Aflatoxin Exposure and Hepatitis C Virus in Advanced Liver Disease in a Hepatitis C Virus–Endemic Area in Taiwan


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Abstract. This community-based study evaluated the role of aflatoxin exposure in advanced liver disease in hepatitis C virus (HCV)-endemic townships. Preventive health examination was performed on 314 adults ≥ 40 years of age recruited from HCV-endemic townships in Tainan, Taiwan. Aflatoxin-albumin in serum was quantified by a new enzyme-linked immunosorbent assay method. After adjusting serum albumin levels and platelet counts, aflatoxin-B1 albumin adducts was still an independent risk factor for advanced liver disease among all 314 residents (> 8 versus ≤ 8 (AFB1)-albumin/albumin; OR = 2.29, 95% CI = 1.23–4.27, P = 0.009) and particularly in anti-HCV-positive subjects (OR = 2.09, 95% CI = 1.09–4.0, P = 0.026). Levels of AFB1-albumin/albumin were significantly related to ultrasonographic parenchyma scores (P < 0.001, one-way ANOVA) in all and anti-HCV–positive subjects. The findings indicated aflatoxin exposure may be associated with advanced liver disease in chronic hepatitis C patients in HCV-endemic regions in Taiwan.

INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, with an estimated 170 million chronic carriers worldwide.1 Chronic HCV infection causes progressive hepatic fibrosis and cirrhosis in up to 20% of patients, and ~10–20% of cirrhotic patients develop hepatocellular carcinoma (HCC) within 5 years.2–7 Chronic hepatitis C with cirrhosis is a major risk factor for HCC,3–7 and 10–30% of Taiwan HCC patients have HCV infection.8 The prevalence of anti-HCV antibody (anti-HCV) is 2–4% among the general population of Taiwan.9,10 However, HCV-related HCC is more prevalent in central and southern Taiwan than in northern Taiwan.9,11–13 Disease progression is influenced by factors including duration of infection, age at infection, sex, coinfection with hepatitis B virus (HBV), and level of HCV viremia and its genotype.4–6 Additionally, dietary factors are known to enhance or attenuate HCC risk in HBV/HCV-infected patients.14–19

Aflatoxin is a mycotoxin produced by a species of Aspergillus that frequently contaminates staple foods. This mycotoxin is metabolized to exo-8,9-epoxide by cytochrome P450(s), and the metabolite reacts with the guanine residue to form the aflatoxin-N7-guanine adducts (AFB1-N7-guanine), resulting in a GC to TA transversion.20,21 Clinical studies have shown that AFB1 selectively targets at the third base position of codon 249 of the human p53 gene, a known mutational hotspot in human HCC.22–25 A significant association between aflatoxin exposure and human HCC has been reported in hyperendemic areas. Residents of sub-Sahara Africa and East and Southeast Asia are the most highly exposed populations.26–28 A synergistic interaction between AFB1 exposure and HBV infection on HCC risk has been reported in several epidemiologic studies.24–28 However, it remains unclear whether aflatoxin exposure is an associated risk factor for advanced liver disease including liver cirrhosis or HCC in patients with chronic hepatitis C.

Aflatoxin B1-albumin adduct (AFB1-albumin) is a biomarker for aflatoxin exposure and can be used to monitor chronic exposure levels for 1–2 months because of its 20-day half-life for serum albumin turnover. Sheabar and others29 developed a radioimmunoassay method for detecting aflatoxin-serum albumin adducts. Wang and others30 developed another radioimmunoassay method, which can be confirmed by high-performance liquid chromatography (HPLC). Furthermore, Kawamura and others31 developed an enzyme-linked immunosorbent assay (ELISA) method to quantify aflatoxin-albumin in serum. These studies showed that serum must normally be digested first with protease and extracted by organic solvent before testing, which is less convenient for clinical routine use. Thus, Wang and others developed and validated a new ELISA method of quantifying aflatoxin-albumin in serum that requires no pretreatment.32

To study the correlation between aflatoxin exposure and HCV infection, a community-based study of 314 residents in HCV-endemic townships was performed to evaluate the role of aflatoxin exposure in advanced liver disease in HCV patients. The ELISA method recently developed by Wang and others was used to quantify serum aflatoxin-albumin levels.

