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Aromatase (*CYP19*) promoter gene polymorphism and risk of non-viral hepatitis-related hepatocellular carcinoma

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Abstract

Background—Experimental studies suggest that sex hormones may induce or promote the development of hepatocellular carcinoma (HCC). Androgens are converted to estrogens by the *CYP19* gene product, aromatase. Hepatic aromatase level and activity have been shown to be markedly elevated in HCC. Aromatase expression in liver tumors is driven by a promoter upstream of *CYP19* exon I.6.

Method—We first identified an *A/C* polymorphism in the exon I.6 promoter of the *CYP19* gene. To determine whether allelic variants in the *CYP19* I.6 promoter differ in their ability to drive gene expression, we carried out an *in-vitro* reporter gene assay. We then studied the association between this polymorphism and HCC risk in two complementary case-control studies: one in high-risk southern Guangxi, China, and another in low-risk US non-Asians of Los Angeles County.

Results—Transcriptional activity was 60% higher for promoter vectors carrying the rs10459592 *C* allele compared to those carrying an *A* allele ($p=0.007$). In both study populations, among subjects negative for at-risk serologic markers of hepatitis B or C, there was a dose-dependent association between number of high activity *C* allele and risk of HCC (p for trend=0.014). Risk of HCC was significantly higher [odds ratio (OR) = 2.25, 95% confidence interval (CI) = 1.18–4.31] in subjects homozygous for the *C* allele compared to those homozygous for the *A* allele.

Conclusion—Our study provides epidemiologic evidence for the role of hepatic aromatization of androgen into estrogen in the development of non-viral hepatitis-related HCC.

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Keywords

Aromatase enzyme; hepatocellular carcinoma; gene polymorphism; viral hepatitis; sex hormones

Hepatocellular carcinoma (HCC) is the predominant form of primary liver cancer, accounting for over 90% of cases in high-risk areas and 75% of cases in low-risk areas. Incidence rates vary internationally more for HCC than for any other major cancer: there is a 40-fold difference between high- and low-risk regions.¹ For example, among Chinese in the high-risk region of Guangxi, China, the age-standardized (world population) incidence is estimated at 120 per 100,000 person-years in men and 30 per 100,000 person-years in women. The corresponding rates among US non-Hispanic white men and women are 3 and 1 per 100,000 person-years respectively.¹⁻² Worldwide, the established risk factors for HCC are chronic infections with either hepatitis B virus (HBV) or hepatitis C virus (HCV), alcohol abuse and exposure to dietary aflatoxin.²⁻³ It is estimated that 80% of HCC worldwide is etiologically associated with chronic HBV infection. In high-risk areas, such as China and Africa, HBV infection is by far the most important risk factor for HCC in humans.⁴⁻⁵ Furthermore, in many of these areas endemic for HBV infection, aflatoxin, a very potent hepatocarcinogen present in moldy foods, plays a synergistic role with HBV in causing HCC.⁶⁻⁸ Conversely, HCV infection is more important in low- to intermediate-risk areas in Japan, North America and Europe.² In Los Angeles County, California, half of HCC cases among non-Asians are related to viral hepatitis, but more predominantly from HCV than HBV, whereas dietary aflatoxin is thought to play a negligible role in HCC development in this low-risk population. Other risk factors such as chronic alcohol abuse and diabetes mellitus have been shown to have independent and synergistic effects on HCC development in the US and possibly in other low-risk populations.⁹

The normal human liver is morphologically and functionally modulated by sex hormones. Whatever the etiologic factor implicated, HCC is consistently 2–3 times higher in men than in women, suggesting that sex hormones, including androgen and estrogen, may be involved in HCC development and progression.¹⁰⁻¹¹ In support of this hypothesis, epidemiologic observations suggest that long-term use of oral contraceptives and of anabolic androgenic steroids are associated with increased risk of benign and malignant liver tumors.¹² In a Taiwanese population, serum testosterone levels were found to be elevated more in HCC patients than in controls regardless of HBsAg status¹³ whereas we reported an elevated serum testosterone level in HBsAg-positive patients among HCC cases compared with controls.¹⁴ Experimental studies using animal models also suggest that androgens and estrogens may induce or promote the process of HCC development.¹⁵

