Hepatocellular carcinoma is one of the leading causes of cancer death worldwide. The etiology of liver cancer is multifactorial, and infection with hepatitis B virus (HBV), whose pathogenesis is exacerbated by the acquisition of mutations that accelerate carcinogenesis, or hepatitis C virus (HCV) and dietary exposure to aflatoxin B1, all contribute to elevating one’s risk for this disease. In this study, we sought to determine the contributions of these agents by measuring the occurrence of an HBV 1762T/1764A double mutation, an aflatoxin-specific 249G→T mutation of the p53 gene, and HCV in plasma of 34 HCC cases and 68 age- and gender-matched controls, and in 25 liver tumors from northern Thailand. In total, 14 cases, 5 controls, and 19 tumors had detectable levels of HBV DNA. All 14 cases, 2 controls (2.9%), and 17 tumors (89.5%) were positive for the HBV double mutation. Nine cases (26.3%), 10 controls (14.7%), and 6 tumors (24%) were positive for the p53 mutation. Five cases (14.7%), no controls, and 4 tumors (16%) had both mutations. The median age of HCC diagnosis in these 5 cases was 34 years versus 51 years for other cases. Five cases (14.7%) and 1 control (1.5%) were HCV enzyme immunoassay positive. Thus, specific HBV, HCV, and aflatoxin biomarkers reveal the complexity of risks contributing to HCC in northern Thailand and suggest further application of these biomarkers as intermediate end points in prevention, intervention trials, and etiologic investigations. (Cancer Epidemiol Biomarkers Prev 2005;14(2):380–4)

Materials and Methods

Case Materials. All HCC cases were diagnosed at Chiang Mai University using serology, ultrasound, and/or histopathology. The age and gender characteristics of the 25 HCC cases and the 102 subjects in the ongoing case-control study are found in Tables 1 and 2, respectively. The overrepresentation of male cases is consistent with previous distributions of HCC
Table 1. Characteristics of the 25 HCC Cases from Chiang Mai, Thailand

<table>
<thead>
<tr>
<th>Case/Control</th>
<th>Cases (n = 25)</th>
<th>Control (n = 68)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (median, y)</td>
<td>54.2</td>
<td>51.5</td>
<td>—</td>
</tr>
<tr>
<td>SD of age at diagnosis (y)</td>
<td>13.5</td>
<td>15.2</td>
<td>—</td>
</tr>
<tr>
<td>Gender, male</td>
<td>18 (72%)</td>
<td>62 (91%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HbsAg+</td>
<td>18 (72%)</td>
<td>62 (91%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HBDNA+</td>
<td>19 (76%)</td>
<td>64 (94%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HBV 1762T/1764A mutation+</td>
<td>14 (41.2%)</td>
<td>12 (17.6%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HCV EIA+</td>
<td>6 (24%)</td>
<td>10 (14.7%)</td>
<td>0.09</td>
</tr>
<tr>
<td>HCV RNA</td>
<td>2 (8%)</td>
<td>3 (4.4%)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Abbreviations: HbsAg, hepatitis B surface antigen; HBDNA, hepatitis B DNA.
*From tissue.
1HCV data available for 20 of 25 cases.

in Asia (6). A case-control study consisting of 34 HCC cases were age and gender matched with 68 hospital-based and liver disease-free controls. Other characteristics of the cases and controls are shown in Table 2. Plasma samples were coded before analysis. The serology studies to ascertain hepatitis B surface antigen and HCV status was done using commercial available assays. This has been studied and approved by the institutional review boards at Johns Hopkins University and Chiang Mai University.

Isolation of DNA from Plasma and Tissue Samples. DNA was isolated from plasma samples using Qiagen columns (Valencia, CA) as previously described (17, 20). DNA was also extracted from paraffin-embedded tissue specimens after sectioning and isolation using the Pinpoint Slide DNA Isolation System (Zymo Research, Orange CA).

