Hormonal Markers and Hepatitis B Virus-Related Hepatocellular Carcinoma Risk: a Nested Case–Control Study Among Men

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Background: **The incidence of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) is higher in men than in women. We examined whether endogenous sex hormone levels or hormone-related factors might affect the risk of HCC in men.** ***Methods:*** **Baseline blood samples were collected from 4841 male Taiwanese HBV carriers without diagnosed HCC from 1988 through 1992. Plasma testosterone and estradiol levels and genetic polymorphisms in the hormone-related factors cytochrome P450c17α (CYP17, A1 versus A2 alleles), steroid 5α-reductase type II (SRD5A2, valine [V] versus leucine [L] alleles), and androgen receptor (AR, number of CAG repeats) were assayed among 119 case patients who were diagnosed with HCC during 12 years of follow-up and 238 control subjects. All statistical tests were two-sided.** ***Results:*** **The risk of HCC increased with increasing concentrations of testosterone (odds ratio [OR]$_{\text{highest versus lowest tertile}}$ = 2.97; 95% confidence interval [CI] = 1.54 to 5.70; P_{trend} <.001) and with increasing number of the V allele of the SRD5A2 V89L polymorphism (OR$_{\text{VV versus LL genotype}}$ = 2.47; 95% CI = 1.21 to 5.03; P_{trend} = .011). Fewer AR gene CAG repeats (<23 repeats) were associated with a 1.64-fold (95% CI = 1.00 to 2.68) increased risk of HCC. Although the CYP17 genotype alone did not increase the risk of HCC, there was evidence of a gene–gene interaction, because the CYP17 A1 allele statistically significantly increased the risk of HCC in the presence of fewer AR gene CAG repeats (OR = 2.51; 95% CI = 1.06 to 5.94). We found a similar interaction between the SRD5A2 VV genotype and fewer AR gene CAG repeats (OR = 5.58; 95% CI = 1.86 to 16.71). Body mass index (BMI) modified the association of HCC with testosterone and SRD5A2 genotype; in men with low BMI, multivariate-adjusted ORs for the highest tertile of testosterone versus the lowest and the SRD5A2 VV genotype versus the LL genotype were 7.63 (95% CI = 2.13 to 27.27) and 8.64 (95% CI = 2.75 to 27.14), respectively. No clear associations were found between estradiol or testosterone-to-estradiol ratio and HCC.** ***Conclusions:*** **Pathways involving androgen signaling may affect the risk of HBV-related HCC among men. [J Natl Cancer Inst 2001;93:1644–51]**

Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) has a major role in the development of hepatocellular carcinoma (HCC). HCC is more prevalent in men than in women throughout the world *(1)*. In Taiwan, where HBV infection is hyperendemic, the incidence of HCC for men is approximately three times that for women, despite similar rates of chronic HBV infection *(2)*. The predominance of males with HCC has also long been observed in various animal models, including transgenic mice expressing HBV or HCV proteins *(3–11)*. Castration of male mice decreased the incidence of chemically induced HCC compared with that of intact males, whereas chronic testosterone administration to female or castrated male animals increased the risk of spontaneous or chemically induced HCC *(3–8)*. In addition, one animal model *(7)* found that ovariectomy increased the incidence of HCC because of partial hepatectomy in chemically induced hepatocarcinogenesis. However, the long-term treatment effects of various synthetic forms of estrogens on hepatocarcinogenesis in animals remain controversial *(3,12–17)*. Although these findings suggest that the pathogenesis of HCC may be influenced by the hormonal environment of the host, data about the role of endogenous sex hormones in human hepatocarcinogenesis are limited *(18–22)*.

In addition to testosterone, proteins involved in androgen transport and metabolism also may play a critical role in the development of HCC. We have begun to explore the genes of three such proteins: the androgen receptor (AR), the steroid 5α-reductase type II (SRD5A2), and the cytochrome P450c17α (CYP17). The AR gene is responsible for androgen transport; the SRD5A2 gene encodes the enzyme responsible for conversion of testosterone to the more active androgen (in terms of AR affinity); dihydrotestosterone, and the CYP17 gene encodes an enzyme that catalyzes critical steps in steroid genesis *(23)*. The AR gene is located on the X chromosome. Its exon 1 contains a polymorphic CAG microsatellite that codes for variable-length polyglutamine in the AR protein *(23)*. Our initial study *(21)* has suggested that higher testosterone levels or fewer number of AR-CAG repeats may increase HBV-related HCC risk in men. There are several SRD5A2 polymorphisms, with the most common being SRD5A2 V89L (valine [V] at codon 89 to leucine [L]) and the least common being SRD5A2 A49T (alanine [A] at codon 49 to threonine [T]) *(23–27)*. Furthermore, the SRD5A2 is expressed in the liver of adults *(28)*. The CYP17 polymorphism was detected by restriction digest with *Msp*A1I. This digestion distinguishes two alleles designated as A1 and A2. The SRD5A2 V89L polymorphism and the CYP17 polymorphism have been associated with circulating sex hormone levels *(23–25,29,30)*.

In this nested case–control study, we dissected the role of endogenous estradiol in the etiology of HBV-related HCC among men who test positive for the HBV surface antigen (HBsAg carriers) and extended our original study *(21)* on the evaluation of the androgen hypothesis concerning hepatocarcinogenesis to the investigation of the possible association between the SRD5A2 and CYP17 polymorphisms and HCC development. We also assessed whether there might be interactions between hormone-related factors, including sex hormone levels and genes involved in androgen biosynthesis (CYP17), activation (SRD5A2), and transport (AR), in determining HCC risk.

Subjects and Methods

Study Population

The cohort consisted of 4841 men, aged 30 years or older, who had tested positive for the HBsAg

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(HBsAg carriers) and who attended a specific clinic for asymptomatic HBsAg carriers at the Liver Unit of Chang-Gung Memorial Hospital or the Government Employee Central Clinics (Taipei, Taiwan) for regular health examinations, from 1988 through 1992 *(21)*. Written informed consent was obtained from all study participants. The investigation was approved by the research ethics committee at the College of Public Health, National Taiwan University, Taipei, and by the appropriate institutional review board.