Aromatase is the enzyme that converts androgens to estrogens, and its hepatic level and activity have been shown to be markedly increased in HCC.¹⁶ Aromatase expression in liver tumors is driven by the *CYP19* gene promoter that lies upstream of the alternate first exon I.6.¹⁷ We have discovered a functional A-to-C polymorphism that lies in this promoter region, 35 base pairs (bp) upstream of the I.6 transcription start site, between the TATA box and the initiation site. In this study, we examined the association between this polymorphism and HCC risk using two complementary case-control studies: one in high-risk southern Guangxi, China, and one in low-risk US non-Asians of Los Angeles County. These two populations represent the extreme ends of the HCC risk spectrum and their risks are explained by different etiologic factors.^{4, 9} It is more appropriate to test the hormonal carcinogenesis hypothesis in the latter with a low background risk for HCC.

Material and Methods

Identification of new single nucleotide polymorphism in CYP19

PCR products containing exon I.6 and its upstream promoter were resequenced in 60 de-identified control samples from multiple ethnicities which are not part of the current study population. Samples were sequenced in both forward and reverse orientation using a standard BigDye Terminator protocol (Applied Biosystems, Foster City, CA) and read on a 2700 ABI PRISM® 2700 DNA Analyzer.

Functional study of polymorphism in the CYP19 promoter (promoter I.6)

To determine whether the two allelic variants of promoter I.6 differed in their ability to drive gene expression, we carried out an *in vitro* reporter gene assay. Using DNA samples from AA and CC individuals, a 157 bp fragment of the I.6 promoter was PCR-amplified using primers 5'-ATTGCAAGTCACAGAACTC-3' (forward) and 5'-GGATGGTAGGTAGTCTGTAGC-3' (reverse). The PCR products were cloned into the pCR2.1-TOPO vector using the TOPO TA Cloning kit (Invitrogen, Carlsbad CA), and subcloned into the pGL3-Basic vector (Promega, Madison WI) using the KpnI and XhoI sites upstream of the firefly luciferase gene. All inserts were sequenced to insure fidelity of the amplified sequences.

Using 24-well plates, six wells were prepared for each of the three reporter plasmids: pGL3-A, pGL3-C, and pGL3-Basic (empty vector), as well as six wells for a non-transfection control. Triplicate plates were prepared using different plasmid preparations. Transient transfections were carried out using the Promega (Madison WI) Tfx-20 liposome reagent in serum-free media. JEG-3 cells (ATCC, Rockville, MD), which are capable of expressing CYP19 exon I.6, were co-transfected with 0.5 ug of the reporter plasmid and, as an internal standard for transfection efficiency, with 0.01 ug of the pRL-TK control plasmid (Promega, Madison WI) containing the Renilla luciferase gene driven by a weak promoter. Cells were maintained in MEME media (ATCC, Rockville, MD) containing 10% FBS and 1% glutamine, penicillin, streptomycin. The cells were incubated with the transfection mixture for 45 minutes, then overlaid with 1 ml of complete medium and incubated for 48 hours.

Cells were washed and lysed with 100 ul of passive lysis buffer, and 20 ul of lysate was assayed for firefly and renilla luciferases using the Dual-Luciferase Reporter Assay System (Promega, Madison WI). Luminescence was measured in a TD20/20 single-channel luminometer (Turner Designs). Background was measured in the non-transfected control. Luciferase activity was expressed as relative light units, dividing the total firefly luciferase activity by total renilla luciferase activity.

Data from the triplicate experiments were analyzed using a nested one-way mixed effect ANOVA to test for differences among the three plasmids (fixed effect) while accounting for differences in transfection efficiency (triplicate plasmid preparations treated as random effect nested within plasmid).¹⁸ The null hypothesis (*i.e.* no difference between the A and C alleles) was tested by conducting an F test with numerator and denominator degrees of freedom of 1 and 6, respectively. Data are presented as means of the triplicate experiments $\pm$ standard errors.

Case-controls studies of HCC in Los Angeles County, California and Guangxi, China

The present study included participants of two case-control studies of HCC, one in low-risk non-Asians in Los Angeles, California, and the other in high-risk Chinese in the southern part of the Guangxi Autonomous Region, China. The designs of the two studies have been described previously.¹⁹ Permission to conduct this study had been obtained from the

Institutional Review Boards at the University of Southern California and the Guangxi Cancer Institute. Separate informed consent forms for interview and biospecimen collection were obtained from each study participant.

