Elevated Serum Testosterone Levels and Risk of Hepatocellular Carcinoma

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ABSTRACT

Serum samples of 9691 male adults had been collected and frozen for a prospective study of hepatocellular carcinoma in Taiwan. After an average follow-up period of 4.6 years, testosterone levels in the stored serum were measured by radioimmunoassay using commercial kits for 35 cases of newly developed hepatocellular carcinoma, 63 hepatitis B surface antigen-negative and 77 hepatitis B surface antigen-positive matched controls. Elevated testosterone levels were found to be associated with an increased risk of hepatocellular carcinoma. The association remained significant after the adjustment for effects of other hepatocellular carcinoma risk factors, including hepatitis B surface antigen carrier status, positivity of serum antibody to hepatitis C virus, cigarette smoking, alcohol drinking, past liver disease history, and dietary habits. The multivariate-adjusted relative risk of hepatocellular carcinoma for men with testosterone levels in the upper tertile was 4.1 (95% confidence interval = 1.3-13.2) compared with those having levels in the middle or lower tertiles (P = 0.016). The results consistent with those observed in animal experiments support the hypothesis that testosterone plays a role in the etiology of human hepatocellular carcinoma.

INTRODUCTION

Liver cancer, largely HCC, is one of the most common fatal cancers in the world (1). Although there has been rapid progress in the understanding of the etiology of HCC during the past 2 decades, the reasons for marked male predominance in HCC incidence observed in both high- and low-risk areas (1, 2) remain to be elucidated. In Taiwan, HB,Ag carrier status has been well documented as the most important risk factor of HCC (3–5). However, HCC is 2–3 times more frequent in males than in females, despite their similarity in HB,Ag carrier rate (2). Differences in life-style habits such as cigarette smoking and alcohol drinking have been implicated in the gender discrepancy (4). Sex hormone and striking male-to-female differences in chronic hepatitis (6) and liver cirrhosis (7) may also be important.

A greater susceptibility of male animals to spontaneous and chemical carcinogen-induced HCC has long been observed in experimental mice and rats (8–14, 15). The increased susceptibility to hepatocarcinogenesis in males has also been observed in HBV transgenic mice (16, 17). These observations are in agreement with epidemiological data suggesting that men are much more prone to HCC than women. Although the marked sex difference in HCC incidence observed in mice and rats may result from the tumor-inhibiting effects of estrogen in female animals as well as the tumor-promoting effects of testosterone in male animals, the persuasive evidence in the animal models indicating that testosterone may enhance the development of HCC suggests that the testosterone may explain, at least in part, the greater incidence of liver cancer observed in men. Male castration significantly decreases the frequency of chemically induced HCC (10, 12, 15, 18, 19), whereas administration of testosterone to castrated male rats or to female mice increases the growth of preneoplastic hepatic foci (11) or the incidence of spontaneous (8) or chemical carcinogen-induced HCC (18). An experiment on rats also showed that hypophysectomy in males inhibits liver tumor induction by aflatoxin (20).

In contrast to the abundant data in animal models, the evidence showing the effect of testosterone on human hepatocarcinogenesis is scanty in spite of several documented HCC cases following androgenic-anabolic steroid treatment (21) and the presence of androgen receptors in human HCC tissues (22, 23). We therefore carried out a nested case-control study using serum samples collected and frozen in 1984–1986 from a cohort of 9691 male adults to investigate the role of elevated testosterone in the etiology of HCC.

SUBJECTS AND METHODS

The study population consisted of 9691 men aged 30–85 years who attended a community-based study carried out in six townships of Taiwan for the early detection of cancer between September 1984 and February 1986. At the initial recruitment examination, a total of 15 ml blood were collected from each study subject. They were also personally interviewed according to a structured questionnaire on sociodemographic characteristics, long-term habits of cigarette smoking, alcohol drinking, and dietary pattern, as well as personal and family history of chronic diseases by well-trained public health nurses in local health centers. Habit of cigarette smoking was defined as having smoked cigarettes more than 4 days a week for at least 6 months. Alcohol drinking was defined as having drunk alcohol more than 3 days a week for at least 6 months. Vegetable consumption frequency was defined as the number of meals including fresh vegetables per week. Vegetarian habit was defined as having one or more meals without eating food from animal sources every day for more than 1 year. The personal and family history of chronic liver diseases diagnosed by physicians was also obtained.