After an initial baseline examination, including ultrasonography measurements and conventional liver function tests, an in-person interview was conducted by trained research assistants with the use of a structured questionnaire to obtain information on demographic and anthropometric characteristics, lifetime habits of alcohol and tobacco use, as well as personal and family histories of major chronic diseases. A blood specimen also was collected at the end of the interview.

Participants were monitored for incident HCC through various channels, including periodic ultrasonography measurements and conventional liver function tests every 6–12 months, a personal telephone interview, abstraction of medical records, and a data linkage to the national death certification and cancer registry systems. After 12 years of follow-up examinations, approximately 70% of the HBsAg carriers who were still alive continued to return for their examinations. Subjects who did not participate in the follow-up examinations were traced and contacted by telephone and by data from the computer files of national cancer registry and death certification systems. In Taiwan, if a patient is diagnosed with or treated for cancer in a hospital with 50 or more beds, then the hospital has a legal obligation to report cancer patients to the national cancer registry. Case ascertainment by the registry through the hospital system is estimated to be 85% complete. Computer files of the death certification system are routinely matched against profiles of members of the cohort. Thus, data on the vital status of all cohort members and the causes of all deaths are complete. When a case patient with HCC was identified, permission was sought from the hospital where the subject was diagnosed with cancer to obtain medical charts and pathology reports. Each case patient was diagnosed on the basis of either a histologic finding or elevated serum α-fetoprotein level (⩾400 ng/mL) combined with at least one positive image on angiography, sonography, and/or computed tomography. By August 31, 2000, we confirmed 146 incident case patients with HCC.

For each case patient, two control subjects were selected at random from the cohort of HBsAg carriers who were alive and remained unaffected with HCC throughout the follow-up period. The control subjects were matched to case patients by date of blood collection (within 3 months) and year of birth (within 5 years, except for one elderly case patient, who was matched within 10 years). These control subjects have been included in a previous nested case–control study on the role of the AR gene CAG repeats and elevated testosterone levels in the development of HCC *(21)*. Five case patients who were included in the previous study *(21)* were not included in the present study because of insufficient DNA samples. Fourteen case patients identified since the end of the follow-up period in the previous study *(21)* were added to the present study. Finally, a total of 119 case patients and 238 control subjects were included in the present study.

Laboratory Analyses

A blood sample was collected from each member of the cohort and processed to isolate white blood cells, serum, and plasma. All components were frozen at –70 °C until analysis.

The status of serum HBsAg was determined by a radioimmunoassay (Abbott Laboratories, Chicago, IL). DNA was extracted from frozen white blood cells according to a standardized protocol basically as described previously *(31,32)* but with some modifications. The CAG trinucleotide repeat found in exon 1 of the AR gene was amplified, and the number of repeats was determined as described previously *(33)*. The CYP17 genotype was determined by use of the polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) method of Feigelson et al. *(29)*, in which restriction digest by *Msp*A1I (New England Biolabs, Inc., Beverly, MA) identifies the presence of the A2 allele. The SDR5A2 V89L polymorphism was determined by use of PCR–RFLP, in which 100 ng of genomic DNA was amplified in the presence of 5 pmol of each of the primers 5′-TCGGGCCACCTGGGACGCTAC-3′ (SRD5A2–960) and 5′-GTTCCTCACAGCGCCCCACGC-3′ (SRD5A2R), 1× *Taq* buffer (i.e., 50 mmol/L potassium chloride, 1.5 mmol/L magnesium chloride, and 10 mmol/L Tris–HCl [pH 9.0]), 5 mmol/L of each deoxynucleoside triphosphate, and 0.5 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ), in a total volume of 40 μL. The first primer contained a mismatched base (underlined) that leads to the loss of a *Rsa*I restriction site in the amplified products. The amplification reaction consisted of an initial denaturation step at 94 °C for 4 minutes, followed by 35 cycles of 40 seconds at 94 °C, 25 seconds at 65 °C, 30 seconds at 72 °C, and a final extension at 72 °C for 10 minutes. The PCR products then were digested overnight with 2 U of *Rsa*I (Roche Molecular Biochemicals, Mannheim, Germany) at 37 °C. The digested fragments were separated on a 2.5% agarose gel and visualized after staining with ethidium bromide. The VV, VL, and LL genotypes resulted in 137 and 40; 177, 137 and 40; and 177-base-pair fragments, respectively. We confirmed PCR–RFLP genotypes by BigDye terminator cycle sequencing of the polymorphic region (Applied Biosystems, Foster City, CA).

Testosterone was measured from the plasma by a competitive immunoassay that uses direct chemiluminescent technology (Chiron Diagnostics Corporation, East Walpole, MA), and estradiol was measured by use of radioimmunoassay kits obtained from Diagnostic Systems Laboratories, Inc., Webster, TX (Catalog #DSL 4800). All assays were carried out and interpreted by individuals blinded to the case–control status of the sample.

Statistical Methods

Odds ratios (ORs) and 95% confidence intervals (CIs) from unconditional logistic regression models were used to evaluate relative risks. Multivariate-adjusted ORs of HCC associated with various hormone-related factors were computed after adjustment for matching variables (e.g., age at recruitment and the time that blood was drawn) and potential confounders. Cigarette smoking and alcohol consumption are risk factors for HCC that might be associated with chronic liver disease *(34)*, and chronic liver disease might influence the phenotype of the SRD5A2 and CYP17 gene by altering the enzymatic activity. In the multivariate analyses on genotype and HCC, cigarette smoking, alcohol consumption, and chronic liver disease were thus also included in the logistic regression models as covariates.