Study subjects of the Los Angeles Case-Control Study of HCC

In Los Angeles, we studied incident HCC in black, Hispanic, and non-Hispanic white residents of Los Angeles County who were between 18 and 74 years of age at diagnosis from January 1984 through December 2001. Cases were identified through the Los Angeles County Cancer Surveillance Program, a population-based cancer registry that records all incident cancers diagnosed in Los Angeles County. Due to the rapidly fatal nature of HCC (the median time interval between diagnosis and death is approximately 3 months), 84% of eligible patients died prior to our attempted contact. Among the 478 patients we contacted, 34 (7%) were too ill to be interviewed, and 325 (73%) of the remaining 444 were interviewed. An experienced hepatopathologist reviewed the histology slides of all interviewed HCC patients; 25 cases judged to be non-HCC were excluded.

For each enrolled patient with HCC (case), we identified up to two consenting control subjects who were matched to index case by age (± 5 years), gender, and race/ethnicity (non-Hispanic white, Hispanic, black). Eligible controls were identified from the neighborhoods in which case patients resided at the time of diagnosis. Using the house of each case as a reference point and proceeding in a systematic and invariable sequence, we canvassed up to a maximum of 150 residential units to identify one to two eligible control subjects. A total of 474 neighborhood control subjects were recruited into the study; most were the first (74%) or second (12%) eligible neighbors.

Study subjects of the Guangxi Case-Control Study of HCC

In Guangxi, China, we identified newly diagnosed HCC from four major hospitals in the city of Nanning. Participating hospitals were comparable in their quality of patient care and diagnosis. Only patients diagnosed during September 1995 through September 1998, between the ages of 20 and 64 years, and residing in Nanning City or its neighboring townships were asked to participate in the study. We began the study in October 1995 and closed enrollment in October 1998 when 250 patients had been recruited into the study.

We identified one consenting control subject per case among all patients admitted to the same hospital within one month of the index case's hospital admission, who had no history of cancer or clinical liver cirrhosis. The matching criteria were age (within 3 years), gender, ethnicity (Han, Zhuang, Yao, other), and district (if resident of Nanning City) or township (if resident of neighboring townships) of residence.

Data Collection

All consenting cases and control subjects in Los Angeles, California and Guangxi, China were interviewed in person by trained interviewers using structured questionnaires. Both the Los Angeles and Guangxi questionnaires solicited demographic information, lifetime use of tobacco and alcohol, medical history, and other lifestyle factors. An alcohol drinker was defined as someone who had drunk alcoholic beverages at least once a week for six months or longer. One drink was defined as 360 g of beer (12.6 g of ethanol), 103 g of wine (12.3 g of ethanol), or 30 g of spirit (12.9 g of ethanol). A smoker was defined as someone who had ever smoked on a daily basis. Smokers were asked the average number of cigarettes smoked per day.

Serum and buffy coat samples were collected from all subjects of the Guangxi study (250 cases and 250 controls). For the Los Angeles study, we collected from study subjects serum

samples beginning in January 1992 and buffy coat samples beginning in July 1993. The buffy coat samples were available on 137 (78%) of 176 eligible HCC cases (i.e., those interviewed after July 1993). For the 283 control subjects from whom DNA donation was sought, 237 (84%) consented and donated blood samples. We examined and found no differences in the distributions by age, gender, level of education, cigarette smoking, alcohol consumption, history of diabetes, and serologic markers for HBV and HCV infections between subjects with with donated DNA (i.e., those included in the present study) and those without donated DNA, both for the HCC case and control subjects groups.

