Aflatoxin B₁ exposure increases the risk of cirrhosis and hepatocellular carcinoma in chronic hepatitis B virus carriers

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The relation between aflatoxin B₁ (AFB₁) and cirrhosis in chronic carriers of hepatitis B virus (HBV) remains inconclusive. This case-control study nested in a large community-based cohort aimed to assess the effect of AFB₁ exposure on cirrhosis and HCC in chronic HBV carriers. Serum AFB₁-albumin adduct levels at study entry were measured in 232 cirrhosis cases, 262 HCC cases and 577 controls. Multivariate-adjusted odds ratios (aORs) and 95% confidence intervals (95% CIs) were estimated using logistic regression. Among all chronic HBV carriers, the time intervals between study entry and diagnosis of HCC, cirrhosis, cirrhotic HCC, and non-cirrhotic HCC were all significantly (p<0.0001) shorter in participants with high serum levels of AFB₁-albumin adducts than those with low/undetectable levels. There were significant dose-response relations with serum AFB₁-albumin adduct level at study entry for cirrhosis (p-trend = 0.0001) and cirrhotic HCC (p-trend < 0.0001) newly diagnosed within 9 years after entry as well as non-cirrhotic HCC (p-trend = 0.021) newly diagnosed within 4 years after entry. The aORs (95% CIs) for high versus undetectable serum AFB₁-albumin adduct levels were 2.45 (1.51–3.98) for cirrhosis (p = 0.0003), 5.47 (2.20–13.63) for cirrhotic HCC (p = 0.0003), and 5.39 (1.11–26.18) for non-cirrhotic HCC (p = 0.0368) HCC, respectively. There remained a significant dose-response relation between serum AFB₁-albumin adduct level and HCC risk (p-trend = 0.0291) in cirrhosis patients, showing an aOR (95% CI) of 3.04 (1.11–8.30) for high versus undetectable serum levels (p = 0.0299). It is concluded that AFB₁ exposure may increase the risk of cirrhosis and HCC in a dose-response manner among chronic HBV carriers.

Liver cancer, primarily hepatocellular carcinoma (HCC), is the sixth most common cancer in the world and the second most common cause of cancer-related death worldwide in 2012.¹ The vast majority of HCC occurs in patients affected with chronic liver disease or cirrhosis.²⁻⁴ Cirrhosis is a late-stage liver disease established in response to liver injury, featuring encapsulation or replacement of injured tissue by scar tissue. Cirrhosis patients are at a greater risk of developing HCC. In chronic hepatitis B virus (HBV) carriers, patients with cirrhosis had a significantly higher annual incidence rate of HCC than patients without cirrhosis (3.5 vs. 0.4%, p < 0.0001).⁵ Cirrhosis led to more than one million deaths globally in 2010.⁶ Cirrhosis is therefore considered an emerging major global health burden.

The development of HCC is characterized by a multistage process and it usually takes decades from the initial liver damage to HCC. Exposures to various etiologic factors induce the liver injury and result in sustained cycles of necrosis, inflammation, and proliferation of hepatocytes. Repeated episodes of liver cell destruction and regeneration consequently foster an environment culminating in fibrosis and cirrhosis and establish a premalignant condition. Many other mediating events such as production of reactive oxygen species, genetic mutations, DNA damages and genetic instability ultimately contribute to the development of HCC.⁷ Cirrhosis and HCC share several common risk factors, including chronic infection with HBV and/or hepatitis C.
Cancer Epidemiology

What's new?
Aflatoxin B₁ (AFB₁), a potent carcinogen produced by fungi, is the most abundant aflatoxin found in contaminated food. Here, the authors examined its association with liver cirrhosis risk. Using an ELISA to measure AFB₁-albumin adducts in the blood, they found that AFB₁ exposure increases the risk of developing cirrhosis and liver cancer, and may also accelerate the progression of chronic hepatitis B virus infection to both conditions. They conclude that AFB₁ may not only contribute to hepatocarcinogenesis directly, but also indirectly by rendering liver cells more vulnerable by promoting cirrhosis.

Material and Methods

Study population
All cases and controls were selected from the participants of a community-based cancer screening project (CBCSP) cohort. Briefly, the CBCSP cohort was established in 1991–1992 when 23,820 residents aged 30 to 65 years were recruited from seven townships in Taiwan. Each participant provided written informed consent. This study was approved by the Institutional Review Board of the College of Public Health, National Taiwan University, Taipei, Taiwan. Detailed information about the CBCSP cohort has been described previously.