The numbers of AR-CAG repeats were originally categorized into four groups (>24, 23–24, 21–22, and ⩽20 repeats) as described in our previous study *(21)*. To gain statistical power and to avoid sparse cells occurring in stratified analyses on the interactions of the AR gene and other hormonal markers, a cut point of fewer than 23 repeats was finally chosen, after combining categories with similar risks. Although the estradiol levels are reported in picograms per milliliters, the ratio of testosterone to estradiol was calculated as the testosterone level in nanograms per milliliters divided by the estradiol level in nanograms per milliliters. Tertile cut points were used for continuous hormone-related variables because of the distribution among the control group. Tertile cut points were chosen to avoid sparse cells so that both their independent effects and their interactive effects with other factors could be determined. Categoric trends were tested in logistic regression by use of an ordered categoric variable. Stratum-specific analyses were compared to evaluate the potential modification effect for each hormone-related factor. Statistical significance of the modification effect of each hormone-related factor on the HCC risk associated with other hormonal factors was determined by comparing the fit of the logistic model that included the main effects and all potential confounders with a fully parameterized model containing all possible two-factor interaction terms for the variables of interest. All *P* values were from two-tailed tests. All analyses were conducted using SAS release 6.12 (SAS Institute, Inc., Cary, NC).

RESULTS

From the cohort of 4841 male HBsAg carriers, 119 who developed HCC during the follow-up period were included in this study. On average, 4.8 years (range, 0.25–11.25 years) elapsed between blood collection and diagnosis. The mean age at recruitment was 50.6 ± 9.3 years for case patients and 50.3 ± 9.0 years for control subjects ($P = .746$). For case patients, the mean age at diagnosis of cancer was 55.5 ± 9.0 years (range, 36–78 years).

To estimate the risk associated with each genetic polymorphism or hormone level, we determined the OR. The crude ORs associated with HCC for the VL and VV genotypes of the SRD5A2 V89L polymorphism were 1.34 (95% CI = 0.81 to 2.21) and 1.61 (95% CI = 0.85 to 3.05), respectively, compared with the LL genotype as the reference group. Compared with HBV carriers in the lowest ter-

tile of testosterone levels, the crude ORs for those in the middle and highest tertile were 1.28 (95% CI = 0.70 to 2.36) and 2.36 (95% CI = 1.35 to 4.12). The crude ORs were 1.43 (95% CI = 0.92 to 2.22) when comparing shorter (<23 repeats) with longer CAG-repeat lengths (data not shown). After adjustment for matching factors (i.e., age at recruitment and the time that blood was drawn), other HCC risk factors (i.e., cigarette smoking, alcohol consumption, and history of chronic liver disease) *(35–37),* and selected sociodemographic characteristics, the VV genotype of the SRD5A2 V89L polymorphism was statistically significantly associated with an increased risk of HCC (OR = 2.17; 95% CI = 1.10 to 4.29) compared with the LL genotype. Inclusion of these covariates did not substantially alter the ORs for testosterone. Case patients were more likely than control subjects to have AR-CAG repeats of fewer than 23 repeats, yet this association was not statistically significant (P = .099) (model 1, Table 1).

For the investigation of the impact of the adjustment for the potential confounding effects by body mass index (BMI) and other hormone-related factors on the OR of HCC for each hormone-related factor, BMI and other hormone-related factors were further added as covariates in the logistic regression model that analyzed the main effect of each hormone-related factor (model 2, Table 1). We found that the association became even stronger for HCC and testosterone or the SRD5A2 V89L polymorphism. An increasing trend in HCC risk was observed with increasing concentrations of testosterone (P_{trend} <.001) and with an increasing number of

Table 1. Association of selected hormone-related factors with risk of HCC among male HBsAg carriers in Taiwan*

Hormone-related factors	Case patients (n = 119)		Control subjects (n = 238)		Model 1† Adjusted OR	Model 1† 95% CI	Model 2‡ Adjusted OR	Model 2‡ 95% CI
CYP17 polymorphism								
A2/A2	43	(36.1%)	90	(37.8%)	1.00	Referent	1.00	Referent
A1/A2	56	(47.1%)	111	(46.6%)	1.07	0.64 to 1.77	1.15	0.67 to 1.96
A1/A1	20	(16.8%)	37	(15.6%)	1.18	0.60 to 2.34	1.23	0.59 to 2.56
SRD5A2 V89L polymorphism								
LL	35	(29.4%)	88	(37.0%)	1.00	Referent	1.00§	Referent
VL	59	(49.6%)	111	(46.6%)	1.45	0.85 to 2.47	1.60	0.92 to 2.80
VV	25	(21.0%)	39	(16.4%)	2.17	1.10 to 4.29	2.47	1.21 to 5.03
Testosterone, ng/mL								
0.87–4.73	25	(21.0%)	78	(32.8%)	1.00	Referent	1.00‖	Referent
4.74–6.47	32	(26.9%)	78	(32.8%)	1.23	0.65 to 2.33	1.41	0.73 to 2.75
6.48–13.99	62	(52.1%)	82	(34.4%)	2.20	1.21 to 3.98	2.97	1.54 to 5.70
Estradiol, pg/mL								
3.7–13.7	32	(27.3%)	74	(31.4%)	1.00	Referent	1.00	Referent
13.8–17.3	38	(32.5%)	77	(32.6%)	1.19	0.64 to 2.20	1.31	0.68 to 2.50
17.4–53.8	47	(40.2%)	85	(36.0%)	1.21	0.67 to 2.19	1.16	0.63 to 2.17
Missing	2		2					
T/E2 ratio¶								
75.7–288.8	35	(29.9%)	78	(33.1%)	1.00	Referent	1.00	Referent
288.9–436.4	40	(34.2%)	77	(32.6%)	1.02	0.57 to 1.82	1.08	0.59 to 1.97
436.5–2539.7	42	(35.9%)	81	(34.3%)	1.03	0.57 to 1.88	1.16	0.61 to 2.17
Missing	2		2					
No. of AR gene CAG repeats								
25–35	33	(27.7%)	73	(30.7%)	1.00	Referent		
23–24	26	(21.8%)	66	(27.7%)	0.80	0.42 to 1.51		
21–22	43	(36.1%)	74	(31.1%)	1.29	0.72 to 2.32		
14–20	17	(14.3%)	25	(10.5%)	1.44	0.66 to 3.14		
Presence of AR alleles with <23 CAG repeats								
No	59	(49.6%)	139	(58.4%)	1.00	Referent	1.00	Referent
Yes	60	(50.4%)	99	(41.6%)	1.48	0.93 to 2.35	1.64	1.00 to 2.68
BMI, kg/m^2								
16.7–22.0	34	(28.6%)	79	(33.2%)	1.00	Referent	1.00	Referent
22.1–24.5	39	(32.8%)	78	(32.8%)	1.20	0.67 to 2.15	1.52	0.81 to 2.87
24.6–32.0	46	(38.7%)	81	(34.0%)	1.42	0.80 to 2.53	1.98	1.05 to 3.74

*HCC = hepatocellular carcinoma; HBsAg = hepatitis B surface antigen; OR = odds ratio; CI = confidence interval; T/E2 ratio = testosterone-to-estradiol ratio; AR = androgen receptor; BMI = body mass index; SRD5A2 = steroid 5α-reductase type II; CYP17 = cytochrome P450c17α.