Laboratory tests

Blood samples from cases and controls were processed and stored (-20°C) in an identical manner. The assays used for testing serologic markers of HBV and HCV infections have been described previously.⁹⁻²⁰ Briefly, we tested all study samples for the presence of hepatitis B surface antigen (HBsAg) in serum using commercialized kits (AUSRIA, Abbott Laboratories, North Chicago, IL), and negative samples (for the Los Angeles study only) were further tested for the presence of antibodies to the hepatitis B core antigen (anti-HBc) using standard testing kits (Corab, Abbott Laboratories, North Chicago, IL). All samples were tested for the presence of antibodies to the hepatitis C virus (anti-HCV) in serum using the ELISA version 2.0 kit manufactured by Ortho Diagnostic Systems, with confirmation of positive samples using RIBA version 2.0 (Chiron, Emeryville, CA). Serum samples were tested blindly, identified only by codes without regard to case/control status.

DNA was purified from buffy coats of peripheral blood using a QIAamp 96 Blood Kit (Qiagen, Valencia, CA). The genomic region containing the polymorphic site was amplified by polymerase chain reaction (PCR) using forward primer (5'-ATATGGTACCATTGCAAGTCACAGAA ACTC-3') and reverse primer (5'-ATATAAGCTTGGATGGTAGGTAGTCTGTAGC-3'). Genotyping was performed by direct sequencing of the PCR products using a standard BigDye Terminator protocol (Applied Biosystems, Foster City, CA) and read on an ABI PRISM 2700 DNA Analyzer (PE Biosystems). Samples were sequenced in both forward and reverse orientation.

Eight HCC cases and 52 controls were non-informative in *CYP19*. These subjects were excluded. Thus, the present analysis involved 379 HCC cases (135 HCC cases of non-Asians from Los Angeles and 244 HCC cases of Chinese from Guangxi), and 435 control subjects (193 of non-Asians from Los Angeles and 242 of Chinese from Guangxi).

Statistical analysis

The *CYP19* promoter genotype distribution was tested for Hardy-Weinberg equilibrium using the chi-square test. The strength of the gene-cancer association was measured by odds ratios (ORs) and their 95% confidence intervals (CIs). Odds ratios were estimated by fitting unconditional logistic regression models,²¹ coding the three genotypes with two dummy variables. Tests of trend were performed by including genotype as a continuous variable (0, 1, 2 minor alleles) and performing a likelihood ratio test. Tests of interaction were performed by including the appropriate cross-product term in the logistic model and conducting a likelihood ratio test. All models were adjusted for age (year) at recruitment, gender, race or ethnicity (Han, Zhuang or Yao in the Guangxi study; and Hispanic, Caucasian or African-American in the Los Angeles study), level of education (below high school, high school graduates, some college/occupational school, college graduates or above), number of cigarettes smoked per day, number of alcoholic drinks per day, and (when the two study populations were combined) study location (Guangxi or Los Angeles). Additional adjustment for previously established risk factors in the two study populations included history of diabetes mellitus for the Los Angeles study⁹ and cytokine gene

polymorphisms for the Guangxi.20 Statistical analyses were carried out using the SAS software Version 9.1 (SAS Institute, Cary NC). All *P* values quoted are two-sided. The two-sided *P* values under 0.05 were considered statistically significant.

Results

We identified two polymorphisms, A/C and A/G at positions 51,536,141 and 51,536,022 respectively (human chromosome 15 primary reference assembly, build 37.1), by resequencing approximately one kilobase of genomic DNA containing exon I.6 from 50 individuals from four ethnic groups: African-American, Hispanic, non-Hispanic white, and Asian. These two polymorphic sites (rs10459592 and rs4775936) were in perfect linkage disequilibrium, with the two haplotypes being CA and AG.

We then conducted a luciferase promoter reporter assay to determine whether the two allelic variants of the promoter I.6 rs10459592 polymorphism differed in their ability to drive reporter gene expression (Figure 1). Transcriptional activity was 60% higher for promoter vectors carrying the C allele compared to those carrying an A allele ($p=0.007$).

In the Los Angeles study, the mean ages (standard deviation) of HCC cases at diagnosis and controls at the time of cancer diagnosis of their index cases were 58.9 (10.2) years and 57.5 (10.9) years. In the Guangxi study, the corresponding figures were 47.3 (9.6) and 47.4 (10.3) years, respectively. In both studies, HCC patients consumed more cigarettes and alcohol than control subjects (Table 1).