Study participants
From study entry in 1991–1992 to June 30, 2004, a total of 303 newly developed cirrhosis cases were identified through regular abdominal ultrasonography examination and computerized linkage to the National Health Insurance database; and 302 newly developed HCC cases were identified through computerized linkage with National Cancer Registry database until December 31, 2011. Because cirrhosis and HCC are both major outcomes in this study, 71 participants with cirrhosis at study entry were excluded (31 cirrhosis only cases and 40 cirrhotic HCC cases). As the cirrhosis status was ascertained only up to June 30, 2004, analyses of the associations with serum AFB₁-albumin adduct levels for cirrhosis, cirrhotic HCC and non-cirrhotic HCC were confined to cases diagnosed before June 30, 2004. Cases and controls were matched on age (±5 years), gender, residence and date of blood sample collection (±3 months). A total of 232 cirrhosis cases, 114 cirrhotic HCC cases, 66 non-cirrhotic HCC cases and controls unaffected by cirrhosis and HCC were included in this analysis.

Data collection, serological testing and follow-up for liver cirrhosis and hepatocellular carcinoma
At the study entry, all participants underwent a health examination including abdominal ultrasonography and serological tests. Blood samples at study entry were tested for hepatitis B e antigen (HBeAg) and HBsAg by immunosayass (Abbott Laboratories), HBV DNA by the COBAS Amplicor HBV monitor test kit (Roche Diagnostics), antibody against HCV (anti-HCV) by enzyme immunosayass using commercial kits (Abbott Laboratories) and alanine aminotransferase (ALT) by serum chemistry autoanalyzer (Model 736, Hitachi) using commercial reagents (Biomerieux).

virus (HCV), heavy alcohol consumption, and non-alcoholic steatohepatitis (NASH)/non-alcoholic fatty liver disease (NAFLD).8–11 Another risk factor for HCC is the intake of aflatoxins found in contaminated food.12–16 It is estimated that four billion people are at the risk of exposure to aflatoxins globally.17 Aflatoxin B₁ (AFB₁), the most potent and abundant aflatoxin found in contaminated foods, exerts its carcinogenicity through its metabolic activation to the reactive AFB₁ 8,9-exo-epoxide by phase 1 cytochrome P450 monooxygenases. The reactive epoxide can interact with DNA to form highly mutagenic DNA lesions such as aflatoxin-N7-guanine adducts.18,19

We have developed the measurement of AFB₁-albumin adducts as a biomarker of biologically effective dose of exposure to AFB₁. This biomarker reflects AFB₁ exposures of liver over several months20,21 and is stable during a long-term deep-frozen storage.22 We previously demonstrated significant associations between the serum level of AFB₁-albumin adducts and the risk of HCC diagnosed within 3 and 13 years after study entry, respectively.15,16 Study participants with both hepatitis B surface antigen (HBsAg) seropositivity and high serum levels of AFB₁-albumin adducts [aOR (95% CI) = 10.4 (5.7–18.8)] had a greater HCC risk than participants with HBsAg seropositivity only [aOR (95% CI) = 7.0 (4.5–11.1)] or high serum levels of AFB₁-albumin adducts only [aOR (95% CI) = 1.6 (0.9–3.0)].16

A previous study suggested that aflatoxin exposure may be involved in the development of cirrhosis,23 but there was very limited epidemiological evidence supporting the causal relation between AFB₁ exposure and cirrhosis. With no clear causation between AFB₁ and cirrhosis, the mutational activity of AFB₁ has been considered to be the primary driver of AFB₁-induced HCC.7 As both AFB₁ exposure and cirrhosis are primary risk factors of HCC, it remains unclear whether AFB₁ also contributes to the earlier stage of HCC progression, i.e., cirrhosis.

Using a case-control study nested in a large community-based cohort with the measurement of serum AFB₁-albumin adduct levels at study entry, this study on chronic HBV carriers aims to (i) evaluate the time interval between the collection of serum sample and the diagnosis of cirrhosis and HCC in which the associations between the serum AFB₁-albumin adduct level and the risk of cirrhosis and HCC were statistically significant; and (ii) elucidate the associations between AFB₁ exposure and the risk of cirrhosis, cirrhotic HCC and non-cirrhotic HCC, respectively.
All participants were personally interviewed by trained public health nurses using a structured questionnaire, and information on sociodemographic characteristics, habits of alcohol consumption and cigarette smoking, and family history of major diseases was collected. During the follow-up period from study entry in 1991–1992 to June 30, 2004, participants seropositive for HBsAg or anti-HCV, with elevated serum levels of ALT, aspartate aminotransferase, or α-fetoprotein (AFP), or with a family history of HCC were regularly followed up by abdominal ultrasonography and serological tests.