†Adjusted for age at recruitment (continuous variable), the time of blood draw (continuous variable), ethnicity (Fukien Taiwanese, Hakka Taiwanese, and Mainland Chinese), years of education (continuous variable), cigarette smoking, alcohol consumption, and history of chronic liver disease.

‡For the T/E2 ratio, ORs have been adjusted for covariates in model 1, BMI, and other hormone-related factors listed in the table, except for testosterone and estadiol. For other hormone-related factors, ORs have been adjusted for the same covariates that we treated for the hormone ratio, but testosterone and estradiol were included as covariates instead of the ratio. All variables were included in the logistic regression models as categorized in the table (the number of AR-CAG repeats was included as a dichotomous variable), except for BMI, which was included as a continuous variable for adjusting for its effect. Two case patients and two control subjects were excluded from analysis because of missing data on plasma estradiol.

§P_{trend} = .011.

‖P_{trend}<.001.

¶T/E2 ratio = testosterone in nanograms per milliliters divided by estadiol in nanograms per milliliters.

V alleles of the SRD5A2 polymorphism (P_{trend} = .011). After adjustment for BMI and other hormone-related factors, male HBsAg carriers with fewer than 23 AR-CAG repeats were found to have a statistically significantly increased risk of HCC (OR = 1.64; 95% CI = 1.00 to 2.68) compared with those with at least 23 AR-CAG repeats (P = .048). Also, the positive association between tertile distribution of BMI and the risk of HCC became statistically significant (highest versus lowest tertile; OR = 1.98; 95% CI = 1.05 to 3.74) after adjustment for other hormone-related factors.

We found no statistically significant association between the risk of HCC and estradiol, testosterone-to-estradiol ratio, or the CYP17 genotype (Table 1). Moreover, when estradiol values were categorized as quartiles in an unconditional logistic regression model adjusted for the matching factors (i.e., age at recruitment and the time that blood was drawn), cigarette and alcohol use, history of chronic liver disease, years of education, ethnicity, BMI, and other hormone-related factors, there was still little evidence for an effect of estradiol (multivariate-adjusted ORs by quartile = 1.00 [referent], 1.01 [95% CI = 0.49 to 2.08], 0.79 [95% CI = 0.37 to 1.68], and 1.39 [95% CI = 0.69 to 2.79]). A similar finding was observed for the testosterone-to-estradiol ratio (multivariate-adjusted ORs by quartile = 1.00 [referent], 1.03 [95% CI = 0.51 to 2.07], 1.13 [95% CI = 0.56 to 2.27], and 1.17 [95% CI = 0.57 to 2.41]).

We also examined the interactions between any two hormone-related factors. Although the strength of the interaction between the SRD5A2 genotype and AR-CAG repeats approached statistical significance ($P_{interaction}$ = .084), the association between the SRD5A2 genotype and HCC appeared to be stronger for male HBsAg carriers with fewer than 23 AR-CAG repeats (Table 2). Among HBsAg carriers with fewer than 23 AR-CAG repeats, the multivariate-adjusted OR of HCC for those with the SRD5A2 VV genotype was 5.58 (95% CI = 1.86 to 16.71) compared with those with the SRD5A2 LL genotype. Conversely, there was no increased risk of HCC associated with the SRD5A2 VV genotype among HBsAg carriers with 23 or more AR-CAG repeats. Similarly, among HBsAg carriers with fewer than 23 AR-CAG repeats, the multivariate-adjusted OR was 2.51 (95% CI = 1.06 to 5.94) for those with the CYP17 A1 allele (A1/A1 and A1/A2 genotypes) compared with those without the A1 allele (A2/A2 genotype). There was no evidence, however, that the presence of the A1 allele increased risk among those with longer repeats. The interaction between the CYP17 genotype and AR-CAG repeats was statistically significant ($P_{interaction}$ = .04) (Table 2). Testosterone, estradiol, or the testosterone-to-estradiol ratio did not appear to modify the effect of any of the other hormonal factors. No notable interaction was also observed between CYP17 and the SRD5A2 genotype (data not shown).

Because high BMI has been associated with hormone-related cancers *(38,39)*, stratified analyses were also performed according to BMI (Table 3). This analysis revealed that the SRD5A2 V89L genotype (VV versus LL genotype; OR = 8.64; 95% CI = 2.75 to 27.14) and testosterone levels (highest versus lowest tertile; OR = 7.63; 95% CI = 2.13 to 27.27) were strong predictors for HCC risk among HBsAg carriers with a BMI less than or equal to the median value among control subjects. However, among those with a BMI above the median value, we identified only a weak positive association for testosterone (highest versus lowest tertile; OR = 2.28; 95% CI = 0.92 to 5.64). Furthermore, there was no association between the SRD5A2 genotype and the risk of HCC in HBsAg carriers with a BMI above the median level. BMI also appeared to modify the effect of CYP17 genotype, but we failed to detect a statistically significant interaction between BMI and CYP17 genotype in the risk of HCC. None of the other hormone-related factors considered showed any meaningful interaction with BMI (Table 3).

We also analyzed the association between BMI and HCC within strata of the SRD5A2 genotype. In this analysis, BMI was treated as a continuous variable.