Mutation Detection by Short Oligonucleotide Mass Analysis. Short oligonucleotide mass analysis for HBV and p53 mutations involved a PCR enrichment step. The primers for the HBV mutation analysis were HBVx7F, 5'-TTT GTT TAA AGA CTT GGA GTG GAG GAG TAG GTT A-3', and HBVx7R, 5'-TGG TGC GCA GAC CAA TTT ATG CTG GAG GCC TTC TAC AA-3'. The PCR primers for the p53 analysis were p53-8F1, 5'-CTACA ACT CAG TGT GAA CAG TGT GGA GGA GGA GAT TAG GTT A-3', and p53-8R1, 5'-CTG GAC GTC TCT CCA CAG TGA CAG TGA CAG GTG AAG TGT GAA-3'. In both cases the thermocycling conditions were 95°C for 2 minutes, then 40 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds followed by a final extension of 72°C for 2 minutes. Negative controls (no DNA added) were included for each set of PCR reactions. PCR product was purified by ethanol precipitation and digested with 8 units of BpmI (New England Biolabs, Beverly, MA) overnight at 37°C in a volume of 50 µL to release internal fragments. These fragments were 7 bp for HBV and 8 bp for p53. A phenol-chloroform extraction followed by an ethanol precipitation in the presence of SeeDNA (Amersham Pharmacia, Piscataway, NJ) was done to purify samples for analysis by electrospray ionization-mass spectrometry.

The digested fragments were resuspended in 10 µL of the high-performance liquid chromatography (HPLC) mobile phase [70:30 (v/v) solvent A/solvent B, in which solvent A was 0.4 mol/L 1,1,1,3,3,3-hexafluoro-2-propanol (pH 6.9) and solvent B was 50:50 (v/v) 0.8 mol/L 1,1,1,3,3,3-hexafluoro-2-propanol/methanol] and 8 µL was introduced into the HPLC coupled to the electrospray-mass spectrometer. HPLC was carried out at 30 µL/min using a 1 x 150-mm Luna C18, 5 µ reversed phase column (Phenomenex, Torrance, CA) and Surveyor pumps (ThermoFinnigan Corp, San Jose, CA). The gradient conditions were 70% A/30% B isocratic 1 minute programmed to 100% B in 3 minutes, in which it was held for 2.5 minutes followed by a return to 70% A/30% B in 1.5 minutes and isocratic elution for the remaining 32 minutes of the chromatography.

Mass spectra were obtained with a LCQ Deca ion-trap mass spectrometer (ThermoFinnigan Corp) equipped with an electrospray ionization source operated in the negative ionization mode. The spray voltage was set at –4.0 kV and the heated capillary was held at 240°C. Each of the oligonucleotide ions was isolated in turn and subjected to collision-induced dissociation at 30% collision energy. Full scan spectra of the resultant fragment ions from relative intensity (m/z) 600 to 2000 were acquired, and signals from up to three specific fragment ions were summed as a function of time for each of the oligonucleotides.

HBV 1762T/1764A Mutation Analysis by Mass Spectrometry. The mass spectrometer was programmed to acquire data in the centroid mode (1 scan, 200 milliseconds, isolation width 3 Da) using scan events monitoring each [M-2H]2+ oligonucleotide individually [scan event 1: WT-s (5'-AACGTTCT-3'), m/z 1099.20–750-2000; scan event 2: WT-as (5'-ACCTTTA-3'), m/z 1086.70–750-2000; scan event 3: Mut-s (5'-ATGATCT-3'), m/z 1078.70–750-2000; scan event 4: Mut-as (5'-ATCATTA-3'), m/z 1078.70–750-2000]. The fragment ions used for each oligonucleotide were WT-s (m/z 803.78 + 1132.22 + 1243.27), WT-as (m/z 910.07 + 1531.29), and Mut-s (m/z 914.30 + 1227.29), and Mut-as (m/z 1084.00 + 1252.00). A sample was considered positive when fragment ions were observed in either or both sense and antisense channels for the mutant allele in at least three scans across the peak.