In Guangxi, a hyper-endemic region for both HBV infection and HCC, positivity for HBsAg, a marker of chronic carrier state, has been shown to be the sole serologic marker of HBV infection predicting HCC risk.^{4, 22} Furthermore, HBV is the predominant etiology in virally related HCC cases in this population; HCV plays a negligible role in this high-risk Asian population.²³ Eighty-two percent of cases versus 14 % of controls in the Guangxi component of this study were positive for HBsAg. In contrast, only 4 percent of the cases versus 1 percent of the controls were positive for anti-HCV (Table 1).

In Los Angeles, a low-risk region for both HBV infection and HCC, two serologic markers of HBV infection, HBsAg (a marker of chronicity) and anti-HBc (a marker of past primary infection), significantly predict risk of HCC.²⁴ Both HBV and HCV significantly contribute to the HCC burden in the U.S. Decades earlier, HBV was responsible for more cases of HCC than HCV.² However, with the increasingly important role of HCV in HCC burden in the U.S. over the past decades, HCV now has overtaken HBV as the principal contributor to virally-related HCC cases in the U.S.²⁵ Twenty-eight percent of cases versus 14% of controls in the Los Angeles component of this study were positive for either HBsAg or anti-HBc. In contrast, 47% of cases versus 0.5% of controls were positive for anti-HCV (Table 1).

To determine whether the I.6 promoter variant is associated with incidence of HCC, we genotyped samples from two existing case-controls studies of HCC. Allele frequencies of the C allele of CYP19 I.6 polymorphism among control subjects were 0.51 in Los Angeles and 0.41 in Guangxi. There was no evidence of departures from Hardy-Weinberg equilibrium in either Guangxi or Los Angeles. The association between genetic polymorphism of in the I.6 region of the *CYP19* I.6 gene and risk of HCC was shown in Table 2. In the Los Angeles County study, subjects possessing at least one copy of the C allele were at approximately 2-fold increased risk compared to those homozygous for the A allele. However, the C allele was not associated with increased risk for subjects in the Guangxi study. Nevertheless, the difference in the *CYP19* genotype-HCC risk association between the two study locations was not statistically significant ($p=0.118$).

Table 3 shows the association between the polymorphism in the *CYP19* gene and HCC risk among subgroups of individuals stratified by the serologic status of HBV and/or HCV and also by study location. In both Los Angeles and Guangxi, among individuals with absence of both HBV and HCV serologic markers, increasing number of *C* alleles of the *CYP19* I.6 genotype was associated with increased risk of HCC (P for trend = 0.014 for pooled results). Overall, compared to those with *AA* genotype, individuals with the *CC* genotype had an OR of 2.25 (95% CI = 1.18–4.31). In Los Angeles, the association was statistically significant. In Guangxi, due to the smaller numbers in this subgroup of subjects, the association did not reach statistical significance. Among the subgroup of subjects negative for HBV and HCV serology, the p value for interaction between *CYP19* genotype and study location was 0.58.

Conversely, in the pooled results from the two studies, there was no statistically significant association between the number of *C* alleles of the *CYP19* I.6 genotype and HCC risk among subjects positive for HBV and/or HCV serological markers. In Los Angeles, no association was observed. In Guangxi, where the virally-related HCC cases were predominantly hepatitis B carriers, a statistically significant, inverse association was noted between the presence of the *C* allele and HCC risk. Among this subgroup of HBV or HCV positive subjects, the *CYP19*-HCC association noted in Los Angeles was not statistically different from that noted in Guangxi; p for interaction between *CYP19* genotype and study location=0.32.

We repeated the analysis shown in Table 3 with additional adjustment for previously established risk factors in the two study populations. In Los Angeles, the additional covariate was history of diabetes mellitus⁹ and in Guangxi, the cytokine gene polymorphisms were the additional covariates.²⁰ No material changes in results were noted (not shown).

We also repeated the analysis using conditional logistic regression with matching by gender and ethnicity and the results remained essentially the same. Among individuals with absence of both HBV and HCV serologic markers, increasing number of *C* alleles of the *CYP19* I.6 genotype was associated with increased risk of HCC (P for trend = 0.010 for pooled results). Overall, compared to those with *AA* genotype, individuals with the *CC* genotype had an OR of 2.35 (95% CI = 1.23–4.51).