Table 2. Association of cytochrome P450c17α (CYP17) and steroid 5α-reductase type II (SRD5A2) genotypes with the risk of HCC by the number of AR gene CAG repeats among male HBsAg carriers*

	No. of AR-CAG repeats							
	⩾23 repeats				<23 repeats			
	Case patients (n = 59)	Control subjects (n = 139)	Adjusted OR†,‡	95% CI	Case patients (n = 60)	Control subjects (n = 99)	Adjusted OR†,§	95% CI
SRD5A2 V89L polymorphism								
LL	18	48	1.00	Referent	17	40	1.00	Referent
VL	33	65	1.72	0.78 to 3.82	26	46	1.46	0.60 to 3.53
VV	8	26	1.08	0.36 to 3.24	17	13	5.58	1.86 to 16.71
Test for interaction: P = .084								
CYP17 polymorphism								
A2/A2	26	48	1.00	Referent	17	42	1.00	Referent
A1/A2	22	66	0.51	0.23 to 1.11	34	45	2.51	1.03 to 6.09
A1/A1	11	25	0.65	0.24 to 1.76	9	12	2.50	0.71 to 8.78
Test for interaction: P = .04								

*HCC = hepatocellular carcinoma; AR = androgen receptor; HBsAg = hepatitis B surface antigen; OR = odds ratio; CI = confidence interval; BMI = body mass index.

†Adjusted for age at recruitment (continuous variable), the time of blood draw (continuous variable), ethnicity (Fukien Taiwanese, Hakka Taiwanese, and Mainland Chinese), years of education (continuous variable), cigarette smoking, alcohol consumption, history of chronic liver disease, BMI, and other hormone-related variable listed in Table 1, except for the ratio of testosterone to estradiol. All variables were included in logistic regression models as categorized in Table 1, except for BMI, which was included as a continuous variable.

‡One control subject was excluded from analysis because of missing data on plasma estradiol.

§Two case patients and one control subject were excluded from analysis because of missing data on plasma eatradiol.

Table 3. Association of selected hormone-related factors with the risk of HCC by BMI among male HBsAg carriers*

Hormone-related factors	BMI ≤23.2† Case patients (n = 59)	Control subjects (n = 119)	Adjsuted OR‡,§	95% CI	BMI >23.2 Case patients (n = 60)	Control subjects (n = 119)	Adjusted OR‡,§	95% CI
Testosterone, ng/mL								
0.87–4.73	4	31	1.00	Referent	21	47	1.00	Referent
4.74–6.47	16	39	2.76	0.74 to 10.31	16	39	0.97	0.40 to 2.35
6.48–13.99	39	49	7.63	2.13 to 27.27	23	33	2.28	0.92 to 5.64
Test for interaction: $P = .023$								
SRD5A2 V89L polymorphism								
LL	13	50	1.00	Referent	22	38	1.00	Referent
VL	30	55	3.22	1.30 to 8.01	29	56	0.86	0.39 to 1.93
VV	16	14	8.64	2.75 to 27.14	9	25	0.74	0.24 to 2.28
Test for interaction: $P = .003$								
CYP17 polymorphism								
A2/A2	27	43	1.00	Referent	16	47	1.00	Referent
A1/A2	25	55	0.73	0.33 to 1.64	31	56	2.44	1.02 to 5.83
A1/A1	7	21	0.38	0.11 to 1.31	13	16	3.75	1.26 to 11.17
Test for interaction: $P = .302$								
No. of AR gene CAG repeats								
≥23	29	66	1.00	Referent	30	73	1.00	Referent
<23	30	53	1.40	0.64 to 3.07	30	46	2.00	0.96 to 4.16
Test for interaction: $P = .708$								
Estradiol, pg/ml								
3.7–13.7	17	46	1.00	Referent	15	28	1.00	Referent
13.8–17.3	20	38	1.92	0.74 to 4.98	18	39	1.35	0.49 to 3.77
17.4–53.8	21	34	1.68	0.65 to 4.38	26	51	0.76	0.30 to 1.97
Test for interaction: $P = .136$								
T/E2 ratio‖								
75.7–288.8	10	31	1.00	Referent	25	47	1.00	Referent
288.9–436.4	24	35	1.63	0.61 to 4.37	16	42	0.99	0.41 to 2.38
436.5–2539.7	24	52	1.55	0.57 to 4.17	18	29	1.38	0.55 to 3.45
Missing	1	1			1	1		
Test for interaction: $P = .067$								

*HCC = hepatocellular carcinoma; BMI = body mass index; HBsAg = hepatitis B surface antigen; OR = odds ratio; CI = confidence interval; AR = androgen receptor; SRD5A2 = steroid 5α-reductase type II; CYP17 = cytochrome P450c17α.

†Cut point was chosen on the basis of median value among control subjects.

‡For the testosterone-to-estradiol ratio, ORs have been adjusted for age at recruitment (continuous variable), the time of blood draw (continuous variable), ethnicity (Fukien Taiwanese, Hakka Taiwanese, and Mainland Chinese), years of education (continuous variable), cigarette smoking, alcohol consumption, history of chronic liver disease, BMI (continuous variable), and other hormone-related variables listed in Table 1, except for testosterone and estradiol. For other hormone-related factors, ORs have been adjusted for the same covariates that we treated for the hormone ratio, but testosterone and estradiol were included as covariates instead of the ratio. All variables were included in logistic regression models as categorized in the table.

§One case patient and one control subject were excluded from analysis because of missing data on plasma estradiol levels.

‖T/E2 ratio = testosterone in nanograms per milliliters divided by estradiol in nanograms per milliliters.

Among HBsAg carriers, there was a positive association in LL homozygotes (multivariate-adjusted OR = 1.37; 95% CI = 1.09 to 1.71) but a negative association among VV homozygotes (multivariate-adjusted OR = 0.66; 95% CI = 0.45 to 0.95). We found no association among the heterozygotes (multivariate-adjusted OR = 1.13; 95% CI = 0.98 to 1.31) (data not shown).