The participants of this study cohort were followed by telephone interviews and home visits annually until March 1990. The person-year under observation for each subject was defined as the period of follow-up from recruitment to the date when he was affected with liver cancer or died from other causes or the date this nested case-control study started, i.e., March 1990. The follow-up period ranged from 0.5 to 5.5 years, with an average of 4.6 years. Death certificates were also reviewed to examine the causes of death for those who died. During the follow-up period, 36 newly diagnosed liver cancer cases were identified. The annual incidence of liver cancer was 91.4/100,000 person-years. Although the coding of liver cancer in death certificates combined cancers of the liver and the intrahepatic bile duct (ICD 155) according to the 8th Revision of the International Classification of Diseases, Injuries and Causes of Death, more than 95% of the deaths classified into this category in Taiwan were HCC. The most accepted diagnostic criteria for HCC in Taiwan are assigned on the basis of either pathological examinations or elevated α-fetoprotein level (>400 ng/ml) combined with at least one positive image on angiography, sonography, liver scan, and/or computerized tomography scans. Among the 36 liver cancer cases, 8 were double-checked with hospital records and the remaining 28 were confirmed histologically.

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3 The abbreviations used are: HCC, hepatocellular carcinoma; HB,Ag, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; RIA, radioimmunoassay; CI, confidence interval; RPHA, reverse passive hemagglutination assay; RR, relative risk.
cancer and most HCC patients are referred to teaching hospitals for diagnosis and treatment in Taiwan, the possibility of misdiagnosis of HCC was rather low.

Because liver cirrhosis has been reported to be frequently associated with hypogonadism (24) and most HCC patients in Taiwan may have a long-term underlying liver cirrhosis before they are affected by HCC, it is essential to evaluate the effect of liver cirrhosis on serum testosterone in the preclinical phase of HCC patients. The deep-frozen serum samples from 15 study subjects who died from liver cirrhosis during the 5-year follow-up period were used to assess whether liver cirrhosis may cause a significant change in serum testosterone levels and distort the association between serum testosterone levels and HCC risk. Since all of these cirrhotic patients died within 5 years after the recruitment but only 3 reported a history of physician-diagnosed liver cirrhosis in the baseline interview, it was believed that these patients already had a subclinical liver cirrhosis at the time of blood collection, the same as most HCC patients identified in this study.

All subjects in the study cohort were tested for their HBsAg carrier status by RPHA at the initial recruitment examination. Because some HBsAg carriers may have chronic liver diseases, it is essential to obtain a valid estimation of serum testosterone level for HBsAg-positive and HBsAg-negative subjects, respectively. Two HBsAg-positive and two HBsAg-negative controls matched with each liver cancer case were selected in order to recruit enough and equal numbers of subjects not affected with HCC. Controls were randomly selected from study subjects who were alive and free from liver cancer on the dates at the diagnosis of liver cancer cases to whom they were matched. Cases and controls were matched with respect to age (within 5 years), date of questionnaire interview and blood collection (within 3 months), and residential township. As the frozen serum sample of one liver cancer case was not enough for the test of testosterone, the statistical analysis was based on the data of 35 matched case-control sets. Serum samples were separated on the same day as blood collection. Two aliquots were frozen in deep freezers and transported in dry ice to the central laboratory at National Taiwan University College of Medicine. The serum samples were kept at −30°C for further examination until this nested case-control study was carried out. Serum samples of 35 liver cancer cases and 140 matched controls were tested in April 1991. The HBsAg status of liver cancer cases and matched controls who were HBsAg-negative in the initial RPHA were retested by RIA using commercial kits (Abbott Laboratories, North Chicago, IL). Seven controls who were HBsAg-negative on RPHA were found to be HBsAg-positive on RIA. In other words, there were 77 HBsAg-positive and 63 HBsAg-negative controls based on RIA results. Anti-HCV was examined in duplicate by enzyme immunoassay (Abbott Laboratories) according to the manufacturer’s instructions. Positive samples at the first test were retested. Only repeatedly positive samples were considered anti-HCV-positive. Serum testosterone levels were measured by RIA using commercial kits (Biomérieux, Marcy l’Etoile, France). Specimens for each liver cancer case and the matched controls were tested blindly in the same batch for all laboratory examinations.