Discussion

This is the first report of an association between polymorphism in the exon I.6 promoter of the *CYP19* gene and risk of human HCC, and provides epidemiologic evidence for the role of hepatic aromatization of androgen into estrogen in the development of HCC. This *CYP19* genotype-HCC risk association was principally confined to non-viral hepatitis-related cases of HCC, suggesting that the carcinogenesis pathway underlying this observed statistical association is independent of any contributory roles of the oncogenic hepatitis viruses.

The human liver is a major site for biotransformation, conjugation and catabolism of sex steroids. Aromatase converts androgen substrates such as testosterone and androstenedione into estrogens. The liver, along with the placenta, is the major source of aromatase expression in fetal tissues.^{26–27} In adult liver, aromatase is nearly undetectable in healthy tissues,^{16, 28–29} but high aromatase expression and activity, along with locally-elevated estrogen levels, has been observed in non-malignant tissues from liver with alcoholic-cirrhosis.²⁹ The locally increased estrogen can act as a tumor promoter by increasing cell proliferation through the amphiregulin-epidermal growth factor receptor signaling pathway,³⁰ or by inducing the formation of potentially mutagenic free radical-mediated DNA and

RNA adducts.^{31–32} In tissues from liver with HCV-cirrhosis, however, neither aromatase activity nor estrogen formation could be detected.²⁹

In line with our hypothesis that increased aromatase activity and local estrogen production play an etiologic role in HCC related to non-viral factors such as alcohol abuse, but not in viral hepatitis-induced HCC, some, but not all HCC cell lines express aromatase. Aromatase-driven estrogen formation is seen in HepG2 and Huh7 cell lines, but not in HA22T cells.²⁹ The latter exhibit autologous production of interleukin-6,³³ which has been implicated in HBV-related HCC development.²⁰ Hence, aromatase-deficient HA22T liver cancer cells may be a model that resembles viral hepatitis-related HCC.

Furthermore, aromatase-deficient mice, which lack the ability to convert androgen into estrogen, develop spontaneous hepatic steatosis, a process which is reversed by estrogen replacement.³⁴ Hepatic steatosis is a characteristic feature of chronic human HBV and HCV infections ^{35–36}, and is implicated in virally-induced liver damage.^{37–38} Estrogen may inhibit the enhanced proinflammatory cytokine production and cell-mediated immune response^{39–40} implicated in HBV-related hepatocellular carcinogenesis.²⁰ In the study in Guangxi, where the virally-related HCC cases were predominantly hepatitis B carriers, a statistically significant, inverse association was noted between the presence of the *CYP19* C allele that drives higher aromatase activity and HCC risk. Our epidemiologic observation therefore corresponds with experimental evidence that suggests a favorable role of estrogen in chronic liver disease with hepatitis B virus infection.⁴¹ However, given the small sample size, this novel observation needs to be replicated in larger studies.

The *CYP19* promoter region examined in this study, the I.6 promoter, was identified in 1998 by Shozu *et al*, and was observed to be expressed at high levels in bone, fetal liver, and especially in liver tumor.¹⁷ Tissue-specific control of aromatase expression is achieved by the use of alternative first exons in the *CYP19* gene, which are spliced onto the mRNA transcript in a tissue-specific manner.⁴² The promoter regions upstream of these alternative untranslated exons contain different constellations of transcription factor binding sites, allowing regulation of estrogen biosynthesis to be uniquely controlled in each tissue. Interestingly, low level aromatase expression in normal adult liver is driven by a different promoter, promoter II, and is not regulated by the same factors as those involved in fetal liver and liver tumor. Sequences in exon I.6 and its promoter regions may therefore be important in driving overexpression of aromatase in HCC. The A/C polymorphism we have described is located in a consensus sequence for a TFIID binding site,¹⁷ suggesting that it may have functional significance. Hence, our finding, that the I.6 promoter allele (C) that more efficiently drives aromatase expression is a risk factor for non-viral hepatitis-related HCC, thus provides epidemiologic evidence for the hypothesis that overexpression of aromatase may be involved in hepatocarcinogenesis.