Discussion

Numerous reports *(35,40)* have suggested that the therapeutic use of androgenic steroids or oral contraceptives may cause benign hepatic adenoma and may increase the risk of HCC. In this study, the positive association between testosterone and HCC risk is consistent with our earlier Taiwanese case–control study, which was nested within another cohort study *(20)*, and with a recent Japanese cohort study of male patients with liver cirrhosis predominantly of HCV origin *(22)*. However, our results are somewhat in contrast with two previous cohort studies *(18,19)*. One study *(18)*, from an area of China with a high-incidence of HCC, reported that male HBsAg carriers with the highest tertile of circulating testosterone levels had a statistically nonsignificant 50% increase in the risk of HCC compared with those in the lowest tertile. However, that study *(18)* included only 50 HBsAg-positive case patients. The other study *(19)* reported no association between testosterone levels and HCC risk in a cohort of 101 male patients with cirrhosis, mainly caused by alcohol abuse. Because alcoholic cirrhosis is frequently associated with hypogonadism *(41)*, the association between testosterone levels and HCC risk observed in such patients with cirrhosis might not be expected to be the same as that observed in HBsAg carriers included in our study.

The positive association between testosterone levels and the incidence of HCC that we observed raises the possibility that genes involved in the regulation of testosterone may also play a role in the etiology of HCC. One such gene is SRD5A2. Two missense mutations have been identified in the SRD5A2 gene *(23–27)*. The valine-

to-leucine substitution at codon 89 appears to be much more common in Asians than in whites or African-Americans *(24,26)*. Studies *(24,25)* have shown a statistically significant association between the SRD5A2 V89L polymorphism and *in vivo* enzymatic activity, with VV homozygotes having substantially higher levels of activity than LL homozygotes and heterozygotes having intermediate levels of activity. We also found that, among male HBsAg carriers, the V allele was statistically significantly associated with an increased risk of HCC, with an allele dosage effect. However, although the SRD5A2 V89L polymorphism appears to have an association with the risk of HCC, it does not appear to be associated with prostate cancer risk, which is thought to be androgen dependent *(26)*. A second missense mutation in the SRD5A2 gene has been identified at codon 49 and results in a substitution of alanine with threonine. This substitution increased enzymatic activity *in vitro* and has been positively associated with advanced-stage prostate cancer *(23,27)*. However, the SRD5A2 A49T allele is rare in various populations, such as African-Americans and Latinos *(23,27)*. A sample size larger than that of the current study may be required to have sufficient power to examine the association of this polymorphism with the risk of HCC.

A second hormone-related gene is the CYP17 gene, which encodes the CYP17 enzyme that functions at key steps in the synthesis of both androgens and estrogens *(42)*. There are conflicting results regarding whether the CYP17 polymorphism, detected by *Msp*A1I digestion, is a risk factor for prostate cancer and female breast cancer *(26,30,43,44)*. Also, whereas the CYP17 A2 allele was associated with higher serum estrogen levels in women *(29,30)*, the A1 allele was associated with higher serum androgen metabolite levels in men *(25)*. In our study, although the CYP17 genotype alone did not influence the risk of HCC, inheritance of at least one CYP17 A1 allele was specifically associated with an increased risk of HCC among male HBsAg carriers who had fewer AR-CAG repeats or higher BMI.

A third hormone-related gene is the AR, which is responsible for the transport of androgens. ARs have been demonstrated in HCC and nontumorous liver tissue, but AR expression is greater in HCC *(45)*. In normal male mice, the growth rate of chemically induced preneoplastic hepatic foci is 20-fold faster than the rate in mice with testicular feminization, which lacks functional ARs *(8)*. This observation suggests that the development of HCC may be AR dependent. In addition, the number of AR-CAG repeats is inversely associated with transcriptional activity by the AR *in vitro (46)*, with an increase in transcriptional activity associated with ARs containing fewer CAG repeats. Fewer AR-CAG repeats also have been associated with an excess risk of prostate cancer *(23,33,47)*. Moreover, a moderate expansion of the CAG-repeat sequence has been suggested to play a role in male infertility *(48)*. In this study and in our previous case–control study involving almost all of the case patients in the present study and a series of hospital male patients with HBV-related HCC *(21)*, we also observed that HBsAg carriers with fewer AR-CAG repeats had an increased risk of HCC, suggesting that increased AR-mediated transcriptional activity may contribute to the development of HBV-related HCC.

We did not find convincing evidence for a modification effect of AR-CAG repeats on the association between testosterone levels and HCC risk. Nevertheless, the effects of SRD5A2 and CYP17 genotypes on the risk of HCC appeared to depend on the AR genotype. For both the SRD5A2 V89L and the CYP17 polymorphisms, the at-risk genotypes statistically significantly increased disease risk among male HBsAg carriers with fewer than 23 CAG repeats but posed no increased risk among those with longer repeats. Regardless of the mechanisms underlying the modifying effect of the AR-CAG repeats on HCC risk associated with the SRD5A2 and CYP17 polymorphisms, it appears that the development of HCC among male HBsAg carriers is mediated by a combination of genes involved in the metabolism and transport of androgens. Thus, HBV-related hepatocarcinogenesis is closely associated with the pathways involving androgen signaling.

The development of HCC may also be associated with obesity. Obesity has been associated with steatohepatitis and non-insulin-dependent diabetes mellitus, which may lead to HCC development *(35,49)*. Patients with non-insulin-dependent diabetes mellitus often manifest insulin resistance, which, in addition to increased adiposity as measured by BMI, has been proposed to be a risk factor for certain hormone-related cancers *(38,39,50)*. After adjusting for other hormone-related factors and potentially relevant covariates, we found that BMI was positively associated with the risk of HCC. However, we also found that the BMI association differed according to the category of the SRD5A2 genotype. Of particular interest is the striking difference in the estimated risks of HCC associated with testosterone levels and the SRD5A2 genotype between male HBsAg carriers with different BMI values (Table 3). This difference raises the intriguing possibility that men with different magnitudes of obesity may have divergent responses to the promoting effect of androgens on hepatocarcinogenesis.

