Associations of Serum Sex Hormone Binding Globulin (SHBG) Levels with SHBG Gene Polymorphisms in the CARDIA Male Hormone Study

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In the sex hormone binding globulin (SHBG) gene, a pentanucleotide-repeat polymorphism [(TAAAA)ₙ] and a single nucleotide polymorphism (D327N) have been associated with circulating SHBG concentrations in women. Only one study, limited to Scandinavians, has examined these associations in men. Using data from the Coronary Artery Risk Development in Young Adults (CARDIA) Male Hormone Study, the authors assessed associations of SHBG polymorphisms with serum SHBG levels in 511 Black men and 698 White men who had SHBG measured in multiple serum samples collected over an 8-year period from 1987 to 1996 and were aged 20–34 years at the time of the first SHBG measurement. Multivariable repeated-measures analyses were used to assess associations of (TAAAA)ₙ and D327N polymorphisms with SHBG concentrations. Results showed statistically significant differences in mean SHBG concentrations for White men with genotypes of (TAAAA)₆/₆ (35.1 nmol/liter), 6/x (30.8 nmol/liter), and x/x (29.6 nmol/liter), where x represents a repeat length greater than 6 (p = 0.001). For Black men, the pattern of association was similar, albeit not statistically significant (p = 0.35). There was no relation between D327N genotype and SHBG levels. These results suggest that the (TAAAA)ₙ repeat length in the SHBG gene, but not the D327N variant, might contribute to the interindividual variability in serum SHBG levels.

Sex hormone binding globulin (SHBG) is the primary plasma transport protein for sex hormones (1). The SHBG gene is located on chromosome 17p12–p13 (2) and encodes a 402-amino-acid polypeptide (3, 4). A signal peptide of 29 amino acids is cleaved, and the resulting 373-amino-acid product undergoes posttranslational modifications (3, 5, 6). The wild-type polypeptide contains one O-linked and two N-linked glycosylation sites (3, 5). In exon 8 of the SHBG gene, a guanine-to-adenine transition (1066G→A) causes substitution of asparagine for aspartic acid at position 327 (D327N), which introduces an additional N-linked glycosylation site (7). In animal models, this substitution decreases the SHBG...
clearance rate (8) but does not appear to affect steroid binding (9, 10). A (TAAAA)$_n$ pentanucleotide polymorphism with a range of 6–11 repeats in humans (11–16) occurs in the promoter region and appears to affect transcriptional activity (11).

At least five studies have assessed relations between SHBG polymorphisms and serum SHBG levels among women with varying metabolic profiles (i.e., premenopausal, postmenopausal, polycystic ovarian syndrome, or hirsutism) (8, 12–15), and one study assessed the relation of the pentanucleotide polymorphism with serum SHBG in younger and older adult Swedish men (16). In women, a higher SHBG concentration was observed among carriers of the variant N327 allele (i.e., the $v$ allele) in comparison with carriers of only the wild-type allele D327 (i.e., the $W$ allele) (8, 13–15). In two studies of women (12, 13) and in the study of men (16), mean SHBG levels were higher for carriers of fewer TAAAA repeat lengths or carriers of the (TAAAA)$_n$ allele, whereas in another study of women (14), lower SHBG levels were observed among carriers of the (TAAAA)$_n$ allele.

Since SHBG affects the bioavailable fraction of circulating testosterone, it is possible that genetic factors associated with serum SHBG concentration contribute to pathologic conditions linked to testosterone in men (i.e., prostate cancer). However, we are aware of no study to date that has examined the distributions of SHBG polymorphisms in a large sample of healthy, young adult Black and White men or that assessed the associations of SHBG polymorphisms with circulating SHBG levels in men. Using data from the Coronary Artery Risk Development in Young Adults (CARDIA) Male Hormone Study, we assessed the associations of the D327N and (TAAAA)$_n$ polymorphisms with serum SHBG levels in 511 Black men and 698 White men.

MATERIALS AND METHODS

Subjects

The CARDIA Study is a multicenter longitudinal study on lifestyle and the evolution of cardiovascular disease risk factors in young Black and White men and women aged 18–30 years (17). At baseline (1985–1986), 1,157 Black men and 1,171 White men were screened at one of four US clinical centers. The CARDIA Male Hormone Study was designed to evaluate 8-year longitudinal changes in hormone levels and SHBG in serum collected at the year 2, year 7, and year 10 CARDIA examinations (18). Only men who had serum available from at least the year 2 and year 10 examinations were included (i.e., 624 Blacks and 796 Whites), with 1,211 having serum available from year 7.