A recent study by Yuan *et al* reported a lack of association between seven functional polymorphisms in genes encoding estrogen metabolizing enzymes, including the *CYP19* gene, and HCC risk in a case-control study among Chinese.⁴³ In this study, a nonsynonymous polymorphism in the coding region of the *CYP19* gene, caused by a substitution of arginine for tryptophan at codon 39 and resulting in a non-functional aromatase protein, was not found to be associated with HCC risk in both HBV positive and negative subjects. However, the variant allele was rare (frequency = 0.03 among controls) and only one subject out of the 416 cases and 480 controls was homozygous for the variant allele.

The strength of the present study is the use of two epidemiologic databases derived from populations at polar ends of the HCC risk spectrum. In both populations, controls were

sampled from the neighborhoods of cases at the time of diagnosis. In the Los Angeles study, we recruited cases from the population-based cancer registry that recorded all incident cancers diagnosed in Los Angeles County. In the Guangxi population, although there was no population-based registry, more than 90 percent of HCC cases in Nanning were diagnosed or treated at the 4 major hospitals in our study. Nevertheless, we recognize that there was a relatively low recruitment rate of cases in the LA study due to the rapidly fatal nature of HCC and patients who were too sick to be interviewed were not included in the Guangxi study. In the LA study, due to differential response rate to our request for biospecimens, the matching by gender and ethnicity between cases and controls was also disrupted. However, the distribution of the CYP19 gene polymorphism did not differ significantly by gender or ethnicity. When we re-did the analysis using conditional logistic regression with cases and controls matched by gender and ethnicity, the results remained essentially unchanged. Finally, small sample size was also a limitation to the study.

In conclusion, we found an association between non-viral hepatitis-related HCC risk and a functional polymorphism in the promoter upstream of the hepatically-expressed alternate first exon I.6 of the *CYP19* gene. The allele driving higher aromatase expression was associated with increased HCC risk, suggesting that increased aromatase expression and activity promote HCC development in non-virally infected individuals. If confirmed, drug development efforts that put a focus on the aromatase-driven pathway as part of prevention and treatment strategies for non-viral hepatitis-related HCC would be warranted.

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Figure 1. Relative transcriptional activity of aromatase exon I.6 allelic variants (rs10459592) in luciferase reporter gene assay

Luciferase activity is reported as relative light units (total firefly luciferase activity/total renilla luciferase activity). Each experiment was performed in triplicate using DNA from three different plasmid preparations. Background was measured in the non-transfected control.

Table 1

Distribution of known or suspected risk factor [mean (standard deviation) or number (percent)] for HCC in the cases and controls in the Los Angeles County and Guangxi studies

	Los Angeles County			Guangxi		
	Cases	Controls	<i>P</i> ^a	Cases	Controls	<i>P</i> ^a
Total number of subjects	135 (100.0)	193 (100.0)		244 (100.0)	242 (100.0)	
Age at interview (year), mean (SD) ^b	58.9 (10.2)	57.5 (10.9)	0.214	47.3 (9.6)	47.4 (10.3)	
Sex (%)						
Males	94 (69.6)	117 (60.6)	0.094	215 (88.1)	213 (88.0)	0.973
Females	41 (30.4)	76 (39.4)		29 (11.9)	29 (12.0)	
Race/Ethnicity (%)						
White	82 (60.7)	158 (81.9)	<0.001	-	-	
Hispanic or African-American	53 (39.3)	35 (18.1)		-	-	
Chinese - Han ethnicity	-	-		195 (79.9)	192 (79.3)	0.874
Chinese - Zhuang/Yao ethnicity	-	-		49 (20.1)	50 (20.7)	
Level of education (%)						
Below high school	26 (19.2)	12 (6.2)	<0.001	34 (13.9)	39 (16.1)	0.639
High school education	39 (28.9)	46 (23.9)		156 (63.9)	146 (60.3)	
Some college/occupational school	46 (34.1)	67 (34.7)		37 (15.2)	34 (14.1)	
College graduates or above	24 (17.8)	68 (35.2)		17 (7.0)	23 (9.5)	
Cigarette smoking (%)						
Never smoker	41 (30.4)	63 (32.6)	0.609	133 (54.5)	160 (66.1)	0.027
Ever smoker, <20 cig per day	30 (22.2)	49 (25.4)		33 (13.5)	28 (11.6)	
Ever smoker, 20+ cig per day	64 (47.4)	81 (42.0)		78 (32.0)	54 (22.3)	
Alcohol drinking (%)						
Non-drinkers	37 (27.4)	66 (34.2)	<0.001	153 (62.7)	203 (83.9)	<0.001
<3 drinks per day	35 (25.9)	90 (46.6)		60 (24.6)	30 (12.4)	
3 or more drinks per day	63 (46.7)	37 (19.2)		31 (12.7)	9 (3.7)	
Risk-defining HBV markers(%) ^c						
Negative	97 (71.9)	167 (86.5)	0.001	44 (18.0)	208 (86.0)	<0.001
Positive	38 (28.1)	26 (13.5)		200 (82.0)	34 (14.0)	