MATERIALS AND METHODS

In a community screening program conducted from July to December 2002, four thousand one hundred twelve residents ≥ 40 years of age underwent the adult preventive health examinations. The subjects were recruited from 11 clinics in HCV-endemic townships in Tainan County in Southern Taiwan. Among the 4,112 residents, 583 had thrombocytopenia (< 150 × 10^3/L). Of these 583, 269 could not be contacted for follow-up. Thus, 314 residents were examined for serum hepatitis B surface antigen (HBsAg), anti-HCV, and α-fetoprotein (AFP) and underwent upper abdominal ultrasonography (US) using a Titan ultrasonograph with a 5- to 2-MHz convex probe (SonoSite, Bothell, WA). Severity of liver parenchyma disease was rated by a modified simple scoring sys-
tem. The examined indicators were change of angle and edge (0: neither, 1: either, and 2: both), coarseness of liver parenchyma (0: normal, 1: mild, and 2: definite), and splanenomalgy (0: none, 1: slight, and 2: definite). Total scores ranged from 0 to 6, and scores of 5 or 6 were defined as liver cirrhosis. All US was performed by five experienced gastroenterologists. Intraobserver variation was minimized by pre-screening training and post-screening photo review.

On identifying hepatic focal lesions, subjects were referred to medical centers elsewhere for further confirmation and treatment. Medical records of medical centers were abstracted. The HCC cases were diagnosed by two criteria: 1) positive findings on cytologic or pathologic examinations or 2) typical images compatible with HCC with an AFP level ≥ 400 ng/mL. The sera were collected and frozen at −80°C before use. The study was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. This study was approved by the Ethical Committee of Chang Gung Memorial Hospital, and sera were sampled only after obtaining informed consent from the participants.

Serology. All HBsAg and anti-HCV antibodies were determined using commercial assay kits (AxSYM, Abbott, IL). The AFP was tested by the microparticle enzyme immunoassay (MEIA) method (AxSYM).

Aflatoxin detection method. Kit and reagents. Serum samples were assayed for aflatoxin-albumin adduct by enzyme immunoassay (EIA) with kits (INER: Institute of Nuclear Energy Research, Taoyuan, Taiwan) and albumin by BCG (bromo cresol green) binding assay with a Hitachi Autoanalyzer. Goat anti-rabbit IgG alkaline phosphatase was obtained from Molecular Probes (Eugene, OR) and p-nitrophenol phosphate from Sigma (St Louis, MO).

Quantification of serum aflatoxin-albumin adducts. Serum aflatoxin-albumin adducts were determined by competitive ELISA using INER kits. Briefly, 20 μL of serum and 160 μL of polyclonal anti-aflatoxin antibodies were pipetted into microtiter plates coated with aflatoxin-albumin adducts. After incubation with shaking for 1 hour at room temperature, the wells were washed four times with 340 μL of wash solution (0.05% Tween in TBS). A 160-μL quantity of goat anti-rabbit IgG alkaline phosphatase (1:2,000) was added to each well and incubated for an additional 1 hour with constant shaking followed by additional washing four times. After the final wash, color development (15 minutes) with 75 μL p-nitrophenol phosphate solution allowed quantification of aflatoxin-albumin. The concentration was achieved by internalizing the optical density (OD) at 405 nm with the calibration curve for serial diluted aflatoxin-albumin adducts. The calibration curve was determined using four parameter logistic curve fitting of the logit-log method using Sigmaplot 2000. The detection limit of this aflatoxin-albumin assay was 39.8 ng/mL. Aflatoxin level

Quantification of albumin. The Hitachi Autoanalyzer and its albumin reagent (BCG, Hitachi, Tokyo, Japan) were used to quantify albumin. Maximum absorption of the albumin BCG complex was 628 nm. The albumin concentration in this sample was generated by internalizing the OD at 628 nm with a linear calibration curve for related fixed albumin concentrations to observe OD at 628 nm. Serum unit of albumin was calculated as milligram/deciliter (mg/dL).

Evaluating aflatoxin exposure. The degree of aflatoxin exposure was determined by calculating the concentration ratio of aflatoxin-albumin to albumin and expressed as nanograms aflatoxin-albumin per milligram albumin.