Our data, in conjunction with past animal studies *(3–11)*, provide strong evidence for a close relationship between HBV-related HCC risk among men and higher levels of androgen signaling, reflected by higher testosterone levels, increased metabolic activation of testosterone, and/or increased AR-mediated transcriptional activity. The predominance of HCC among males may be attributed to the higher androgenic activity and/or the lower estrogenic activity in men than in women. Of note, similar to two earlier cohort studies *(19,22)*, we did not find a relationship between estradiol levels and the risk of HCC. In addition, the results of animal studies have been inconsistent regarding the role of estrogens in the development of HCC *(3,7,12–17)*. The major source of estrogens in men is from the conversion of androgens to estrogens in peripheral tissues, such as adipose tissues. Unlike the small cohort study of Japanese patients with cirrhosis, which reported a strong positive association between the testosterone-to-estradiol ratio and HCC risk *(22)*, we did not find a statistically significant association in any of our analyses. Instead, plasma testosterone levels appeared to be a better predictor than the hormone ratio for HCC risk. This is presumably because absolute rather than relative hormone amounts have a more profound effect on HCC development. If so, pharmacologic approaches to decrease androgen action may warrant investigation as a strategy specifically targeted at male HBsAg carriers for the treatment or prevention of HCC.

REFERENCES

(1) Yu MW, Chen CJ. Hepatitis B and C viruses in the development of hepatocellular carcinoma. Crit Rev Oncol Hematol 1994;17:71–91.

(2) Yu MW, Tsai SF, Hsu KH, You SL, Lee SS, Lin TM, et al. Epidemiologic characteristics of malignant neoplasms in Taiwan. II. Liver cancer. J Natl Public Health Assoc 1988;8:125–38.

(3) Agnew LR, Gardner WU. The incidence of spontaneous hepatomas in C3H, C3H (low milk factor), and CBA mice and the effect of estrogen and androgen on the occurrence of these tumors in C3H mice. Cancer Res 1952; 12:757–61.

(4) Vesselinovitch SD, Mihailovich N. The effect of gonadectomy on the development of hepatomas induced by urethan. Cancer Res 1967; 27:1788–91.

(5) Toh YC. Effect of neonatal castration on liver tumor induction by *N*-2-fluorenylacetamide in suckling BALB/c mice. Carcinogenesis 1981; 2:1219–21.

(6) Firminger HI, Reuber MD. Influence of adrenocortical, androgenic, and anabolic hormones on the development of carcinoma and cirrhosis of the liver in A×C rats fed *N*-2-fluorenyldiacetamide. J Natl Cancer Inst 1961;27:559–95.

(7) Vesselinovitch SD, Itze L, Mihailovich N, Rao KV. Modifying role of partial hepatectomy and gonadectomy in ethylnitrosourea-induced hepatocarcinogenesis. Cancer Res 1980;40: 1538–42.

(8) Kemp CJ, Leary CN, Drinkwater NR. Promotion of murine hepatocarcinogenesis by testosterone is androgen receptor-dependent but not cell autonomous. Proc Natl Acad Sci U S A 1989;86:7505–9.

(9) Dunsford HA, Sell S, Chisari FV. Hepatocarcinogenesis due to chronic liver cell injury in hepatitis B virus transgenic mice. Cancer Res 1990;50:3400–7.

(10) Kim CM, Koike K, Saito I, Miyamura T, Jay G. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. Nature 1991;351: 317–20.

(11) Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4: 1065–7.

(12) Mishkin SY, Farber E, Ho RK, Mulay S, Mishkin S. Evidence for the hormone dependency of hepatic hyperplastic nodules: inhibition of malignant transformation after exogenous 17β-estradiol and tamoxifen. Hepatology 1983;3: 308–16.

(13) Taper HS. The effect of estradiol-17-phenylpropionate and estradiol benzoate on *N*-nitrosomorpholine-induced liver carcinogenesis in ovariectomized female rats. Cancer 1978;42: 462–7.

(14) Yager JD Jr, Yager R. Oral contraceptive steroids as promoters of hepatocarcinogenesis in female Sprague-Dawley rats. Cancer Res 1980; 40:3680–5.

(15) Cameron RG, Imaida K, Tsuda H, Ito N. Promotive effects of steroids and bile acids on hepatocarcinogenesis initiated by diethylnitrosamine. Cancer Res 1982;42:2426–8.

(16) Yager JD, Zurlo J, Sewall CH, Lucier GW, He H. Growth stimulation followed by growth inhibition in livers of female rats treated with ethinyl estradiol. Carcinogenesis 1994;15: 2117–23.

(17) Shimizu I, Yasuda M, Mizobuchi Y, Ma YR, Liu F, Shiba M, et al. Suppressive effect of oestradiol on chemical hepatocarcinogenesis in rats. Gut 1998;42:112–9.

(18) Yuan JM, Ross RK, Stanczyk FZ, Govindarajan S, Gao YT. A cohort study of serum testosterone and hepatocellular carcinoma in Shanghai, China. Int J Cancer 1995;63:491–3.

(19) Ganne-Carrie N, Chastang C, Uzzan B, Pateron D, Trinchet JC, Perret G, et al. Predictive value of serum sex hormone binding globulin for the occurrence of hepatocellular carcinoma in male patients with cirrhosis. J Hepatol 1997; 26:96–102.

(20) Yu MW, Chen CJ. Elevated serum testosterone levels and risk of hepatocellular carcinoma. Cancer Res 1993;53:790–4.

(21) Yu MW, Cheng SW, Lin MW, Yang SY, Liaw YF, Chang HC, et al. Androgen-receptor gene CAG repeats, plasma testosterone levels, and risk of hepatitis B-related hepatocellular carcinoma. J Natl Cancer Inst 2000;92:2023–8.

(22) Tanaka K, Sakai H, Hashizume M, Hirohata T. Serum testosterone : estradiol ratio and the development of hepatocellular carcinoma among male cirrhotic patients. Cancer Res 2000;60: 5106–10.

(23) Ross RK, Pike MC, Coetzee GA, Reichardt JK, Yu MC, Feigelson H, et al. Androgen metabolism and prostate cancer: establishing a model of genetic susceptibility. Cancer Res 1998;58:4497–504.

(24) Makridakis N, Ross RK, Pike MC, Chang L, Stanczyk FZ, Kolonel LN, et al. A prevalent missense substitution that modulates activity of prostate steroid 5α-reductase. Cancer Res 1997; 57:1020–2.