	Los Angeles County			Guangxi		
	Cases	Controls	<i>P^a</i>	Cases	Controls	<i>P^a</i>
Anti-HCV positive (%) ^d						
Negative	72 (53.3)	192 (99.5)	<0.001	235 (96.3)	239 (98.8)	0.072
Positive	63 (46.7)	1 (0.5)		9 (3.7)	3 (1.2)	

^a*P*-values (2-sided) were derived from *t*-test (for means) or χ^2 (for frequencies) statistics.

^bSD, standard deviation

^cIn Guangxi (high-risk region), the sole risk-defining marker was HBsAg. In Los Angeles (low-risk region), the risk-defining markers were HBsAg and anti-HBc (see text in Results for explanation).

^d*P*-values (2-sided) were derived from Fisher's exact test.

CYP19 exon 1.6 (rs10459592) genotype in relation to risk of hepatocellular carcinoma (HCC), the Los Angeles County and Guangxi case-control studies

Table 2

	Los Angeles		Guangxi		Total	
	CA/CO ^a	OR (95% CI) ^b	CA/CO ^a	OR (95% CI) ^b	CA/CO ^a	OR (95% CI) ^c
CYP19 1.6 genotype						
AA	19/43	1.00	92/85	1.00	111/128	1.00
AC	82/104	2.00 (1.00–3.97)	110/115	0.85 (0.56–1.27)	192/219	1.08 (0.77–1.50)
CC	34/46	2.25 (1.02–4.96)	42/42	0.88 (0.52–1.50)	76/88	1.10 (0.72–1.68)
P for trend		0.056		0.538		0.637

^aCA/CO, number of cases/number of controls.

^bAdjusted for age, sex, race/ethnicity, level of education, number of cigarettes smoked per day and number of alcoholic drinks per day.

^cAdditionally adjusted for study location.

Table 3

CYP19 exon I.6 genotype (rs10459592) and HBV/HCV status in relation to risk of hepatocellular carcinoma (HCC), the Los Angeles County and Guangxi case-control studies

	<u>Los Angeles</u>		<u>Guangxi</u>		<u>Total</u>		
	<i>CYP19</i> I.6 genotype	CA/CO ^a	OR (95% CI) ^b	CA/CO ^a	OR (95% CI) ^b	CA/CO ^a	OR (95% CI) ^c
<u>HBV and HCV negative</u>							
AA	9/40	1.00	1.00	14/76	1.00	23/116	1.00
AC	32/86	1.75 (0.73–4.19)	1.14 (0.52–2.52)	19/97	1.37 (0.77–2.44)	51/183	1.37 (0.77–2.44)
CC	22/41	2.79 (1.07–7.24)	2.14 (0.82–5.55)	11/33	2.25 (1.18–4.31)	33/74	2.25 (1.18–4.31)
<i>P</i> for trend		0.032	0.150		0.014		
<u>HBV or HCV positive</u>							
AA	10/3	1.00	1.00	78/9	1.00	88/12	1.00
AC	50/18	1.20 (0.21–6.74)	0.50 (0.21–1.21)	91/18	0.61 (0.29–1.30)	141/36	0.61 (0.29–1.30)
CC	12/5	1.17 (0.15–9.28)	0.33 (0.11–0.94)	31/9	0.48 (0.20–1.19)	43/14	0.48 (0.20–1.19)
<i>P</i> for trend		0.905	0.032		0.107		

^aCA/CO, number of cases/number of controls.

^bAdjusted for age, sex, race/ethnicity, level of education, number of cigarettes smoked per day and number of alcoholic drinks per day.

^cAdditionally adjusted for study location