, is associated with an increased half-life of the protein and, in turn, with increased serum SHBG levels in Asn allele carriers [13,14].

Recently, a pentanucleotide repeat polymorphism [PNRP (TAAAA)*n*] (*rs*35785886) in the 5 promoter region of the SHBG gene has been determined [13,15], with alleles varying from six to 11 TAAAA repeats. The relationship between (TAAAA)*n* repeats and the SHBG concentrations remains an area of controversy. In two studies of women [13,15] and in two studies of men [10,16], mean serum SHBG concentrations were higher for carriers of fewer TAAAA repeat lengths or carriers of the six-repeat allele, whereas in another study of women [17], lower SHBG levels were demonstrated among carriers of the six-repeat allele.

To our knowledge there is only one study in the literature addressing the role of the (TAAAA)*n* repeat polymorphism on male factor fertility. In that study in men with normal sperm count and motility, those with short SHBG alleles had higher sperm concentration than men with long SHBG alleles [18].

The aim of the present study was to investigate the possible interactive role of the SHBG gene Asp327Asn and (TAAAA)*n* polymorphisms in the idiopathic male infertility, since both polymorphic variants may alter androgen and estrogen bioactivity by influencing androgen and estrogen availability and action.

2. Materials and methods

2.1. Study subjects

One hundred sixty-eight men (mean age 32.4 ± 6.4 years, range 25–45 years) affected by oligoasthenoteratozoospermia (OAT) and history of infertility of unknown etiology were selected for this study. They were recruited after screening process for eligibility. Infertility was defined as failure of the same female partner to conceive after 2 years of regular and unprotected intercourse. They were referred or addressed themselves to our Urology facility for infertility workup. Male infertility was diagnosed if one or more standard semen parameters were below the cutoff levels proposed by World Health Organization (WHO, 1999) [19]. At least 2 semen analyses were performed, 4 weeks apart. Normal WHO values included, sperm density greater than 20×10^6/ml, sperm motility greater than 50%, normal morphology greater than 30%, and semen volume greater than 2 ml. The equal number of healthy normal fertile men ($n = 168$) (mean age 32.8 ± 6.7 years, range 25–45 years) selected from the general population of Tehran served as controls. They were matched to cases according to the age distribution. All of the controls had fathered children. Informed consent was obtained from all participants prior to participation in the study. This study was performed in accordance with the guidelines in the Declaration of Helsinki for the appropriate treatment of human subjects.

2.2. Inclusion/exclusion criteria

The participants met the following criteria: abnormal semen analysis in all three sperm parameters (density, motility, and morphology), normal fertile female partner, and a history of infertility for at least two years without known reasons for their infertility. The exclusion criteria were as follows: azoospermia; a history of previous epididymo-orchitis, prostatitis, genital trauma, and testicular torsion; a testicular volume of less than 12 ml; inguinal or genital surgery; genital disease such as cryptorchidism, urinary tract infection or varicocele; presence of any endocrinopathy; use of cytotoxic drugs, immunosuppressants, or anticonvulsives; Y chromosome microdeletions or karyotype abnormalities; leukocytospermia (more than 10^6 white blood cells per ml), or positive mixed agglutination reaction test; tobacco use; and drug, alcohol or substance abuse. Patients were also excluded from study if they had concomitant medical problems known to be associated with decreased fertility; hepatobiliary disease; significant renal insufficiency; a body mass index (BMI) of 30 kg/m^2 or greater; and occupational and environmental exposures to potential reproductive toxins. Subjects who were taking any medication that affects serum hormones, such as androgens, antiandrogens, selective estrogen receptor modulators such as tamoxifen, aromatase inhibitors, GnRH analogs, and glucocorticoids were excluded from the study.

2.3. Evaluations

An extensive medical history was obtained and physical examination was done for each subject. In-person interviews were conducted with study participants by one of the investigator (MRS). A self-administered questionnaire was used to gather detailed information on demographic factors (ethnicity, education, weight, height, and occupational status), reproductive history, history of prior medical and surgical conditions, exogenous hormone use, physical activity, and tobacco and alcohol use. The body mass index (BMI) of each patient, calculated as weight (kg)/height^2 (m). The urogenital examination included the site and presence of the testes, with the testicular volume calculated using ultrasonography. A volume of less than 12 ml was considered small. Blood was drawn after overnight fasting for the measurement of serum luteinizing hormone (LH), follicle stimulating hormone (FSH), total testosterone (T), free testosterone (free T), prolactin (PRL), estradiol (E2), free estradiol (free E2), SHBG, thyroid stimulating hormone (TSH), thyroxin (T4), triiodothyronin (T3), and Inhibin B levels.

Hematological and routine biochemistry analyses were also done. The laboratory evaluation also included, at least two semen analyses after 3 days of abstinence with an interval of 4 weeks between them, Karyotype analysis and Y chromosome microdeletion evaluation. The presence of antisperm antibodies was also determined using antibodies mixed agglutination reaction (MAR) test.

Fertile female partners was diagnosed after extensive infertility evaluation, including baseline body temperature; mid-luteal progesterone concentrations; karyotyping; serum chemistry, hematology and hormonal profile, including: FSH, LH, PRL, TSH, and thyroxine (T4); sperm immobilizing antibodies in serum; antiphospholipid and anticardiolipin antibodies; lupus anticoagulant; hysterosalpingography; and cervical cultures for Ureaplasma, Mycoplasma, Chlamydia and bacterial vaginosis as needed. Female partners with abnormal findings on hysterosalpingography underwent laparoscopy and/or hysteroscopy.