A age-adjusted serum testosterone value was used when we compared the mean levels of the hormone of liver cirrhosis cases to those of controls. Since the distribution of the age-adjusted testosterone values showed no substantial deviation from the normal distribution, judging from histogram and coefficients of skewness and kurtosis, a t test was applied to compare the mean levels of serum testosterone between groups. To assess the effect of elevated serum testosterone on the risk of HCC, the serum testosterone level was dichotomized using the lowest value of the upper tertile of testosterone levels in controls, i.e., 5.69 ng/ml, as the cutoff point. The relative risk of liver cancer for the higher testosterone level was estimated by the odds ratio using the lower testosterone level as the referent group. Conditional logistic regression analyses were used to calculate matched crude and multivariate-adjusted odds ratios and their 95% CI for various risk factors of HCC. Statistical significance of the risk estimates was examined by Z test. All P values for tests of statistical significance were based on two-tailed tests. This study was conducted with the approval of the Department of Health and in compliance with regulations for the protection of human research subjects.

RESULTS

There were 20 HBsAg-positive and 15 HBsAg-negative liver cancer cases. The age of liver cancer cases at the initial recruitment examination ranged from 40 to 74 years. The mean age ± SD at recruitment was 60.1 ± 8.8, 58.6 ± 8.9, 58.3 ± 9.8, and 59.2 ± 9.2 years, respectively, for HBsAg-negative controls, HBsAg-positive controls, HBsAg-negative liver cancer cases, and HBsAg-positive liver cancer cases. The age distribution of liver cancer cases was similar to that of their matched controls.

The age of patients who died from liver cirrhosis ranged from 38 to 66 years, with a mean ± SD of 52.5 ± 9.6 years. They were younger than controls. Since the serum testosterone level in men has been reported to decline gradually with age after 40 years (25), age-adjusted testosterone values were derived for comparison. The age and testosterone level of controls were used to build a regression line. A residual was first calculated by subtracting the predicted value obtained through the regression equation from the observed testosterone level. The residual of each study subject was then added to the mean testosterone level of controls to derive an age-adjusted value of serum testosterone. Since the regression coefficient (b = 0.00056, P = 0.98) of this regression line indicated that the testosterone level did not significantly change with age, the adjustment would not alter the observed association between liver cirrhosis and serum testosterone levels. The age-adjusted serum testosterone levels of liver cirrhosis cases and controls by HBsAg carrier status were compared in Table 1. There were 12 HBsAg-positive and 3 HBsAg-negative cases of liver cirrhosis. The HBsAg-negative liver cirrhosis cases had the lowest testosterone level among the four groups. But the difference in mean serum testosterone level was not significant between any two groups. The proportion of a testosterone level above 5.69 ng/ml was significantly higher in HBsAg-positive controls than in HBsAg-negative controls (P = 0.045). The proportion was slightly higher in HBsAg-positive controls than HBsAg-positive liver cirrhosis cases.

The mean ± SD of serum testosterone levels was 5.20 ± 2.52 ng/ml for liver cancer patients. Among the HBsAg-positive matched case-control sets, 55.0% of liver cancer cases and 47.5% of matched controls had a testosterone level greater than 5.69 ng/ml, giving a matched RR of 1.5. Among the HBsAg-negative matched sets, 46.7% of liver cancer cases and 23.3% of matched controls had an elevated testosterone level, giving a matched RR of 2.3. In order to increase the statistical power of detecting a significant effect of elevated testosterone, all controls (2 HBsAg-positive and 2 HBsAg-negative) matched with each liver cancer case were included in the analyses. Because the HBsAg carrier rate of the liver cancer cases (57%) was similar to that of controls (55%), the association between serum testosterone level and HCC observed in this study is acceptable. The proportion of elevated testosterone level was compared between liver cancer cases and matched controls, as shown in Table 2. Eighteen (51.4%) of 35 liver cancer cases and 48 (34.3%) of 140 matched controls had a serum testosterone level greater than 5.69 ng/ml. There was a significant association between elevated serum testosterone level and liver cancer, with a RR of 2.3 (95% CI = 1.01–5.14) and a P value of 0.046.

<table>
<thead>
<tr>
<th>Table 1 Age-adjusted serum testosterone levels (ng/ml) of liver cirrhosis cases and controls by HBsAg carrier status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
</tr>
<tr>
<td>HBsAg n</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Negative 63</td>
</tr>
<tr>
<td>Positive 77</td>
</tr>
</tbody>
</table>

* The lowest value of upper tertile in the testosterone distribution of controls.