Table 2. Characteristics of case-control study

<table>
<thead>
<tr>
<th>Case/Control</th>
<th>Cases (n = 34)</th>
<th>Control (n = 68)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at hospitalization (median, y)</td>
<td>51.7</td>
<td>51.5</td>
<td>—</td>
</tr>
<tr>
<td>Age at HCC diagnosis (median, y)</td>
<td>51.5</td>
<td>51.7</td>
<td>—</td>
</tr>
<tr>
<td>Gender, male</td>
<td>28 (82.4%)</td>
<td>56 (82.4%)</td>
<td>NS</td>
</tr>
<tr>
<td>Ethnicity, Thai</td>
<td>33 (97.1%)</td>
<td>67 (98.5%)</td>
<td>NS</td>
</tr>
<tr>
<td>Current residence, Chiang Mai</td>
<td>19 (55.9%)</td>
<td>39 (57.4%)</td>
<td>NS</td>
</tr>
<tr>
<td>History of liver disease</td>
<td>4 (11.8%)</td>
<td>10 (14.7%)</td>
<td>0.09</td>
</tr>
<tr>
<td>HbsAg+</td>
<td>25 (73.5%)</td>
<td>40 (58.8%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HBDNA+</td>
<td>14 (41.2%)</td>
<td>2 (2.9%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HBV 1762T/1764A mutation+</td>
<td>14 (41.2%)</td>
<td>2 (2.9%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Codon 249 p53 mutation+</td>
<td>9 (26.5%)</td>
<td>10 (14.7%)</td>
<td>0.15</td>
</tr>
<tr>
<td>HCV EIA+</td>
<td>5 (14.7%)</td>
<td>1 (1.5%)</td>
<td>0.02</td>
</tr>
<tr>
<td>HCV RNA</td>
<td>3 (8.8%)</td>
<td>1 (1.5%)</td>
<td>0.02</td>
</tr>
<tr>
<td>α-Fetoprotein &gt;400 ng/mL</td>
<td>27 (81.8%)</td>
<td>48 (70.6%)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Abbreviation: NS, not significant.
*Cases and controls matched by age and gender.
1From plasma.
HBV Mutations, HCV, p53, and HCC

Table 3. Analysis of HCC cases from the case-control study for HBV 1762T/1764A, codon 249 p53 mutations, and HCV infection from plasma samples

<table>
<thead>
<tr>
<th>Codon 249 p53 mutation</th>
<th>HBV 1762T/1764A mutation</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>16 (4; 25% HCV+)</td>
<td>9 (1; 11% HCV+)</td>
</tr>
<tr>
<td>Yes</td>
<td>4 (1; 25% HCV+)</td>
<td>5 (0 HCV+)</td>
</tr>
<tr>
<td></td>
<td>20 (5; 25% HCV+)</td>
<td>14 (1; 7% HCV+)</td>
</tr>
</tbody>
</table>

NOTE: Among the subjects who were negative for HBV 1762/1764 and codon 249 p53 mutations, 4 were HCV EIA positive, 10 were HBsAg positive, 8 had >20 years of tobacco smoking, 6 had >20 years of heavy alcohol use, and 2 each used betel nut or had type 2 diabetes. Collectively, only 4 subjects had none of these possible risk exposures.

Codon 249 p53 Mutation Analysis by Mass Spectrometry. The mass spectrometer was programmed to acquire data in the centroid mode (1 ms, 200 milliseconds, isolation width 3 Da) using four scan events monitoring each [M-2H]- oligonucleotide individually [scan event 1: AGG-s (5'-CGGAGCC-3'), m/z 1256.3—600-2000; scan event 2: AGG-as (5'-CCTTCGGT-3'), m/z 1219.8—600-2000; scan event 3: AGT-s (5'-CGGAGTC-3'), m/z 1244.3—600-2000; scan event 4: AGT-as (5'-ACTTCGGT-3'), m/z 1231.8—600-2000]. Reconstructed ion chromatograms were generated and smoothed from this raw data using an isolation width of 1.0 Da. The fragment ions used for each oligonucleotide were AGG-s (m/z 1047.3 + 1180.7), AGG-as (m/z 1268.6 + 1347.8 + 1637.2), AGT-s (m/z 1437.4 + 1542.4), and AGT-as (m/z 1075.0). A sample was considered positive when fragments were observed in either or both sense and antisense channels for the mutant allele in at least three scans across the peak.

Detection of HBV and HCV Infections. Serologic evidence of HBV infection was documented using the licensed enzyme immunoassays for hepatitis B surface antigen, hepatitis B core antigen, and anti–hepatitis B core (Abbott Laboratories, Abbott Park, IL). Antibodies to HCV were measured using a third-generation licensed HCV enzyme immunoassay (ELIA, Abbott Laboratories); HCV RNA was amplified using HCV core E1 primers according to methods previously reported (21, 22).

Data Analysis. All plasma samples were coded to mask the case status for short oligonucleotide mass analysis and interpretation. Standard descriptive analyses were conducted. Frequency distributions and proportions were calculated for categorical variables; medians and SDs were calculated for continuous variables. Cases and controls were compared using simple conditional logistic regression to account for the matching design.