Data analysis. Categorical variables were analyzed by chi-square and Fisher exact tests. Student t test and Mann-Whitney U test were used for continuous variables with normal and skewed distribution, respectively. Pearson correlation was used to evaluate correlations between AFB1-albumin/albumin ratio and advanced liver disease. Receiver operator characteristic (ROC) curve analysis was used to determine the best cut-offs for factors differentiating advanced liver disease from mild or no parenchymal liver disease. Multivariate logistic regression was performed to assess factors affecting advanced liver disease. One-way analysis of variance (ANOVA) was applied to test the linear trend between the ultrasonographic parenchyma scores and AFB1-albumin/albumin levels. P < 0.05 was considered statistically significant.

RESULTS

The 314 subjects included 163 men and 151 women, and the mean age was 66.9 ± 9.7 years. Twenty-five subjects were positive for HBsAg, 167 were positive for anti-HCV, 24 were positive for both HBsAg and anti-HCV, and 98 were negative for HBsAg and anti-HCV. Furthermore, advanced liver disease was diagnosed ultrasonographically in 85 subjects (72 and 13 with liver cirrhosis and HCC, respectively), and no cases of mild or no parenchyma were noted in the 229 subjects. Table 1 shows clinical features, hepatitis virus markers, and aflatoxin exposure levels for all patients with and without advanced liver disease. The patients with advanced liver disease included more women than men and significantly higher levels of alanine aminotransferase (ALT), AFP, and AFB1-albumin/albumin ratios. These patients also had lower albumin levels and platelet counts than those with mild or no liver parenchyma change. Moreover, patients with advanced liver disease had a higher prevalence of HBsAg and/or anti-HCV. Stepwise multiple logistic analysis was performed for all variables in Table 1. Independent factors for advanced liver disease (Table 2) were sex (men versus women; OR = 1.97, 95% CI = 1.02–3.82, P = 0.044), aflatoxin level (> 8 versus ≤ 8 AFB-albumin/albumin; OR = 2.29, 95% CI = 1.23–4.27, P = 0.009), albumin (> 42 versus ≤ 42 g/dL; OR = 0.37, 95% CI = 0.18–0.6, P < 0.001), platelet count (> 10 versus ≤ 10).
Multivariate analysis of risk factors for advanced liver disease in all patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>Comparison</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male vs. female</td>
<td>1.97</td>
<td>1.02-3.82</td>
<td>0.044</td>
</tr>
<tr>
<td>Platelet</td>
<td>&gt; 10 vs. &lt; 10 × 10^9/L</td>
<td>0.17</td>
<td>0.09-0.32</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Albumin</td>
<td>&gt; 4.2 vs. &lt; 5.2 g/dL</td>
<td>0.37</td>
<td>0.18-0.61</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Aflatoxin</td>
<td>&gt; 8 vs. ≤ 8</td>
<td>2.29</td>
<td>1.23-4.27</td>
<td>0.009</td>
</tr>
<tr>
<td>Viral hepatitis marker</td>
<td>Non B, Non C</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HBsAg</td>
<td>3.82</td>
<td>0.86-16.9</td>
<td>0.078</td>
</tr>
<tr>
<td></td>
<td>Anti-HCV</td>
<td>5.26</td>
<td>1.90-14.53</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>AFB1-albumin/albumin</td>
<td>16.09</td>
<td>4.37-59.31</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

≤ 10 × 10^5/L; OR = 0.17, 95% CI = 0.09-0.32, P = 0.001), anti-HCV (OR = 5.26, 95% CI = 1.9-14.53, P = 0.001), and HBV or HCV co-infection (OR = 16.1, 95% CI = 4.37-59.31, P = 0.001). Eleven of the 13 HCC patients were positive for anti-HCV, and 2 were positive for both HBsAg and anti-HCV. Compared with all subjects without HCC, those with HCC had a higher rate of anti-HCV, significantly lower plateau counts (9.6 ± 3.7 versus 11.4 ± 2.8, P = 0.023) and albumin (3.9 ± 0.5 versus 4.3 ± 0.4, P < 0.001), and higher AFB1-albumin levels (10.99 ± 2873.6 versus 12.3 ± 38.2, P = 0.001). Subjects with and without HCC did not significantly differ in age, sex, HBsAg-positive rate, ALT levels, or ratio of AFB1-albumin/albumin (10.8 ± 3.8 versus 9.6 ± 11.2, P = 0.7).