** P = 0.045 compared to HBsAg-negative controls.
diagnosed by physicians (17.1%) than matched controls (4.3%), with CI = 0.7-6.7). Liver cancer cases had a lower percentage of fresh 0.10). There were more vegetarians who consumed mostly preserved 25.7%) than matched controls (13.6%), with a RR of 2.6 The percentage of habitual alcohol drinking was higher among liver 5.69 ng/ml was 2.7 (95% CI = 1.04-6.80).

Because subclinical HCC might be present at the time of blood collection and the observed elevation of serum testosterone might be due to the presence of cancer, the association between serum testosterone level and HCC was further examined for two distinct follow-up periods by stratifying the cases into two groups: early-onset cases who were diagnosed within 1 year after blood collection (7 cases) and late-onset cases who were diagnosed more than 1 year after blood collection (28 cases). The effect of elevated testosterone on developing HCC was stronger after the exclusion of the early-onset cases who were diagnosed within 1 year after blood collection. The matched RR for developing HCC for a serum testosterone level greater than 5.69 ng/ml was 2.7 (95% CI = 1.04-6.80).

Table 3 shows the frequency distribution of other HCC risk factors in liver cancer cases and controls. Liver cancer cases had a significantly higher proportion of anti-HCV-positive status (20.0%) than matched controls (2.9%), with a RR of 11.8 (95% CI = 2.4-57.7). More liver cancer cases (68.6%) than matched controls (57.9%) were diagnosed within 1 year after blood collection (7 cases). The effect of elevated testosterone on developing HCC was stronger after the exclusion of the early-onset cases who were diagnosed within 1 year after blood collection. The matched RR for developing HCC for a serum testosterone level greater than 5.69 ng/ml was 2.7 (95% CI = 1.04-6.80).

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Table 3 Relative risk of factors associated with HCC

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases</th>
<th>Controls</th>
<th>Relative risk (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HCV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>28(80.0)</td>
<td>136(97.1)</td>
<td>1.0</td>
</tr>
<tr>
<td>Positive</td>
<td>7(20.0)</td>
<td>4(2.9)</td>
<td>11.8 (2.4-57.7)</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>11(31.4)</td>
<td>59(42.1)</td>
<td>1.0</td>
</tr>
<tr>
<td>Yes</td>
<td>24(68.6)</td>
<td>81(57.9)</td>
<td>1.6 (0.7-3.5)</td>
</tr>
<tr>
<td>Alcohol drinking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>26(74.3)</td>
<td>121(86.4)</td>
<td>1.0</td>
</tr>
<tr>
<td>Yes</td>
<td>9(25.7)</td>
<td>19(13.6)</td>
<td>2.6 (1.0-7.0)</td>
</tr>
<tr>
<td>Vegetarian habit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>29(82.9)</td>
<td>127(90.7)</td>
<td>1.0</td>
</tr>
<tr>
<td>Yes</td>
<td>6(17.1)</td>
<td>13(9.3)</td>
<td>2.2 (0.7-6.7)</td>
</tr>
<tr>
<td>Vegetable consumption (meals/week)*&lt;6</td>
<td>27(79.4)</td>
<td>131(93.6)</td>
<td>1.0</td>
</tr>
<tr>
<td>&lt;6</td>
<td>7(20.6)</td>
<td>9(6.4)</td>
<td>4.1 (1.3-13.4)</td>
</tr>
<tr>
<td>Past liver disease history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>29(82.9)</td>
<td>134(95.7)</td>
<td>1.0</td>
</tr>
<tr>
<td>Yes</td>
<td>6(17.1)</td>
<td>6(4.3)</td>
<td>5.0 (1.4-18.2)</td>
</tr>
</tbody>
</table>

*One case without data on vegetable consumption frequency.

DISCUSSION

The striking male:female ratios in the incidence of HCC in ethnically and geographically diverse populations (1, 2) as well as the greater susceptibility of male animals to spontaneous and chemical-induced HCC (8-14, 18) suggest that sex-related factors may be involved in its development. The male predominance in HCC is independent of the hereditary diversity of different races and animal species, as well as the varying environmental exposures in different geographical areas and animal experiments. It seems reasonable to assume that sex hormone is primarily responsible for the gender discrepancy. This study shows that elevated serum levels of testosterone were associated with an increased risk of developing HCC. The association remained significant after adjusting for the effect of HBsAg carrier status, anti-HCV positivity, alcohol drinking, cigarette smoking, past liver disease history, and dietary habits. These results reveal that the association between testosterone level and HCC was independent of other HCC risk factors. They are also compatible with the hypothesis that testosterone has a role in the etiology of human HCC.