Incident liver cirrhosis was identified through abdominal ultrasonography and computerized data linkage to the National Health Insurance database and medical chart reviews until June 30, 2004. Cirrhosis was determined by a quantitative scoring system deriving from (i) morphological appearance of the liver surface (normal, irregular, undulated); (ii) liver parenchymal texture (normal, heterogeneous, coarse); (iii) intrahepatic vascular structure (normal, obscure, narrowing) and (iv) spleen size (normal, enlarged). Newly developed HCC cases were ascertained by regular health examinations including abdominal ultrasonography and AFP testing before June 30, 2004 and computerized data linkage to the National Cancer Registry database until December 31, 2011. Medical chart reviews were also performed to confirm the diagnosis of HCC according to the following criteria: (i) histopathologically confirmed HCC lesion; (ii) detection of HCC lesion by two imaging techniques (abdominal ultrasonography, angiogram, or computed tomography or (iii) detection of HCC lesion by one imaging technique in combination with elevated serum AFP level (≥400 ng/mL).

Measurement of serum AFB1-albumin adduct levels

AFB1-albumin adduct levels were measured in serum samples collected at study entry. Albumin extraction from serum and determination of AFB1-albumin adduct levels by ELISA were performed as previously described.12,24 In brief, 50 μL of proteinase K-digested albumin extract (200 μg) was applied into 96-well plates coated with 3 ng of AFB1, epoxide-modified human serum albumin. A standard curve was prepared from serial dilution of enzymatically digested AFB1, epoxide-modified human serum albumin. Polyclonal antiserum 7 (1:2 × 105 dilution) and goat anti-rabbit IgG alkaline phosphatase (1:750 dilution) were used as primary and secondary antisera, respectively. Samples with <15% inhibition were considered undetectable and assigned a value of 1 fmol/mg. All samples were assayed in duplicate with laboratory staff blinded to disease status. Two controls were assayed with the test samples in each batch: a pooled sample of plasma from nonsmoking subjects from the United States and serum from a rat treated with 1.5 mg of AFB1 as negative and positive controls, respectively. The baseline serum samples were analyzed in three different batches during the follow-up period.

The percentages of samples with undetectable levels in control subjects in the three batches were 41.3, 45.6 and 49.7%. The median detectable adduct levels among control subjects, 21.5 fmol/mg was used as the cutoff for low (<21.5 fmol/mg) and high (≥21.5 fmol/mg) levels of AFB1-albumin adduct.

Statistical analysis

To describe the demographic and clinical features of the case and control groups, means and standard deviations were used for continuous variables, and percentages were used for categorical variables; t-tests and χ2 tests were used to examine differences in the characteristics between cases and controls for continuous variables and categorical variables, respectively. Univariate logistic regression was used to calculate the crude odds ratios (ORs) and 95% confidence intervals (CIs) for associations between the serum AFB1-albumin adduct level and the risk of cirrhosis or HCC. Variables showing significant associations (p < 0.05) with serum AFB1-albumin adduct level in controls and with the outcome specified in univariate logistic regression were further incorporated into the corresponding multivariate logistic regression to estimate the adjusted ORs (aORs) and 95% CIs. To assess the dose-response relation, serum AFB1-albumin adduct level was analyzed as a continuous variable in the logistic regression model. To test for the equality of distribution of time intervals from study entry to the diagnosis of cirrhosis and HCC in participants with different AFB1 exposure levels, Kolmogorov–Smirnov test was performed. t-test was used to test the difference of time intervals from study entry to the diagnosis of cirrhosis and HCC between cases with high and low/undetectable serum levels of AFB1-albumin adducts. SAS 9.4 (Cary, NC) was employed for data management and all statistical analyses.

Results

To assess the time-dependent associations between the serum level of AFB1-albumin adducts and the risk of HCC, the frequency distributions of the year at HCC diagnosis after study entry were compared between HCC cases with high and low/undetectable serum levels of AFB1-albumin adducts at study entry as shown in Figure 1. All 262 HCC cases newly diagnosed until December 31, 2011 were included in the analysis. The percentage of HCC cases newly diagnosed within 9 years after study entry was significantly higher (p < 0.0001) for cases with high serum levels of AFB1-albumin adducts than those with low/undetectable levels (Fig. 1a). The time intervals from study entry to HCC diagnosis were significantly shorter (p < 0.0001) for those who had high levels (mean ± SD, 7.5 ± 5.6) than those with low/undetectable levels (mean ± SD, 10.9 ± 4.5) as shown in Figure 1b.