2.4. Hormonal assays

Total serum T was measured using radioimmunoassay (RIA) kit (3H Testosterone, Biomerieux, Lyon, France) with a sensitivity of detection of 4 pg/ml. The normal reference range for T was 3.5–9.5 ng/ml. Serum LH and FSH levels were assayed using time-resolved immunofluorometric assay kits (DELFIA hLH for LH and

DELFIA hFSH for FSH; Wallac Co., Turku, Finland). The intra- and interassay coefficients of variation of the individual immunofluorometric assay method were below 9% within the reference ranges. The reference ranges were as follows: LH, 1.0–8.4 IU/l; and FSH, l.0–10.5 IU/l. SHBG concentrations were measured in serum using 1235 AutoDELFIA automatic system based on a time-resolved fluoroimmunoassay (AutoDELFIA SHBG, Wallac Co). The between-assay coefficient of variation is 2.3–3.0%. The reference range for SHBG is 15–50 nmol/l. Serum E2 was quantified using a RIA (Esoterix, Inc., Calabasas Hills, CA, USA), in which intra- and inter-assay coefficients of variation were 5.2% and 8.0%, respectively. The normal reference range for E2 was 0–50 pg/ml. Serum levels of PRL were measured by commercial RIA kit. This commercial kit has been used previously with inter- and intra-assay variations of less than 10%. The reference ranges for PRL was 92–697 pmol/l. Serum Inhibin B was determined by ELISA method using kit reagents and Inhibin B standard (Oxford Bio-innovation Ltd., Oxon, UK). The assay sensitivity was 4 pg/ml and the between-assay variation was 15%. Ciba Corning kits (Ciba Corning Diagnostics SA, Spain) were used to determine by chemoluminescence TSH (intra-assay < 4.7%, interassay < 6.25%), FT3 (intra-assay < 3.8%, interassay < 6.2%), and FT4 (intra-assay < 3.26%, interassay < 4.95%). The reference ranges were as follows: 0.35–5.5 mU/l for TSH, 3.5–6.5 pmol/l for FT3, and 10.3–23.2 pmol/l for FT4.

FT and free E2 (FE2) were determined using the method described by Vermeulen et al. [20] and van den Beld et al. [21] taking the blood levels of total T, total E2, and SHBG into account and assuming a fixed albumin concentration of 43 g/l.

2.5. Genotyping of the Asp327Asn polymorphism

Genomic DNA was extracted from buffy coat fractions using the Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN) following the protocol of the manufacturer. The genotype assays for the SHBG polymorphism were done using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. The primers for the PCR amplification were 5′-TTCTGGATCCGAGCCACCT-3′ (forward) and 5′-AGTGCCTGGTACATTGCTAG-3′ (reverse). The PCR reactions were performed in a Biometra T Gradient Thermocycler. The PCR reaction was performed using standard protocol in which each 25 μl of PCR mixture included 10 ng DNA, 1× PCR buffer, 1.5 mmol/l $MgCl_2$, 0.4 μmol/l of each primer, 0.16 mmol/l each of deoxynucleotide triphosphate, and 1 unit of DNA polymerase. Amplification condition was initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and final extension step at 72 °C for 7 min. Each PCR product (10 μl) was then digested with 10 units of HinfI (New England BioLabs, Beverly, MA) at 37 °C for 3 h. The separation of the DNA fragments was accomplished by electrophoresis on 3% agarose gel containing ethidium bromide, and visualized under UV light. The PCR product (294 bp) digestion will, therefore, result in three fragments (139, 92, and 63 bp) for subjects carrying the Asp allele (G allele), and in two fragments (155 and 139 bp) for the Asn allele (A allele) carriers. Study subjects were then assigned to three genotypes: homozygotes for the wild-type (Asp/Asp) or the variant allele (Asn/Asn) or heterozygotes (Asp/Asn). The laboratory staff was blind to the identity of the participants and replicate quality control samples. Ten quality control samples were taken from the samples of randomly selected subjects of the study and assayed three to five times per genotype. The consistency rate was 100% for quality control samples.

2.6. Genotyping of the (TAAAA)n polymorphism

DNA was extracted from the buffy coat fraction using the QIAGEN 96 DNA Spin Kit (QIAGEN, Valencia, CA). The (TAAAA)*n* repeat polymorphism genotyping was performed as follows. PCR amplification of the fragment was generated using PCR forward primer 5′-GATCGCTTGAACTCGAGAGG-3′ and reverse primer 5′-GCCTGTCTCCCAAGAAGGTA-3′.

This resulted in a PCR product of 275 bp (eight TAAAA repeats). Two forward primers, one 5′-labeled with a fluorescent dye, and one without labeling were employed in the reaction. Thirty nanograms of genomic DNA were utilized per 20 μl reaction, consisting 200 nmol of each forward and reverse primer (20 nmol 5′-labeled and 180 nmol unlabeled forward primers), $MgCl_2$ (25 mm), deoxy-NTPs (10 mm), 10× PCR buffer, and 0.5 U QIAGEN Hot-StarTaq. The reaction mixtures were denaturated at 95 °C for 5 min and then subjected to 40 cycles of 1 min at 95 °C, 1 min at 60 °C, and 1 min at 72 °C, and a final extension of 10 min at 72 °C. The size of the fragment length was determined using an ABI 377 sequencer and GeneScan software. Ten replicate samples were placed in to assess quality control. The concordance was 100%.