The testosterone levels in this study were determined from serum samples collected 6 months to 5 years (average, 2.4 years) before the diagnosis of HCC. To investigate whether subclinical testosterone-HCC relationship, the association between elevated testosterone and HCC was further examined according to the interval between blood collection and the diagnosis of HCC. Inasmuch as the effect of elevated testosterone on the development of HCC was stron-
ger after the exclusion of the early-onset cases diagnosed within 1 year after blood collection, it is unlikely that the elevated testosterone levels in HCC cases were a result of this cancer. However, preclinical disease may also have an effect on serum testosterone. Previous studies have shown that most male patients with chronic liver disease had clinical and biochemical evidence of hypogonadism, and their serum testosterone levels fell further as the disease progressed (24). One study also reported that patients of alcoholic cirrhosis combined with HCC had significantly lower levels of circulating testosterone than patients affected with alcoholic cirrhosis alone (26). HCC usually occurs in cirrhotic livers (27). Chronic hepatitis, liver cirrhosis, and HCC may be successive sequelae of chronic HBV infection (28). In this study, we cannot exclude the possibility that underlying cirrhosis has an effect on testosterone levels in the preclinical phase of some HCC cases and thus leads to an underestimation of the relative risk of HCC associated with elevated testosterone levels. In other words, the relative risk estimate in this study was obtained under conservative circumstances. After HB,Ag carrier status and past liver disease history were adjusted for by the conditional logistic regression analysis, an increase in RR associated with elevated testosterone levels was observed.

It has been well established that testosterone plays a prominent role in mouse hepatocarcinogenesis (8, 10–12, 14, 19), but the importance of this male hormone in the development of HCC was reported less frequently in rats (15, 18). In animal models, male castration decreased the incidence of chemically induced HCC (10, 12, 15, 18, 19), while chronic administration of testosterone to female or castrated male animals increased the growth of preneoplastic hepatic foci (11) or the risk of spontaneous (8) or chemically induced HCC (18). These results suggest that testosterone may promote the development of HCC, even at physiological levels. This is consistent with our data suggesting that men with elevated testosterone levels are at an increased risk for HCC. The mechanism of testosterone involvement in the genesis of HCC remains to be elucidated. However, in the light of animal studies, this may be related to the ability of testosterone to promote the growth of preneoplastic hepatic lesions (11, 19). The marked sex difference in susceptibility to chemically induced hepatocarcinogenesis may also be related to the sexual dimorphism in the metabolism of environmental carcinogens (12, 20, 29–31). The pathway leading to the active metabolite of aflatoxin was reported to be more active in male animals than in females (29). Recent animal studies also indicated that male hormone may mediate the activity of sulfotransferase (30) and the expression of cytochrome p450 enzymes (31). However, the role of testosterone in carcinogenic metabolism in humans remains to be explored. A HBV transgenic mouse model also suggested that sex hormones may play a role in HBV gene expression (32). HBV-associated chronic hepatitis (6) and liver cirrhosis (7) are found more frequently and may have a more aggressive behavior in men than in women (28). It would be worthwhile to determine whether the effect of testosterone on the development of HCC occurs through the regulation of HBV gene expression, which causes a higher incidence of chronic hepatitis and liver cirrhosis in males than in females.

A significantly higher proportion of serum testosterone levels above 5.69 ng/ml was also observed among HB,Ag-positive controls than HB,Ag-negative controls in this study. There was no established mechanism to explain this phenomenon. The higher testosterone levels in HB,Ag carriers than in noncarriers may result from some unexplored factors which were associated with serum testosterone levels and distributed differently between the two groups. It might also be possible that chronic HBV infection itself increases the circulating testosterone level. Whether increased testosterone production is also one of the mechanisms for HBV-related hepatocarcinogenesis remains to be studied.

Although our study suggests that the positive association between serum testosterone level and risk of HCC observed in many animal experiments may apply to human beings, there may be some uncontrolled factors such as genetic factors, environmental factors not studied here, or circulating hormones other than testosterone which may also partly result in an association between elevated testosterone and HCC risk in this study. Because testosterone is converted into estrogen and other metabolites in peripheral tissues, the circulating level of testosterone may not completely reflect the level of testosterone functioning directly at the target site. It is worthwhile to examine the interindividual variation in the rate of testosterone conversion into estrogen and other metabolites and to assess the importance of this individual discrepancy in the induction of HCC. This is the first report on the role of serum testosterone level in the development of HCC; further studies on other populations are needed to validate this finding.

REFERENCES

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