(25) Makridakis N, Reichardt J, Pike M, Stanczyk F, Yu M, Kolonel L, et al. Genetic control of human androgen metabolism by the CYP17 and SRD5A2 genes. Am J Hum Genet (Suppl) 1997;61:A257.

(26) Lunn RM, Bell DA, Mohler JL, Taylor JA. Prostate cancer risk and polymorphism in 17 hydroxylase (CYP17) and steroid reductase (SRD5A2). Carcinogenesis 1999;20:1727–31.

(27) Makridakis NM, Ross RK, Pike MC, Crocitto LE, Kolonel LN, Pearce CL, et al. Association of mis-sense substitution in SRD5A2 gene with prostate in African-American and Hispanic men in Los Angeles, USA. Lancet 1999;354: 975–8.

(28) Thigpen AE, Silver RI, Guileyardo JM, Casey ML, McConnell JD, Russell DW. Tissue distribution and ontogeny of steroid 5 α-reductase isozyme expression. J Clin Invest 1993;92: 903–10.

(29) Feigelson HS, Shames LS, Pike MC, Coetzee GA, Stanczyk FZ, Henderson BE. Cytochrome P450c17α (CYP17) polymorphism is associated with serum estrogen and progesterone concentrations. Cancer Res 1998;58:585–7.

(30) Haiman CA, Hankinson SE, Spiegelman D, Colditz GA, Willett WC, Speizer FE, et al. The relationship between a polymorphism in CYP17 with plasma hormone levels and breast cancer. Cancer Res 1999;59: 1015–20.

(31) Buffone GJ, Darlington GJ. Isolation of DNA from biological specimens without extraction with phenol [letter]. Clin Chem 1985;31: 164–5.

(32) Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:1215.

(33) Giovannucci E, Stampfer MJ, Krithivas K, Brown M, Brufsky A, Talcott J, et al. The CAG repeat within the androgen receptor gene and its relationship to prostate cancer. Proc Natl Acad Sci U S A 1997;94:3320–3.

(34) Yu MW, Hsu FC, Sheen IS, Chu CM, Lin DY, Chen CJ, et al. Prospective study of hepatocellular carcinoma and liver cirrhosis in asymptomatic chronic hepatitis B virus carriers. Am J Epidemiol 1997;145:1039–47.

(35) Yu MC, Tong MJ, Govindarajan S, Henderson BE. Nonviral risk factors for hepatocellular carcinoma in a low-risk population, the Non-Asians of Los Angeles County, California. J Natl Cancer Inst 1991;83:1820–6.

(36) Yu MW, Gladek-Yarborough A, Chiamprasert S, Santella RM, Liaw YF, Chen CJ. Cytochrome P450 2E1 and glutathione *S*-transferase M1 polymorphisms and susceptibility to hepatocellular carcinoma. Gastroenterology 1995;109: 1266–73.

(37) Yu MW, Yang SY, Chiu YH, Chiang YC, Liaw YF, Chen CJ. A p53 genetic polymorphism as a modulator of hepatocellular carcinoma risk in relation to chronic liver disease, familial tendency, and cigarette smoking in hepatitis B carriers. Hepatology 1999;29:697–702.

(38) Ziegler RG, Hoover RN, Nomura AM, West DW, Wu AH, Pike MC, et al. Relative weight, weight change, height, and breast cancer risk in Asian-American women. J Natl Cancer Inst 1996;88:650–60.

(39) Potischman N, Hoover RN, Brinton LA, Siiteri P, Dorgan JF, Swanson CA, et al. Case–control study of endogenous steroid hormones and endometrial cancer. J Natl Cancer Inst 1996;88: 1127–35.

(40) Farrell GC, Joshua DE, Uren RF, Baird PJ, Perkins KW, Kronenberg H. Androgen-induced hepatoma. Lancet 1975;1:430–2.

(41) Van Thiel DH, Lester R, Sherins RJ. Hypogonadism in alcoholic liver disease: evidence for a double defect. Gastroenterology 1974;67: 1188–99.

(42) Picado-Leonard J, Miller WL. Cloning and sequence of the human gene for P450c17 (steroid 17α-hydroxylase/17,20 lyase): similarity with the gene for P450c21. DNA 1987;6: 439–48.

(43) Spurdle AB, Hopper JL, Dite GS, Chen X, Cui J, McCredie MR, et al. CYP17 promoter polymorphism and breast cancer in Australian women under age forty years. J Natl Cancer Inst 2000;92:1674–81.

(44) Habuchi T, Liqing Z, Suzuki T, Sasaki R, Tsuchiya N, Tachiki H, et al. Increased risk of prostate cancer and benign prostatic hyperplasia associated with a CYP17 gene polymorphism with a gene dosage effect. Cancer Res 2000;60:5710–3.

(45) Nagasue N, Ito A, Yukaya H, Ogawa Y. Androgen receptors in hepatocellular carcinoma

and surrounding parenchyma. Gastroenterology 1985;89:643–7.

(46) Irvine RA, Ma H, Yu MC, Ross RK, Stallcup MR, Coetzee GA. Inhibition of p160-mediated coactivation with increasing androgen receptor polyglutamine length. Hum Mol Genet 2000; 9:267–74.

(47) Ingles SA, Ross RK, Yu MC, Irvine RA, La Pera G, Haile RW, et al. Association of prostate cancer risk with genetic polymorphisms in vitamin D receptor and androgen receptor. J Natl Cancer Inst 1997;89:166–70.

(48) Dowsing AT, Yong EL, Clark M, McLachlan RI, de Kretser DM, Trounson AO. Linkage between male infertility and trinucleotide repeat expansion in the androgen-receptor gene. Lancet 1999;354:640–3.

(49) Powell EE, Cooksley WG, Hanson R, Searle J, Halliday JW, Powell LW. The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. Hepatology 1990;11:74–80.

(50) Bruning PF, Bonfrer JM, van Noord PA, Hart AA, de Jong-Bakker M, Nooijen WJ. Insulin resistance and breast-cancer risk. Int J Cancer 1992;52:511–6.

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