Results

HBV 1762T/1764A and Codon 249 p53 Mutations in Liver Tumors. Initial studies in this investigation involved the assessment of the status of both HBV and p53 mutations in 25 liver tumors. Nineteen (76%) of 25 tumors contained detectable integrated HBV. Of these 19 HBV positive tumors, 17 (89.5%) had an HBV 1762T/1764A double mutation. Tumor and adjacent normal liver tissue pairs were available for four of these cases and, in each instance, the HBV mutation was present in both samples. The median age of the HCC cases in this group was 54.2 years; the SD was 13.5 years (Table 1).

In contrast to the HBV mutation data, the codon 249 p53 guanine to thymine transversion mutation was detected in 6 (24%) of 25 tumors. In the four tumor/normal pairs, the p53 mutation was detected in 3 of the tumors but not in any of the adjacent normal tissues. Furthermore, the median age of the liver cancer cases containing the codon 249 p53 mutation was 50.1 years, which was nearly 4 years younger than the whole group. Both the HBV and p53 mutations were detected in 4 (16%) of the samples. HCV virus status was measured in 20 of the tumor samples from paired plasma specimens and 4 (20%) of the 20 were positive for HCV antibody; in each of these cases the tumor contained the HBV 1762T/1764A double mutation. The high prevalence of all of these markers of HCC risk encouraged the design of a case-control investigation.

HBV 1762T/1764A and Codon 249 p53 Mutations in Plasma from Liver Cancer Cases and Controls. Plasma samples from 14 (41.2%) of the 34 HCC cases were found to have detectable levels of HBV DNA. All 14 of the plasma HCC samples were positive for the HBV 1762T/1764A mutation. Five (7.4%) of the 68 control plasma samples had detectable levels of HBV DNA; 2 (2.9%) were positive for the HBV double mutation. Among HBV DNA positive samples, a statistically significant (P = 0.01, two-tailed) overrepresentation of the HBV double mutation was observed among cases. Nine (26.5%) of the 34 cases and 10 (14.7%) of the 68 controls were positive for the codon 249 p53 mutation. Five cases (14.7%) and no controls were positive for both mutations. Six (17.7%) of the cases and 1 (1.5%) of the controls were EIA positive for HCV. Three cases (8.8%) and 1 control (1.5%) were HCV-RNA positive. Although the occurrence of the HBV 1762T/1764A mutation had been previously reported in patients with chronic liver disease in Thailand, this case-control study extends these findings to HCC patients. Five of the HCC cases contained both the HBV 1762T/1764A and codon 249 p53 mutations in plasma and the median age of diagnosis of cancer in these patients was 34.1 years compared with 51.5 years for the other HCC cases. A liver cancer case comparison analysis was done as shown in Table 3. At the time of the liver cancer diagnosis, 41.2% and 26.5% of the cases were positive for the HBV 1762T/1764A and codon 249 p53 mutations in plasma, respectively. Of the 16 cases containing neither the HBV or p53 mutation, 3 (18.8%) were EIA positive for HCV biomarker. In addition, none of the cases had all three markers and only one of the cases showed the presence of the HBV mutation and coinheritance with HCV. None of the controls had both mutations. Among the 13 HCC cases who were negative for HBV DNA, HCV, and a p53 mutation, 11 were hepatitis B surface antigen seropositive, 9 had a >20-year history of tobacco smoking, 6 had used alcohol heavily for >20 years, 2 used betel nut, and 2 were type 2 diabetics.

Discussion

Major risk factors for HCC include chronic infection with HBV or HCV and dietary exposure to aflatoxin B1, a potent, naturally occurring liver carcinogen (6, 23). Because the etiology of HCC is complex, the disease commonly progresses through a multistage process, with most cases involving liver cirrhosis (23). Studies in some populations in Asia and Africa suggest that a specific double mutation in the HBV X gene may be associated with accelerated...
The pathobiology of HBV infection is modulated through the selection and expression of several common viral mutants that affect a number of key viral proteins (9, 10, 30, 31). One of these common mutations is HBV 1762T/1764A that affects the expression of both the hepatitis B e antigen, because the mutation lies in the basic core promoter, and the X gene (32). This double mutation induces an increased inflammatory response that becomes stronger as the liver damage progresses from chronic hepatitis to cirrhosis (33). The underlying mechanism of the effects of HBV e antigen on the biology of inflammation and cirrhosis is still unclear, but substantial data suggest immune tolerance in the presence of this protein (32-34). The HBV 1762T/1764A double mutation also affects the amino acid sequence of the HBV X gene because it resides in codons 130 and 131, thereby inducing lysine to methionine and valine to isoleucine alterations, respectively (35). The X gene protein has been found to have numerous biological activities, but its specific role and that of this mutant protein in the pathogenesis of liver cancer have yet to be elucidated (36). The 1762T/1764A double mutation has been reported to occur more frequently in people infected with the genotype C strains of HBV, which is the most common genotype found in East Asian patients (37-39).