Serum SHBG levels were normally distributed for both Black men and White men. We used $\chi^2$ analyses to assess Hardy-Weinberg equilibrium for each polymorphism and to test for potential linkage disequilibrium. First, we examined linkage disequilibrium between the presence of the (TAAAA)$_n$ and N327 $v$ alleles, because two other studies found significant linkage disequilibrium between these two alleles (14, 15). However, because in our study SHBG levels were highest for the (TAAAA)$_n$ homozygous group, we also

Data collection

At each examination, weight and height were measured using a balance-beam scale and a vertical ruler. Body mass index was calculated as weight (kg) divided by height squared (m$^2$). Age and race were self-reported. Fasting blood samples were collected by venipuncture between 7:30 a.m. and 12 noon from over 95 percent of CARDIA Male Hormone Study participants. Serum aliquots were stored at $-70^\circ$C. DNA was extracted from white blood cells and stored at $-80^\circ$C.

SHBG was measured with a chemiluminescent assay (Immulite kit; Diagnostic Products Corporation, Los Angeles, California). For each participant, all three serum samples were analyzed in the same batch and each participant was randomly assigned to a batch, reducing the likelihood of systematic difference in genotype frequency across batches. Assay variability was monitored by including 10 percent blind quality control samples in each analyzed batch. The intra- and interbatch variations were 7.9 percent and 11.2 percent, respectively.

(TAAAA)$_n$ genotype was determined using polymerase chain reaction (PCR). The forward primer was 5'-ATC GCT TGA ACT CGA GAG GC-3', and the reverse primer was 5'-5HEX/-CAG GCC CTA AAC AGT CTA GC-3'. The size of the PCR product was determined using an ABI 377 sequencer and GeneScan software (Applied Biosystems, Inc., Foster City, California). TAAAA repeat length was determined by comparing the length of the PCR product with a standard characterized by direct sequencing.

The 1066G $\rightarrow$ A single nucleotide polymorphism resulting in the D327N amino acid polymorphism was determined by real-time PCR. The forward primer was 5'-CGA GCC ACC TTA ATG CTC TA-3', the reverse primer was 5'-GAT CTC ATG GCT TCT GTT CAG G-3', and the wild-type probe was 5'-56-FAM/TCC CTC TAG GAG AAG ACT CCT CCA CCT CT/3BHQ-1/-3' (BHQ, black hole quencher). The variant probe was 5'-5HEX/TCC CTC TAG GAG AAA ACT CCT CCA CCT CT/3BHQ-1/-3' (Integrated DNA Technologies, Coralville, Iowa). Control plasmids containing the wild-type or variant cDNA were included with 14 of 33 total 96-well plates. Interpretation of real-time PCR results was confirmed through direct sequencing of samples selected from four of the 33 plates and restriction-fragment-length polymorphism analysis of samples selected from one of the 33 plates. HinfI digests the wild-type allele and produces two products of 54 and 89 base pairs but does not digest the variant allele.

For quality control, 5 percent of the DNA samples were randomly selected for reanalysis. Six of 136 (4.4 percent) (TAAAA)$_n$ alleles and two of 136 (1.5 percent) D327N alleles did not show consistent agreement.

Statistical analyses

Serum SHBG levels were normally distributed for both Black men and White men. We used $\chi^2$ analyses to assess Hardy-Weinberg equilibrium for each polymorphism and to test for potential linkage disequilibrium. First, we examined linkage disequilibrium between the presence of the (TAAAA)$_n$ and N327 $v$ alleles, because two other studies found significant linkage disequilibrium between these two alleles (14, 15). However, because in our study SHBG levels were highest for the (TAAAA)$_n$ homozygous group, we also
assessed linkage disequilibrium between the presence of the (TAAAA)₆ allele and the N327 v allele.

SHBG concentrations were compared among three groups of men who were homozygous for the (TAAAA)₆, (TAAAA)₈, and (TAAAA)₉ alleles and among three groups classified according to the total number of (TAAAA)ₙ alleles (i.e., 6/6 homozygosity vs. 6/x and x/x, where x represents a repeat-length allele greater than 6). For Black men and White men, analysis of covariance was used to examine the cross-sectional relations of the (TAAAA)ₙ and D327N genotypes with SHBG concentration after adjustment for age and body mass index as continuous variables. In repeated-measures analyses, the year (e.g., 2, 7, or 10) × (TAAAA)ₙ genotype and the D327N genotype interactions were tested; the pattern of changes in SHBG over time did not differ between the genotypes. A test for interaction showed no significant Black-White differences in the association of SHBG with either the pentanucleotide repeat genotype (p = 0.73) or the D327N genotype (p = 0.82). Therefore, analyses were also conducted for all men combined in which race was treated as a dichotomous dummy variable. Because there were no Black men with a 6/6 genotype and a W/v or v/v genotype, the interaction between the pentanucleotide repeat and the D327N polymorphisms was assessed for White men only and for Black and White men combined.