2.7. Statistical analysis

Desired power of the present study was set at >80% as analyzed by power genetic association analysis software (http://dceg.cancer.gov/bb/tools/pga) at the level of significance $\alpha = 0.05$ (2-sided significance). With 168 men in each group we had power of 80% to show a correlation as low as 0.2 between the SHBG gene polymorphisms and the infertility. All results are expressed as the mean ± SD. To determine the difference in the distribution of SHBG allele types and genotypes between cases and controls χ^2 statistics was applied. Normal distribution of continuous parameters was examined by Kolmogorov–Smirnov test. Differences in continuous parameters between genotypes were assessed with analysis of variance test (ANOVA) and were confirmed with the non-parametric Kruskal–Wallis test. SHBG concentrations were expressed as geometric means and 95% confidence intervals (CI), and differences between cases and controls and between carriers and noncarriers of the variant allele types and genotypes were analyzed with the use of *t*-test. Univariate associations were assessed using Pearson's correlation. Regression analyses including SHBG genotype as the independent predictor were used to examine the associations between genotype and levels of SHBG. Regression analyses treating the (TAAAA)*n* polymorphism as a continuous variable were used to determine the biological effects of increasing number of repeats. Analyses were adjusted for the following variables: age, duration of infertility, body mass index, occupational status, and level of education. Unconditional logistic regression was applied to estimate odds ratios (OR) and 95% CIs adjusting for age and other potential confounders. To examine Hardy–Weinberg equilibrium for each polymorphism and to test for potential linkage disequilibrium, χ^2 analyses were used. All tests were two tailed and conducted at the 5% significance level. All analyses used the SPSS statistical package (version 16.0, SPSS Inc., Chicago, IL).

3. Results

The characteristics of infertile men and the control group are presented in Table 1. Cases and controls were well matched on age, and other demographic characteristics. The mean for SHBG in control subjects (24.7 ± 7.9 nmol/l) was significantly higher than the means for cases (21.1 ± 7.2 nmol/l, $P = 0.04$).

The mean for total T (6.1 ± 2.14 ng/ml) in control subjects was higher than the mean for infertile patients (4.8 ± 1.3 ng/ml). But, the difference between these two groups was not statistically significant $P = 0.06$). The mean for free T (149.4 ± 52.4 pg/ml) in cases was higher than the means for controls (134.4 ± 46.2 pg/ml). The means for the two groups did not differ significantly from

Table 1
Baseline demographics, serum hormones, and semen parameters of study groups.

Characteristics	Patients (n = 168)	Controls (n = 168)	P value
Age (year)	32.4 ± 6.4	32.8 ± 6.7	0.1
Duration of marriage	7.4 ± 3.2	7.6 ± 2.9	0.1
Infertility duration (year)	4.3 ± 2.1	4.4 ± 2.2	0.1
BMI (kg/m^2)	26.8 ± 2.7	26.7 ± 2.6	0.1
Occupational status no. (%)			
Employed	146 (86.9)	145 (86.3)	0.1
Unemployed	22 (13.1)	23 (13.7)	0.1
Educational level			
None	0	0	–
Primary school	9 (5.4)	8 (4.8)	0.08
High school	106 (63.1)	105 (62.5)	0.09
Graduate	53 (31.5)	55 (32.7)	0.07
Serum hormones			
Testosterone (ng/ml)	4.8 ± 1.30	6.1 ± 2.14	0.06
Free testosterone (pg/ml)	149.4 ± 52.4	134.4 ± 46.2	0.06
LH (IU/l)	6.2 ± 2.4	6.7 ± 2.2	0.08
FSH (IU/l)	6.8 ± 2.2	6.2 ± 2.4	0.08
Estradiol (pg/ml)	18.4 ± 6.8	20.2 ± 7.0	0.04
Free estradiol (pg/ml)	0.45 ± 0.21	0.38 ± 0.16	0.04
PRL (pmol/l)	352 ± 117	356 ± 116	0.1
TSH (mlU/ml)	2.1 ± 1.2	2.2 ± 1.2	0.1
Free thyroxine (pmol/l)	14.2 ± 2.8	14.4 ± 2.6	0.1
Free triiodothyronine (pmol/l)	3.7 ± 1.2	3.6 ± 1.1	0.1
SHBG (nmol/l)	21.1 ± 7.2	24.7 ± 7.9	0.04
Inhibin B (pg/ml)	172.4 ± 20.6	183.4 ± 21.3	0.06
Semen parameters			
Ejaculate volume (ml)	2.8 ± 1.3	2.8 ± 1.3	0.1
Total sperm/ejaculate (×10^6)	42.2 ± 10.7	174.6 ± 24.5	0.001
Sperm density (×10^6/ml)	13.0 ± 3.2	57.3 ± 11.7	0.001
Motility (% motile)	21.4 ± 4.7	63.0 ± 8.4	0.001
Morphology (% normal)	20.1 ± 4.3	57.5 ± 8.6	0.001

Abbreviations: BMI: body mass index; LH: luteinizing factor; FSH: follicle stimulating hormone; PRL: prolactin; TSH: thyroid stimulating hormone.

each other (P = 0.06). The mean total E2 level in normal subjects (20.2 ± 7.0 pg/ml) was significantly higher than the mean for patients (18.4 ± 6.8 pg/ml) (P = 0.04). In contrast, the mean for free E2 in healthy fertile men (0.38 ± 0.16 pg/ml) was significantly lower than the mean for infertile men (0.45 ± 0.21 pg/ml, P = 0.04).

3.1. Genotyping

The Asp237Asn and (TAAAA)n genotype distributions were not influenced by age, BMI, duration of infertility, occupational status and educational level. Genotype analysis of SHBG Asp327Asn, and (TAAAA)n polymorphisms showed no significant deviation from Hardy–Weinberg equilibrium in any group (P = 0.36 and P = 0.34, respectively).