Of the 314 residents, ultrasonography scores for liver parenchyma disease in Groups 1 (N = 133), 2 (N = 110), and 3 (N = 71) were 0–1, 2–4, and 5–6, respectively. Mean AFB1-albumin/albumin (ng/mg) levels were 5.8 ± 6.7, 10.8 ± 12.5, and 14.5 ± 14.1 in Groups 1, 2, and 3, respectively. The AFB1-albumin/albumin levels increased significantly with ultrasonographic parenchyma scores (P < 0.001).

Table 3 shows clinical characteristics for all HCV infection cases. The clinical characteristics for 191 anti-HCV–positive subjects (including HBV and HCV co-infection) were compared between subjects with and without advanced liver disease. Subjects with advanced liver disease had higher AFP and aflatoxin, lower albumin, and lower platelet counts than those with mild or no liver parenchyma change. Furthermore, aflatoxin level (> 8 versus ≤ 8 AFB1-albumin/albumin; OR = 2.09, 95% CI = 1.09-4.0, P = 0.026), albumin level (> 4.2 versus ≤ 4.2 g/dL; OR = 0.4, 95% CI = 0.21-0.76, P = 0.005), and platelet count (> 10 versus ≤ 10 × 10^9/L; OR = 0.25, 95% CI = 0.13-0.48, P < 0.001) were major determinants of advanced liver disease in HCV patients. The 191 anti-HCV-positive subjects were grouped by ultrasonography scores as described above. The mean AFB1-albumin/albumin (ng/mg) levels in Groups 1, 2, and 3 (N = 48, 76, and 67, respectively) were 5.9 ± 7.1, 10.5 ± 11.3, and 14.8 ± 12.0, respectively. The AFB1-albumin/albumin levels also increased significantly with ultrasonographic parenchyma scores (P < 0.001).

Of the 123 subjects without anti-HCV, albumin level (> 4.2 versus ≤ 4.2 g/dL; OR = 0.05, 95% CI = 0.007-0.37, P = 0.003) and platelet count (> 10 versus ≤ 10 × 10^9/L; OR = 0.02, 95% CI = 0.002-0.21, P = 0.001) were independent factors for advanced liver disease. Aflatoxin levels were not associated with advanced liver disease according to univariate and multivariate analysis among participants without HCV infection.

**DISCUSSION**

This community-based study revealed an association between advanced liver disease and HCV. However, further study is need to determine whether advanced liver disease is a confounder affecting aflatoxin adduct metabolism and causing higher levels of aflatoxin rather than HCV infection. In humans, aflatoxin is metabolized in the liver by cytochrome P450(s), which activates the AFB1 to the AFB1 exo-8,9-epoxide. The AFB1 exo-8,9-epoxide is highly unstable and binds with high affinity to guanine bases in DNA to form aflatoxin-N-guanine. The AFB1 exo-8,9-epoxide is also capable of binding to lysine residues in serum albumin (AFB1-albumin). Thus, altered expression of cytochrome P450(s) enzymes in hepatocytes may increase AFB1-albumin levels. Experimental animal studies have shown that liver injury resulting from HBV and/or other factors can increase cytochrome P450 enzyme, which can activate aflatoxin metabolism. A recent study also showed that the highest of

**TABLE 3**

Comparison of clinical features and aflatoxin levels between anti-HCV-positive patients with and without advanced liver disease

<table>
<thead>
<tr>
<th>Variables</th>
<th>Advanced liver disease (n = 76)</th>
<th>No or mild liver parenchyma change (n = 115)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>68.6 ± 6.4</td>
<td>67.7 ± 8.0</td>
<td>0.41</td>
</tr>
<tr>
<td>Sex (male:female)</td>
<td>29:47</td>
<td>51:64</td>
<td>0.39</td>
</tr>
<tr>
<td>HBsAg</td>
<td>13</td>
<td>11</td>
<td>0.12</td>
</tr>
<tr>
<td>ALT (U/L) [median (range)]</td>
<td>52.5 (8–192)</td>
<td>48.5 (5–292)</td>
<td>0.12</td>
</tr>
<tr>
<td>Platelet (10^12/L)</td>
<td>9.6 ± 3.0</td>
<td>11.6 ± 2.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.0 ± 0.4</td>
<td>4.3 ± 0.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>AFP (ng/mL) [median (range)]</td>
<td>10.1 (1.1–10020)</td>
<td>4 (1.1–353)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Aflatoxin (AFB-alb/alb)</td>
<td>11.4 (0–51.4)</td>
<td>6.3 (0–61.4)</td>
<td>0.007</td>
</tr>
</tbody>
</table>