RESULTS

We identified six alleles ranging from 6 to 11 for the (TAAAA)ₙ repeat (figure 1). Although the 6, 8, and 9 alleles were most common, the distributions of alleles between Blacks and Whites were significantly different (p < 0.0001). For both Blacks and Whites, the 8 allele was most common, but the frequency was greater in Black men than in White men. Among Whites, the frequencies of the 6 (27.4 percent) and 9 (22.1 percent) alleles were greater than those for Blacks (20.9 percent and 14.0 percent, respectively). For Black men and White men, the (TAAAA)ₙ allele frequencies were in Hardy-Weinberg equilibrium (χ² = 8.91 (p = 0.88) and χ² = 18.99 (p = 0.59), respectively).

Cross-sectionally, serum SHBG concentrations were highest for the 6/6 group and, except for Black men at year 2, were similar between the 8/8 and 9/9 groups for both Black men and White men (table 1). There were no differences in longitudinal change in SHBG across genotypes (p > 0.05). In repeated-measures analysis, the differences in serum SHBG level across genotype groups were statistically significant for White men and for all men. For Black men, the patterns of association were similar to those for White men but were not statistically significant. Similarly, SHBG was highest for the 6/6 group and was comparable between the 6/x and x/x groups for Blacks and Whites; differences among genotype groups were statistically significant for White men and all men.

The frequency of the N327 v allele was 12.9 percent in White men and 4.7 percent in Black men. Among 511 Black men, one was homozygous for the v/v genotype (0.2 percent), as compared with nine of the 698 homozygous White men (1.3 percent). The D327N genotype frequencies adhered closely to Hardy-Weinberg equilibrium for both Blacks (χ² = 0.02, p = 0.90) and Whites (χ² = 0.77, p = 0.38). SHBG concentrations were not different across strata of D327N genotypes for any group (table 2), nor was there any genotype difference in the longitudinal change in SHBG over the 8-year follow-up period.

For Black men, there was no evidence of linkage disequilibrium between N327 v and either the (TAAAA)₉ allele (p = 0.94) or the (TAAAA)₉ allele (p = 0.75). However, there

were 156 White men who possessed at least one (TAAAA)$_8$ allele and one N327 $v$ allele. On the basis of random allelic assortment, one would predict this combination in 104 persons ($\chi^2 = 87.5, p < 0.0001$). Similarly, 50 White men had at least one (TAAAA)$_6$ allele and one N327 $v$ allele, whereas 79 were expected ($\chi^2 = 25.6, p < 0.0001$).

To assess the interaction between the polymorphisms, we stratified the men according to the presence or absence of the N327 allele (i.e., $WW$ vs. $Wv$ or $vv$) and the total number of (TAAAA)$_n$ alleles (6/6 vs. other) (table 3). Among White men with the $WW$ genotype, mean SHBG level was higher for those with the 6/6 genotype than for those with other (TAAAA)$_n$ genotypes, whereas for the $Wv$ and $vv$ groups, mean SHBG concentration was lower among men with the 6/6 genotype. The test of interaction was statistically significant ($p = 0.045$). Among Blacks, mean SHBG levels were similar to those among Whites; however, there were no Black men who were 6/6 homozygous and carriers of the N327 variant allele, precluding a test of genotype interaction. For all men combined, there was only a marginally significant interaction ($p = 0.066$) between the pentanucleotide repeat and the D327N polymorphism.