3.1.1. Asp237Asn polymorphism

The distributions of Asp237Asn genotypes in cases and controls are shown in Table 2. The frequency of Asp/Asn genotype of SHBG was significantly higher in controls (26.8%) when compared to the cases (17.9%) resulting in a reduced risk of more than 50% (OR: 0.46, 95% CI: 0.25–0.80, P = 0.001). As the frequency of the homozygous Asn/Asn genotype was very rare both in the cases (1.2%) or the controls (1.8%), the Asn/Asn and Asp/Asn genotypes were combined together and are referred to as variant genotypes (Asn/Asn + Asp/Asn) of SHBG. The frequency of the variant genotypes of SHBG (Asp/Asn + Asn/Asn) was found to be higher in controls (28.6%) when compared to the cases (19.1%), and resulted in a significant decreased risk to infertility in the cases (OR: 0.45, 95% CI: 0.28–0.78, P = 0.004).

3.1.2. (TAAAA)n repeat polymorphism

In both patients and controls, six (TAAAA)n alleles with 6–11 repeats in the SHBG gene were identified. They theoretically have resulted in 21 genotypes; however, no patients with genotypes 7/11, 8/11, or 11/11 were actually found in our population. Thus, according to their TAAAA repeat genotype, the patients could be assigned to one of the following 18 genotype groups: 6/6, 6/7, 6/8, 6/9, 6/10, 6/11, 7/7, 7/8, 7/9, 7/10, 8/8, 8/9, 8/10, 9/9, 9/10, 9/11, 10/10, or 10/11. The genotypic classes with outlying values [genotypes 6/11 (n = 4), 9/11 (n = 2), 10/10 (n = 1), and 10/11 (n = 1)] had no effect on statistical analysis; the significance level remained P < 0.05 when one or all of these genotypic classes were removed from the analysis. The six- and eight-repeat alleles were the most common alleles in whole study participants. Both had similar frequency of 42.9%. However, the six-repeat allele (46.4%), and the nine-repeat allele (42.9%) were the most common alleles among controls and cases, respectively. The study population was further subdivided into two subgroups. As a result, eight repeats were used as a cut point in additional analyses, and six, seven, and eight repeat alleles were classified as short (S) and nine or more repeat alleles were classified as long (L).

The association of the (TAAAA)n polymorphism with infertility was strongly influenced by the presence of six-repeat allele, as well as other short repeat alleles (seven, and eight repeat alleles). Compared with the no. 6 genotype, the 6/X variant was related to a significantly decreased risk of infertility (OR: 0.47, 95% CI: 0.21–0.76, P = 0.01) (Table 2). The frequency of S/L genotype was nearly similar in cases (35.1%) and controls (36.3%), and the frequencies of genotypes were 48.2% and 57.7% for S/S (P = 0.003) and 16.7% and 6.0% for L/L (P = 0.002), in infertile cases and controls, respectively. A decreased risk for infertility associated to heterozygotes S/L (OR: 0.68, 95% CI: 0.45–0.82), and homozygotes S/S (OR: 0.48, 95% CI: 0.25–0.72), was observed when it was compared to L/L homozygotes. The multivariate analysis including the potential prognostic factors (age, and S/S, S/L and L/L genotypes) followed by the logistic regression of (TAAAA)n genotypes on infertility adjusted by, age, BMI, duration of infertility, occupational status

Table 2
Risk of infertility and polymorphisms in sex hormone binding globulin gene among cases and controls.

Genotype frequency	Controls ($N=168$)	Cases ($N=168$)	Crude OR (95% CI)	P value	Adjusted OR[a] (95% CI)	P value
Asp237 Asn						
Asp/Asp	120 (71.4%)	136 (80.9%)	1.0 (Ref.)			
Asp/Asn	45 (26.8%)	30 (17.9%)	0.72 (0.44–1.12)	0.08	0.46 (0.25–0.80)	0.001
Asn/Asn	3 (1.8%)	2 (1.2%)	1.26 (0.37–4.49)	1.0	1.76 (0.40–8.4)	0.49
Asp/Asn + Asn/Asn	48 (28.6%)	32 (19.1%)	0.73 (0.42–1.08)	0.14	0.45 (0.28–0.78)	0.004
TAAAA repeat						
No. 6	90 (53.6%)	102 (60.7%)	1.0 (Ref.)			
6/X	64 (38.1%)	57 (33.9%)	0.42 (0.26–0.72)	0.01	0.47 (0.21–0.76)	0.01
6/6	14 (8.3%)	9 (5.4%)	0.84 (0.56–1.42)	0.07	0.87 (0.51–1.12)	0.08
6/X + 6/6	78 (46.4%)	66 (39.3%)	0.47 (0.31–0.82)	0.01	0.43 (0.27–0.76)	0.01
No. 7	110 (65.5%)	115 (68.5%)	1.0 (Ref.)			
7/X	48 (28.6%)	45 (26.8%)	0.82 (0.54–1.26)	0.08	0.75 (0.48–1.16)	0.07
7/7	10 (6.0%)	8 (4.8%)	1.18 (0.57–2.31)	0.09	1.13 (0.51–2.15)	0.08
7/X + 7/7	58 (34.5%)	53 (31.5%)	0.84 (0.57–1.26)	0.07	0.78 (0.56–1.38)	0.06
No. 8	92 (54.8%)	100 (59.5%)	1.0 (Ref.)			
8/X	57 (33.9%)	51 (30.4%)	1.21 (0.83–1.77)	0.08	1.18 (0.65–1.84)	0.06
8/8	19 (11.3%)	17 (10.1%)	1.06 (0.52–1.84)	0.09	0.98 (0.56–1.91)	0.08
8/X + 8/8	76 (45.2%)	68 (40.5%)	0.64 (0.32–0.85)	0.04	0.60 (0.35–0.75)	0.02
No. 9	110 (65.5%)	96 (57.1%)	1.0 (Ref.)			
9/X	51 (30.4%)	58 (34.5%)	2.78 (1.44–4.67)	0.04	2.82 (1.27–4.79)	0.01
9/9	7 (1.8%)	14 (8.3%)	2.70 (1.41–4.86)	0.03	2.84 (1.55–5.18)	0.003
9/X + 9/9	58 (34.5%)	72 (42.5%)	3.40 (2.25–5.65)	0.003	3.54 (2.32–5.71)	0.001
No. 10	154 (91.7%)	145 (86.3%)	1.0 (referent)			
10/X	14 (8.3%)	23 (13.7%)	2.72 (1.40–4.87)	0.02	2.68 (1.29–4.82)	0.01
No. 11	166 (98.8%)	162 (96.4%)	1.0 (Ref.)			
11/X	2 (1.2%)	6 (3.5%)	1.91 (0.67–1.35)	0.08	1.88 (0.59–1.38)	0.07

Abbreviations: OR: odds ratio; CI: confidence interval; Ref.: referent.