Chronic infections with either HBV or HCV have been reported to be associated with an increased risk of HCC. HBV is not cytotoxic for infected hepatocytes and these cells synthesize and secrete high levels of the S gene product (hepatitis B surface antigen), which appears in blood before the onset of symptoms. Plasma hepatitis B surface antigen levels peak during the symptomatic phase and then decline to undetectable levels within 6 months after infection in persons who clear the acute infection. HCV carrier status is then defined by hepatitis B surface antigen positivity in sequential samples obtained 6 months apart (8). Hepatitis B e antigen, HBV DNA, and DNA polymerase also appear in the serum soon after hepatitis B surface antigen and these are all biomarkers of active viral replication (40). Thus, these plasma HBV specific biomarkers reflect an intrinsic risk for the future development of HCC, but <10% of all chronic carriers of HBV will develop this cancer. In contrast, HCV has a much higher chronicity rate and ~80% of all infected individuals become carriers (4, 13). It is predicted that between 20% and 25% of all HCV carriers who develop cirrhosis will develop HCC subsequently.

The use of a biomarker in blood for the early detection of HCC is well established using α-fetoprotein (41). Whereas the use of α-fetoprotein as a HCC diagnostic marker is widely used in high-risk areas because of its ease of use and low cost, this marker suffers from both low specificity and sensitivity (42, 43). This lack of specificity and sensitivity has contributed to the identification of other molecular biomarkers that are possibly more mechanically associated with HCC development. Jackson et al. (19) have showed the potential use of specific p53 mutations in blood as a biomarker of HCC risk. In a recent investigation, the temporality of detecting the HBV 1762T/1764A mutation and wild-type HBV in plasma and tumor specimens, both before and after the clinical diagnosis of HCC was examined (20). Similar to previous reports, almost all of the HCC tumors contained either the HBV tandem mutation or integrated HBV DNA; the HBV 1762T/1764A double mutation in plasma was a predictive biomarker for HCC development.

Previously, in patients with chronic liver disease the HBV 1762T/1764A mutation has been examined in plasma from hepatitis B e antigen positive and negative individuals (44). These double mutations at positions 1762 and 1764 of the core promoter were found in 25 (60.4%) of 41 and 19 (76%) of 25 of the hepatitis B e antigen positive and negative individuals, respectively. Follow-up analysis of an
additional 80 cases of chronic liver disease confirmed the high prevalence of this mutation in hepatitis B e antigen negative individuals (45). The study reported in this investigation extends these findings for the first time to HCC cases in Thailand.

At the time of the liver cancer diagnosis, 38% and 20.6% of the cases were positive for the HBV 1762T/1764A and codon 249 p53 mutations in plasma, respectively. In contrast, in Qidong, People’s Republic of China, a similar case study found 73.3% and 60% of the samples had the HBV 1762T/1764A and codon 249 p53 mutations in plasma, respectively (17, 20). This suggest the occurrence of similar etiologic factors but at a lower prevalence in the Thailand population compared with the liver patients with cancer in Qidong, China. In contrast, the prevalence of markers of HCV infection were more frequent among HCC cases in Thailand. Because both changes are induced mutations that contribute in some fraction to the pathogenesis of HCC, these biomarkers could be useful for the early detection of an increased risk of HCC. Thus, specific biomarkers of HBV, HCV, and aflatoxin-induced changes in p53 reveal the complexity of the risk factors that contribute to hepatocellular carcinoma in northern Thailand and suggest the further evaluation of these biomarkers.

References
Hepatitis B 1762^T/1764^A Mutations, Hepatitis C Infection, and Codon 249  \textit{p53} Mutations in Hepatocellular Carcinomas from Thailand

Shuang-Yuan Kuang, Suree Lekawanvijit, Niwat Maneekarn, et al.


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