**DISCUSSION**

In the CARDIA Male Hormone Study, we found differences in allele frequencies for the TAAAA pentanucleotide repeat length and the D327N single nucleotide polymorphism in the $SHBG$ gene between young adult Black men and White men. Additionally, we observed significantly higher serum SHBG levels for men who were homozygous for the (TAAAA)$_n$ allele than for men with other genotypes. In contrast, SHBG was not associated with the D327N polymorphism. There was weak evidence of an interaction between the pentanucleotide repeat length and the D327N polymorphisms for White men in particular.
Importantly, there were no associations between genotype and change in serum SHBG level over 8 years of follow-up. In our study, the 6, 8, and 9 pentanucleotide repeats were the most common, with frequencies of 14 percent or more for both Blacks and Whites. The (TAAAA)₈ allele was more frequent in Blacks (56 percent) than in Whites (38 percent), whereas the (TAAAA)₉ allele was more common in Whites. Similar results were observed in a study of postmenopausal women (14), where the 6, 8, and 9 repeat lengths also were most commonly observed (i.e., 14 percent), but the (TAAAA)₈ allele was more common and the (TAAAA)₆ allele was less common in Blacks than in Whites. In our study, the N327v allele constituted approximately 13 percent of alleles among White men and 4.7 percent among Black men. Similarly, in another study (10), the frequency of the variant allele was lower in persons from Zaire (3 percent) than in persons from Belgium (12 percent) or Finland (7.8 percent). Among postmenopausal women, the frequency of the N327v allele was 2 percent in African Americans and 10 percent in Whites (14). These differences in the N327v allele frequency between persons of African descent and persons of European descent suggest that the D327N polymorphism might constitute an ancestry-informative marker.

Comparing our results with those of other studies is somewhat complex, for several reasons: 1) there is a large number of possible genotypes for the pentanucleotide repeat length; 2) not all studies that assessed the association of (TAAAA)ₙ with serum SHBG concentration classified participants using the same criteria; and 3) most studies were conducted in women with varying metabolic profiles. Nonetheless, our finding of a significantly higher mean SHBG concentration among men who were homozygous for the (TAAAA)₆ allele is similar to that of Eriksson et al. (16), who observed 17 percent and 22 percent higher serum SHBG levels in 3,000 younger and 1,068 older Swedish men who were homozygous for the (TAAAA)₆ allele as compared with men who were not carriers of the (TAAAA)₆ allele. In our study, serum SHBG concentrations were 14–16 percent higher in men

| TABLE 2. Adjusted* least-square mean concentrations of sex hormone binding globulin (nmol/liter) among men in the CARDIA Male Hormone Study, by D327N genotype, over an 8-year period from 1987 to 1996 |
|----------|----------|----------|----------|----------|------------------|
| Black men | | | | | |
| WW | 464 | 31.8 | 29.9 | 29.1 | 30.4 (0.5)† |
| Wv | 46 | 31.4 | 29.4 | 28.6 | 29.6 (1.7) |
| v/v | 1 | 42.7 | 28.7 | 34.5 | 35.8 (11.5) |
| p value | | | | | 0.81 |
| White men | | | | | |
| WW | 527 | 31.2 | 30.8 | 30.4 | 30.7 (0.5) |
| Wv | 162 | 30.9 | 30.3 | 29.3 | 30.2 (0.9) |
| v/v | 9 | 29.8 | 27.2 | 27.7 | 28.4 (3.6) |
| p value | | | | | 0.71 |
| All men | | | | | |
| WW | 991 | 31.6 | 30.5 | 29.9 | 30.7 (0.4) |
| Wv | 208 | 31.3 | 30.0 | 29.0 | 30.0 (0.8) |
| v/v | 10 | 31.3 | 27.0 | 27.9 | 29.0 (3.5) |
| p value | | | | | 0.67 |

* Adjusted for age, body mass index (weight (kg)/height (m)²), and race.
† Numbers in parentheses, standard error.

| TABLE 3. Results from repeated-measures analysis of serum sex hormone binding globulin concentration* over an 8-year period from 1987 to 1996 for combined genotype groups among men in the CARDIA Male Hormone Study |
|----------|----------|----------|----------|----------|
| (TAAAA)ₙ genotype | D327N genotype | WW | Wv or v/v |
| (TAAAA)ₙ genotype | No. of subjects | Mean SHBG level (nmol/liter) | No. of subjects | Mean SHBG level (nmol/liter) |
| Black men | | | | |
| 6/6 | 21 | 33.9 (2.5)‡ | 0 | |
| Other | 443 | 30.2 (0.5) | 47 | 29.7 (1.7) |
| White men | | | | |
| 6/6 | 58 | 35.8 (1.4) | 4 | 24.6 (5.4) |
| Other | 469 | 30.1 (0.5) | 167 | 30.2 (0.8) |
| All men | | | | |
| 6/6 | 79 | 35.4 (1.3) | 4 | 24.7 (5.6) |
| Other | 912 | 30.3 (0.4) | 214 | 30.2 (0.8) |

* Adjusted for time-dependent age, body mass index (weight (kg)/height (m)²), and race.
‡ SHBG, sex hormone binding globulin.
§ Numbers in parentheses, standard error.