[a] Adjusted OR: adjusted in multivariate logistic regression models including age, duration of infertility, body mass index, occupational status, educational level and SHBG genotypes.

and educational level increased the prior risk for infertility, and remained statically significant.

3.1.3. Association of combined Asp237Asn and (TAAAA)n polymorphisms with infertility

Gene–gene interactions were evaluated considering only genotypic combinations for each polymorphism pairs, due to the limitations of the sample size of this study. The data revealed that 41.6% of the controls and patients were found to be carriers for genotype of either Asp/Asp or S/S (Table 3). About 27% of the controls, and 29% of cases carried the genotype of either Asp/Asp or S/L, which increased nonsignificantly the risk to 1.24-fold (95% CI: 0.61–2.42, $P=0.75$) in the cases carrying both genotypes. Combination of Asp/Asn genotype with S/S genotype was found to be present in 6.0% of the patients as compared to the controls (15.5%), which resulted in more than 50% (OR: 0.47, 95% CI: 0.26–0.81, $P=0.006$) decrease in the risk to infertility. Also, when genotype combinations of Asp/Asn and S/L were studied, it was found that 8.9% of the controls and 6.0% of the patients were carriers of Asp/Asn and S/L genotype combination and the risk associated with this genotype combination was found to be nonsignificantly decreased (OR: 0.88, 95% CI: 0.64–1.86, $P=0.08$). In addition, when we used the Asp/Asp and S/S genotype combination as the reference, we found that the Asp/Asp and L/L genotype combination was associated with a 3.47-fold increased risk of infertility (OR: 3.47, 95% CI: 2.82–5.70, $P=0.001$). When combinations of Asp/Asn and L/L were studied, this genotype combination was found to be present in 2.4% of the controls while 6.0% of the patients were carriers of this genotype combination, which did not reach statistically significance.

3.1.4. Influence of SHBG gene polymorphisms on plasma SHBG concentration

We found individual Asp237Asn polymorphism to contribute significantly to the overall prediction of SHBG levels (by ANCOVA, $P=0.006$; Table 4). There was a statistically significant difference in serum SHBG levels between subjects with Asp allele and those with Asn allele. The mean values ± SD of SHBG (nmol/l) was 24.41 ± 3.4 for the combined Asp/Asn and Asn/Asn genotypes and 22.44 ± 3.6 for the Asp/Asp genotype ($P=0.008$).

We detected consistent pattern of association between SHBG levels and number of (TAAAA)n repeats when comparing men with two short versus two long alleles (Table 4). Compared with

Table 3
Distribution of double sex hormone binding globulin genotypes among infertile patients and controls.

Genotype frequency	Control ($N=168$) (%)	Infertile patients ($N=168$) (%)	Odds ratio (OR)	95% CI	P value
Asp/Asp and (TAAAA)n					
Asp/Asp and S/S	70 (41.6)	70 (41.6)	1.0 (Ref.)		
Asp/Asp and S/L	45 (26.8)	48 (28.5)	1.24	0.61–2.42	0.75
Asp/Asp and L/L	5 (3.0)	18 (10.7)	3.47	2.82–5.70	0.001
Asp/Asn and (TAAAA)n					
Asp/Asn and S/S	26 (15.5)	10 (6.0)	1.0 (Ref.)		
Asp/Asn and S/L	15 (8.9)	10 (6.0)	0.88	0.64–1.86	0.08
Asp/Asn and L/L	4 (2.4)	10 (6.0)	1.57	0.85–1.72	0.08
Asn/Asn and (TAAAA)n					
Asn/Asn and S/S	1 (0.6)	1 (0.6)	1.0 (Ref.)		
Asn/Asn and S/L	1 (0.6)	1 (0.6)	1.0	0.85–1.07	1.0
Asn/Asn and L/L	1 (0.6)	0 (0.00)	1.09	0.84–1.42	0.87

Abbreviations: CI: confidence interval; Ref.: referent.

Table 4
Relationship between different SHBG genotypes and serum levels of SHBG, total and free testosterone, and estradiol.

SHBG genotype	*n*	Sex hormone binding globulin[a]			Total testosterone (mean)			Free testosterone (mean)			Total estradiol (mean)			Free estradiol (mean)		
		nmol/l	95% CI[b]	*P* value	ng/ml	95% CI[b]	*P* value	pg/ml	95% CI[b]	*P* value	pg/ml	95% CI[b]	*P* value	pg/ml	95% CI[b]	*P* value
Asp237 Asn																
Asp/Asp	256	22.44	1.0 (Ref.)		5.17	1.0 (Ref.)		144.1	1.0 (Ref.)		18.9	1.0 (Ref.)		0.43	1.0 (Ref.)	
Asp/Asn	75	24.38	20.56–28.12	0.032	6.3	6 4.6–5.9	0.035	135.4	126–151	0.024	20.4	17.6–21.1	0.021	0.38	0.36–0.51	0.017
Asn/Asn	5	25.84	19.32–28.52	0.006	7.11	4.0–5.3	0.006	127.5	117–140	0.004	22.1	15.7–18.6	0.008	0.32	0.33–0.49	0.001
TAAAA repeat																
S/S	178	24.50	1.0 (Ref.)	6.24	1.0 (Ref.)	135.4	1.0 (Ref.)	20.1	1.0 (Ref.)	0.40	1.0 (Ref.)					
S/L	120	22.0	18.32–26.36	0.027	4.69	3.5–6.7	0.031	147.5	127–189	0.027	18.4	15.4–21.1	0.022	0.43	0.36–0.51	0.014
L/L	38	18.44	15.06–22.42	0.001	4.40	3.7–6.1	0.006	154.7	115–199	0.002	18.24	15.4–21.2	0.018	0.46	0.34–0.57	0.002