**FIGURE 1.** Median aflatoxin-albumin/albumin levels and interquartile range according to ultrasonographic scores for liver parenchyma disease for all patients.
Moreover, this study showed that aflatoxin levels have been observed in acutely HBV-infected children, in less high levels in chronic carriers, and was lowest in uninfected children. However, it remains unclear whether advanced liver disease (liver cirrhosis or HCC) increases aflatoxin activation. A previous study of human liver biopsies indicated that cases classified as mild chronic active hepatitis exhibited modestly increased aflatoxin activation, but no increase was observed in severe chronic hepatitis or cirrhosis. Moreover, this study showed that aflatoxin levels were associated with advanced liver disease in anti-HCV-positive patients. However, aflatoxin level was not an important factor in advanced liver disease in patients without anti-HCV. Thus, advanced liver disease is apparently not a factor causing increased aflatoxin levels. Conversely, exposure of aflatoxin exposure may induce advanced liver disease, especially in HCV-infected patients. Additionally, assessment of dietary aflatoxin exposure in HCV-infected patients may clarify this question. The patients in this study were not assessed for dietary aflatoxin exposure. However, the AFB1-albumin in peripheral blood is a reliable marker of dietary aflatoxin exposure during the previous 2–3 months.

Possible associations between chronic hepatitis B and increased activation of AFB1-albumin adducts have been examined in epidemiologic studies. A synergistic interaction between AFB1 exposure and HBV infection on HCC risk has also been reported. However, this study revealed no significant difference in serum aflatoxin levels between advanced and non-advanced liver disease in HBV patients. The small population of HBsAg carriers in this study may be the major cause of this discrepancy. Additionally, no significant relationship was observed between HBV infection and advanced liver disease. Thus, HBV apparently plays a relatively minor role in the prevalence of advanced liver disease in HCV-endemic areas, particularly in cases of thrombocytopenia.

Several factors are known to affect the progression of HCV-related liver diseases. However, it remains unclear whether aflatoxin exposure is an associated risk factor for developing advanced liver disease in patients with chronic hepatitis C. Previous community-based studies in Taiwan have revealed that anti-HCV-positive status does not significantly affect temporal variability in AFB1-albumin adducts. However, these studies were performed in HBV-endemic areas, and anti-HCV-positive case numbers were limited. A recent study in an HCV-endemic country elsewhere found that the incidence of HCC in HCV-infected patients is strongly associated with the food and the seeds contaminated with aflatoxins. This study showed that aflatoxin exposure levels were associated with HCV infection and was an independent risk factor for advanced liver disease in patients with HCV infection. Furthermore, the level of aflatoxin exposure was significantly related to ultrasonographic parenchyma scores. These scores reflect the severity of liver fibrosis. Thus, aflatoxin exposure might be associated with advanced liver fibrosis in patients with chronic hepatitis C. The number of HCC cases in this study was limited (power: 0.051); thus, no significant difference in the level of AFB1-albumin adducts was noted between patients with and without HCC. A further large-scale study is needed to evaluate the effect of aflatoxin exposure on HCV-related HCC.

In conclusion, a higher AFB1-albumin/albumin level is an independent risk factor for advanced liver disease in HCV-infected patients. Ultrasonographic parenchyma scores were significantly related to AFB1-albumin levels. Although the exact mechanism is unclear, the analytical results of this study suggest that aflatoxin exposure may be associated with progression of advanced liver disease in patients with chronic hepatitis C in HCV-endemic areas of Taiwan. The biologic mechanism underlying the interaction between chronic HCV infection and increased activation of AFB1-albumin adducts in humans merits further study.

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