SHBG, sex hormone binding globulin.
whose TAAAA genotype was 6/6 than in men with other pentanucleotide repeat genotypes. Importantly, while the associations of SHBG with pentanucleotide repeat genotypes were not statistically significant for Black men, a test of interaction showed no differences in the patterns of association between Black men and White men. In a study of 303 hirsute French women, SHBG concentration was higher for women with the 6/6 genotype than for those with the 8/8 or 9/9 genotype; however, the highest SHBG levels were observed in two women with the 10/10 genotype (13). Among 185 Greek women with polycystic ovarian syndrome, mean serum SHBG concentration was higher for women who had one allele with eight or fewer TAAAA repeats than for women who were homozygous for the (TAAAA)_8 repeat or repeats longer than 8 (12). Conversely, in a study of 372 healthy postmenopausal women, there were marginally significant lower SHBG concentrations for carriers of the 6 allele than for other women (14). Findings were similar, albeit not statistically significant, for the 81 African-American women included in that analysis. Reasons for inconsistencies across studies in the association between serum SHBG and pentanucleotide repeat-length polymorphisms are unclear. In vitro, the presence of the (TAAAA)_6 allele reduces SHBG gene expression (11), supporting the finding of lower SHBG levels with the (TAAAA)_6 repeat, as was observed in the study of postmenopausal women (14). However, it is possible that variations in sex hormone profiles across study participants could differentially affect gene expression. Among the three other studies that found higher SHBG levels for carriers of the (TAAAA)_6 allele, the study participants (i.e., women with hirsutism, women with polycystic ovarian syndrome, and men) are expected to have higher mean androgen levels than healthy postmenopausal women.

We observed no association between serum SHBG concentration and the D327N genotype for either Black men or White men. In two studies of postmenopausal women (14, 15) and in two studies of hirsute French women (8, 13), serum SHBG levels were higher for carriers of the variant allele than for women who were homozygous for the wild-type allele. In the only other study of women to include African Americans, there were only three women who were carriers of the N327_v allele (14), thus limiting an ethnicity-specific analysis. Similar to the pentanucleotide repeat length, the physiologic consequence of the N327_v allele also could differ between women and men. Indeed, in vitro, the variant N327 protein showed a significantly longer half-life than the wild type, and the variant protein had a significantly longer half-life if purified from a woman than if purified from a man (8). Furthermore, an inverse association between the N327_v variant and estradiol:SHBG ratios among postmenopausal women has been reported (15). These observations are consistent with a possible differential effect of the variant allele in women as compared with men.

Whether there are interactions between the two SHBG genotypes in relation to serum SHBG level is unclear. In the study of healthy postmenopausal women, there was a statistically significant interaction (p = 0.006) such that among women who were homozygous for the wild-type D327 allele, there was no difference in SHBG concentration between carriers and noncarriers of the (TAAAA)_6 allele, but for carriers of the N327_v allele, serum SHBG concentration was lowest among carriers of the (TAAAA)_6 allele (14). Similarly, our findings showed the lowest SHBG concentrations among men who were carriers of the N327_v allele and were homozygous for the (TAAAA)_6 allele. These results should be interpreted cautiously, given the small number of men with these genotypes.

Similarly to investigators in other studies of White populations (14, 15), we observed strong linkage disequilibrium between the presence of the N327_v allele and either the (TAAAA)_8 allele or the (TAAAA)_6 allele in White men. However, similarly to the study of postmenopausal African-American women (14), there was no linkage disequilibrium among the Black men in our study. This might be due to the fact that there were only 47 Black carriers of the variant N327_v allele in our study and in the study of healthy postmenopausal African-American women there were only three (14), which limited the power to detect significant linkage disequilibrium. Moreover, at least 12 single nucleotide polymorphisms map between TAAAA and D327N, and they could be in strong linkage disequilibrium with the two markers assayed in this study. Three of the 12 variants are nonsynonymous changes (rs9282845, rs6260, and rs6258) and might be functional. However, these variants are rare, and their impact in the population is probably small.

The strengths of the current study were the availability of genotype information and the collection of longitudinal data on serum SHBG concentration and anthropometric measures for a large number of young adult Black and White men. Despite the large sample size, power to detect potential interactions between genotypes was limited.

In summary, our results showed differences between Black men and White men in allele frequencies for the SHBG gene pentanucleotide repeat length and the D327N single nucleotide polymorphism. They also showed the highest SHBG concentrations among men who were homozygous for the (TAAAA)_6 allele but no difference in SHBG level according to D327N genotype. The absence of an association between SHBG level and D327N genotype in men contrasts with studies in women, suggesting a potential gender difference in the physiologic effect of the N327_v allele.

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