Abbreviations: CI: confidence interval; Ref.: referent.
[a] Geometric least-squares means adjusted for, age, BMI, duration of infertility, occupational status, and educational level.
[b] 95% CI for mean.

short-repeat carriers (23.48 nmol/l), carriers of the long-repeat allele (21.14 nmol/l) had significantly lower plasma levels of SHBG (Table 4). This association was influenced by the six-repeat allele heterozygotes (6/X), who had significantly higher mean SHBG levels than noncarriers of the six-repeat allele ($P = 0.006$).

Individuals with the S/S genotype (24.50 ± 4.4 nmol/l) had the highest serum levels of SHBG, and individuals with the L/L genotype had the lowest (18.44 ± 4.2 nmol/l) in both groups (Table 4). Men homozygous for the seven- and eight-repeat alleles were also observed to have higher levels of SHBG (versus noncarriers: 9/9 genotype, +11.6%, $P = 0.08$; 9/10 genotype, +14.1%, $P = 0.08$), although these associations did not reach statistical significance.

Also, we explored the combined effects of the Asp237Asn and the (TAAAA)*n* polymorphisms in predicting SHBG levels. The test for interaction between carriers and noncarriers of the Asp/Asp and the L/L genotypes was statistically significant ($P = 0.006$). Compared with noncarriers of the Asp/Asp and the L/L genotypes (24.88 nmol/l, 95% CI: 20.3–28.8), carriers of both of these alleles had significantly lower SHBG levels (20.45 nmol/l, 95% CI: 18.2–26.7, $P = 0.001$).

3.1.5. Relationship between of SHBG gene polymorphisms and androgens and estrogens

We found significant differences in total and free T levels with the Asp/Asn and Asn/Asn genotypes compared to the Asp/Asp genotype ($P = 0.008$, $P = 0.004$, respectively). The mean values ± SD of total testosterone was 6.4 ± 1.7 ng/m1 for the combined Asp/Asn and Asn/Asn genotypes and 5.17 ± 1.4 ng/ml for the Asp/Asp genotype ($P = 0.006$) (Table 4). A statistically significant difference was also found in free T levels between these two groups, with the combined Asp/Asn and Asn/Asn genotype group having lower free T levels (134.5 ± 14.14 pg/ml), compared with the Asp/Asp genotype group (144.1 ± 17.12 pg/ml) ($P = 0.004$). The mean values ± SD of estradiol (pg/ml) were 20.5 ± 4.5 for the Asp/Asn and Asn/Asn genotypes and 18.9 ± 4.7 for the Asp/Asp genotype (Table 4). However, we observed a lower level of free E2 in subjects with the Asp/Asn and Asn/Asn genotypes (0.37 ± 0.11 pg/ml) compared to the Asp/Asp genotype (0.43 ± 0.09 pg/ml) ($P = 0.002$). Regarding (TAAAA)*n* polymorphism, participants with L/L genotype had lower levels of total T and E2, but higher levels of free T and E2, compared to the S/S subjects (for details, see Table 4).

3.1.6. Multivariate analysis of the effects of SHBG gene polymorphisms on semen parameters

To assess the interaction between the polymorphisms, we stratified the men according to the presence or absence of the Asp allele (Asp/Asp genotype versus combined Asp/Asn and Asn/Asn genotype) and the total number of short- and long-repeat TAAAA alleles (S/S genotype versus S/L, and L/L genotype) (Table 5).

The sperm density ($14.8 \pm 3.2 \times 10^6$/ml), sperm motility ($24.0 \pm 4.2\%$), and sperm with normal morphology ($22.4 \pm 3.4\%$), in the infertile patients with combined Asp/Asn and Asn/Asn genotypes was significantly greater than the levels in the cases with Asp/Asp genotype ($12.6 \pm 3.3 \times 10^6$/ml, 20.8%, and 19.6%, respectively) ($P = 0.01$, $P = 0.008$, and $P = 0.006$, respectively). The levels of sperm density ($15.2 \pm 3.6 \times 10^6$/ml), sperm motility ($23.1 \pm 4.4\%$), and sperm with normal morphology ($22.1 \pm 3.3\%$), in the infertile patients with S/S genotype were significantly greater than the levels in the infertile subjects with L/L genotype ($10.8 \pm 3.1 \times 10^6$/ml, 17.6%, and 15.8%, respectively) ($P = 0.007$, $P = 0.004$, and $P = 0.003$, respectively). The same relationship between semen parameters and different SHBG genotypes also exited in normal controls (for details, see Table 5).

Table 5
Relationship between SHBG gene polymorphisms and semen parameters in fertile and infertile men.

Genotypes	*n*	Density ($\times 10^6$/ml)	95% CI[a]	*P* value	Motility (%)	95% CI[a]	*P* value	Morphology (%)	95% CI[a]	*P* value
Infertile men										
Asp237 Asn										
Asp/Asp	136	12.6	1.0 (Ref.)	20.8	1.0 (Ref.)	19.6	1.0 (Ref.)			
Asp/Asn	30	14.7	11.2–17.2	0.025	23.7	21.4–25.6	0.034	22.2	18.0–20.7	0.024
Asn/Asn	2	17.4	15.3–19.2	0.009	28.2	25.8–28.1	0.006	25.3	22.7–24.6	0.004
TAAAA repeat										
S/S	81	15.2	1.0 (Ref.)	23.1	1.0 (Ref.)	22.1	1.0 (Ref.)			
S/L	59	11.1	9.4–13.8	0.017	20.9	17.8–24.2	0.027	19.5	16.7–22.4	0.018
L/L	28	10.8	8.1–12.4	0.007	17.6	14.8–22.1	0.004	15.8	12.3–18.4	0.003
Fertile men										
Asp237 Asn										
Asp/Asp	120	56.4	1.0 (Ref.)	60.8	1.0 (Ref.)	56.6	1.0 (Ref.)			
Asp/Asn	45	59.2	51.4–67.3	0.037	68.2	61.3–77.6	0.036	59.4	55.7–63.4	0.044
Asn/Asn	3	63.4	55.2–73.4	0.002	70.2	62.8–78.4	0.004	62.4	57.8–67.2	0.006
TAAAA repeat										
S/S	97	59.1	1.0 (Ref.)	64.1	1.0 (Ref.)	59.0	1.0 (Ref.)			
S/L	61	55.7	44.5–66.4	0.023	61.5	54.8–68.2	0.026	55.8	48.7–62.4	0.034
L/L	10	49.2	38.2–60.4	0.001	60.5	53.8–68.9	0.004	52.5	45.2–59.9	0.006

Abbreviations: CI: confidence interval; Ref.: referent.
[a] 95% CI for mean.

3.1.7. Secondary analysis

To investigate whether the effects of SHBG genotype on levels of androgens and estrogens were mediated via SHBG levels, serum levels of SHBG were added to regression models investigating the effects of (TAAAA)*n* and Asp237 Asn polymorphisms on the above mentioned parameters. With the (TAAAA)*n* repeat polymorphism, the values for T measured by RIA were $\beta = -0.079$ and $P = 0.0001$ before and $\beta = -0.057$ and $P = 0.004$ after inclusion of SHBG levels. For E2, the values were $\beta = -0.105$ and $P = 0.008$ and $\beta = -0.089$ and $P = 0.02$, respectively. For free testosterone, the values were $\beta = -0.109$ and $P = 0.005$ and $\beta = -0.128$ and $P = 0.002$, respectively. For free E2, the values were $\beta = -0.112$ and $P = 0.004$ and $\beta = -0.124$ and $P = 0.001$, respectively. When this analysis was done for investigating the effects of Asp237Asn polymorphism the following results were observed: for measured T were $\beta = -0.091$ and $P = 0.001$ before and $\beta = -0.072$ and $P = 0.006$ after inclusion of SHBG levels. For E2, the values were $\beta = -0.112$ and $P = 0.009$ and $\beta = -0.086$ and $P = 0.03$, respectively. For free testosterone, the values were $\beta = -0.104$ and $P = 0.007$ and $\beta = -0.121$ and $P = 0.006$, respectively. For free E2, the values were $\beta = -0.97$ and $P = 0.006$ and $\beta = -0.118$ and $P = 0.004$, respectively.

SHBG level negatively correlated (Spearman correlation coefficient) significantly with the presence of Asp237 ($r = -0.618$, $P = 0.001$) and nine repeat TAAAA allele ($r = -0.733$, $P = 0.001$). Infertility status correlated with the presence of Asp237 ($r = 0.678$, $P = 0.001$) and long repeat TAAAA allele ($r = 0.743$, $P = 0.001$), and negatively correlated with the presence of Asn ($r = -0.678$, $P = 0.001$) and short repeat TAAAA alleles ($r = -0.743$, $P = 0.001$).

There were strong and significant positive correlations between plasma SHBG and sperm count ($r = 0.672$, $P = 0.01$), sperm motility ($r = 0.721$, $P = 0.01$) and sperm morphology ($r = 0.574$, $P = 0.02$).

4. Discussion

In the present study, we investigated the possible effect of SHBG gene polymorphism in the pathogenesis of idiopathic male infertility. In particular, we examined the combined effect of two functional polymorphisms, the SHBG Asp237Asn and the SHBG (TAAAA)*n* repeat polymorphisms on the development of male infertility. The distribution and frequency of SHBG (TAAAA)*n* repeat and Asn237 alleles vary widely across populations [14]. In our population, the SHBG gene had at least six alleles with between six and 11 TAAAA repeats, with six, eight, and nine repeats being the most common. This is in consistence with the study in which, the authors assessed associations of SHBG polymorphisms with serum SHBG levels in 698 White men [16]. On comparing the distribution of the polymorphic (TAAAA)*n* repeat alleles between the infertile and control groups, we found that infertile men had alleles with longer repeats (more than eight repeats) more frequently than normal men who had shorter alleles (less than eight repeats), in a much higher frequency. Furthermore, in both groups, genotypes with long alleles were associated with lower SHBG levels and higher free T and E2 levels than those with shorter alleles. This finding is consistent with previous studies, in which individuals with longer repeat alleles were reported to have significantly lower circulating SHBG levels than those with shorter repeat alleles [13,15,16]. It has been shown that the biological half-life is significantly higher for SHBG obtained from subjects homozygous for the variant Asn allele than for that from subjects homozygous for the wild-type Asp allele [22]. In the present study, we also observed that the variant Asn allele in the SHBG gene was associated with elevated plasma SHBG levels and a reduced infertility risk in men. The association of the Asn allele with higher serum SHBG concentration was also demonstrated in previous studies [13,23]. These polymorphic variants are known to influence androgen and estrogen availability. Increased production of SHBG, caused by genetic variations in the SHBG gene, results in an increase in the levels of inactive, bound sex-steroids such as T and a decrease in the concentration of active, unbound, or free sex-steroids such as free T. The importance of circulating SHBG in regulating bioavailable sex hormones levels has been studied in a variety of hormone-related disorders/diseases such as breast and prostate cancer; and in other diseases [22,24–27]. However, very little information is available on the effect and underlying mechanism(s) of these polymorphisms on male infertility. A decrease in testosterone/estrogen ratio has been demonstrated to be associated with infertility [2]. In this study, the testosterone/estradiol ratio was 0.30 and 0.26, in fertile and infertile men, respectively. It is doubtful, however, whether this modest increase in circulating free E2 levels is sufficient to importantly alter intratesticular E2 concentrations, and gonadotropin secretion.

The new finding of this study was the synergistic effect of the combined genotypes on the development of male infertility. Because the combinations of the different alleles might have masked the effects of some of them, we examined their effects in homozygous patients and found significant differences in SHBG levels between 6/6 and 9/9 and between Asn/Asn and Asp/Asp homozygous subjects. Focusing on the distribution of the combined polymorphic variants of the two polymorphisms, men with infertil-

ity tended to have more frequently the combination of long SHBG (TAAAA)*n* alleles with Asp/Asp genotype and less frequently the combination of short SHBG (TAAAA)*n* alleles and Asn/Asn genotype compared with healthy controls. The protective effect of the Asn allele was much stronger when accompanied with S/S genotype, and the interactions were statistically significant ($P = 0.006$). After adjusting for age, BMI and duration of infertility, infertile men with the combination of long SHBG (TAAAA)*n* allele and Asp/Asp genotype had the lowest SHBG, and total T and the highest free T, and free E2 levels compared with other patient subgroups. A similar and statistically significant trend was also seen among the healthy fertile men. Conversely, men with short SHBG (TAAAA)*n* allele and Asn/Asn genotype were found to have the greater total T and E2 profile, but lesser free T, and E2 profile. We cannot fully explain the underlying mechanism(s), which results impaired spermatogenesis with long-repeat and Asp alleles of the SHBG gene. Men with lower plasma SHBG, had also higher plasma free T, and free estrogen, concentrations. The beneficial effects of free bioavailable T on spermatogenesis are well known. Perhaps, elevated levels of plasma free E2, have deleterious effect on the process of spermatogenesis. A decrease in testosterone/estrogen ratio has been demonstrated to be associated with infertility [2]. Estrogens can provoke acute oxidative damage by increasing the oxidative metabolism in spermatozoa [28]. Down regulation of DNA repair enzymes [29] and antioxidant enzymes [30] by estrogens is also an assumed mechanism. Aromatase inhibitors, such as testolactone is used for the treatment of idiopathic oligozoospermia. This enzyme is responsible for the conversion of testosterone and androstenedione to estradiol [31]. Data from a large cohort of patients with abnormal testosterone-to-estradiol ratio levels treated with testolactone have demonstrated beneficial effects on the balance of serum androgens and estrogens, as well as semen parameters [2].

Previous studies have demonstrated that interindividual differences in circulating SHBG concentrations may in part be genetically determined [13,15,32]. Given the role of SHBG in reducing the risk of male infertility and the functionality of the Asp327Asn, and (TAAAA)*n* repeat polymorphisms in this gene, it is conceivable that men carrying the Asn and short-repeat alleles may be at a reduced risk of infertility. This hypothesis, however, has not been tested in epidemiologic studies. In summary, the results of this study suggested that men with infertility are more frequently carriers of long (TAAAA)*n* repeat and Asp alleles, in the promoter of the SHBG gene, and these are associated with lower plasma SHBG concentrations. These SHBG polymorphisms were also associated with serum total T and E2, and free T, and E2, levels in both fertile and infertile men.

The present study has several limitations and the results should be interpreted with caution. This study was somewhat limited by its moderate sample size ($n = 168$); after several stratifications, the numbers become small. For this reason, our finding regarding an interaction between SHBG genotypes, and infertility should be viewed as exploratory; replication of these results in larger, population-based, racially diverse cohorts is warranted. The cross-sectional nature of our study limits conclusions about the temporal relation of SHBG gene polymorphisms and the male infertility. Another limitation of the study is the low power (80%) of the study for gene–gene interactions and stratified analyses. Therefore the probability of chance findings in these analyses should be considered. The easiest way to improve precision is to increase the number of subjects and patients in the genetic studies. However, this may not be applicable to all studies due to such factors as additional costs, poorer availability of resources, lower population, which compromises the number of subjects eligible for investigation. Multiple statistical testing might also be a concern, mainly when examining the interactions of SHBG genotypes with other factors. We have not corrected *P*-values for multiple testing. Clearly, significant associations can arise from multiple testing though it is argued that correction is inappropriate as it risks type II errors [33]. In addition, as we know, the power of a case–control research to determine interactions is low compared to the power to detect main effect [34]. Thus, the interaction between SHBG genotypes and the infertility risk found in present study is still worth reporting.

5. Conclusion

In summary, our data suggest that the (TAAAA)9 repeat and Asp alleles of the SHBG gene may be associated with significant lower circulating SHBG levels and an increased risk for infertility in men. Our sample size, however, is not large enough to definitively draw clinically relevant conclusions.

Additional multicenter large studies with enough power are needed to confirm our results and to further characterize the biological effects of genetic variation in SHBG on spermatogenesis.

Conflict of interest

The authors declare that they have no conflict of interest whether of a financial or other nature.

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