Further analyses of the time intervals from study entry to the diagnosis of newly developed cirrhosis, cirrhotic HCC and non-cirrhotic HCC by serum level of AFB1-albumin adducts are shown in Figure 2. A total of 232 cirrhosis cases, 114 cirrhotic HCC cases and 66 non-cirrhotic HCC cases newly diagnosed until June 30, 2004 were included in the
analyses. Similarly, cases with high serum levels of AFB1-albumin adducts at study entry had a significantly higher percentage of cases diagnosed in earlier years after entry than those with low/undetectable levels for cirrhosis (Fig. 2a, \( p < 0.0001 \)), cirrhotic HCC (Fig. 2b, \( p = 0.0001 \)), and non-cirrhotic HCC (Fig. 2c, \( p = 0.0025 \)). The time intervals from study entry to the diagnosis of cirrhosis, cirrhotic HCC and non-cirrhotic HCC were much shorter for those who had high serum AFB1-albumin adduct levels than those with low/undetectable levels as shown in Figure 2d (mean ± SD, 3.6 ± 3.7 vs. 6.5 ± 3.8, \( p < 0.0001 \)), 2E (mean ± SD, 6.0 ± 3.7 vs. 8.6 ± 9.8, \( p < 0.0001 \)), and 2F (mean ± SD, 3.6 ± 3.5 vs. 7.7 ± 3.8, \( p < 0.0001 \)), respectively. The time intervals from cirrhosis to cirrhotic-HCC were similar for cirrhosis patients with high and low/undetectable serum levels of AFB1-albumin adducts (mean ± SD, 2.7 ± 3.2 vs. 2.1 ± 2.6, \( p = 0.27 \)).

Table 1 shows the comparisons of the associations with serum AFB1-albumin adduct levels for HCC, cirrhosis, cirrhotic HCC and non-cirrhotic HCC by the year of disease onset since study entry. During a long follow-up period of 20 years, the association between the serum AFB1-albumin adduct level at study entry and the risk of HCC was not statistically significant for all HCC cases (\( n = 262 \)) newly developed from 1991 to 2011. But there was a significant dose-response relation with between the level of serum AFB1-albumin adducts and the risk of HCC newly diagnosed within 9 years (\( n = 112 \)) after study entry (p-trend = 0.0007). High serum AFB1-albumin adduct levels were significantly associated with an increased risk of HCC (crude OR = 2.17; 95% CI = 1.26–3.73) compared with the undetectable levels.

There was a significant dose-response relation between the serum level of AFB1-albumin adducts at study entry and the risk of cirrhosis newly diagnosed from 1991 to 2004 (\( n = 232 \)) with the p-trend of 0.0068 and a crude OR (95% CI) of 1.60 (1.08–2.37) for high levels of AFB1-albumin adducts versus the undetectable levels. For cirrhosis diagnosed in 9 years of follow-up (\( n = 177 \)), the dose-response relation was more striking (p-trend < 0.0001) with a crude OR (95% CI) of 2.29 (1.44–3.64) for high levels of AFB1-albumin adducts versus the undetectable levels.

When stratifying 180 HCC cases diagnosed before June 30, 2004 into 114 cirrhotic HCC cases and 66 non-cirrhotic HCC cases, the serum AFB1-albumin adduct levels were not significantly associated with the long-term risk of HCC. However, significant dose-relations with serum AFB1-albumin adduct levels were observed for cirrhotic HCC within 9 years (\( n = 67 \)) of follow-up and non-cirrhotic HCC within 4 years (\( n = 21 \)) of follow-up with the p-trends of <0.0001 and 0.0063, respectively.

Further analyses were carried out to estimate multivariate-adjusted OR of the serum level of AFB1-albumin adducts at study entry for cirrhosis, and cirrhotic HCC developed within 9 years of follow-up, and non-cirrhotic HCC developed within 4 years of follow-up with adjustment for other major risk factors. Table 2 shows the frequency distributions of major risk factors collected at study entry of 577 control participants unaffected with cirrhosis or HCC, 110 cirrhosis only cases, 67 cirrhotic HCC cases, and 21 non-cirrhotic HCC cases. Compared with control participants, newly diagnosed cases of cirrhosis, and cirrhotic HCC had higher percentages of HBeAg seropositivity and higher serum levels of ALT, HBsAg, and HBV DNA; and non-cirrhotic HCC cases had higher percentages of cigarette smoking, alcohol drinking, family history of HCC, HBeAg seropositivity, and elevated serum levels of ALT and HBV DNA. The percentages of high serum AFB1-albumin adduct levels were higher in cases...
of cirrhosis, cirrhotic HCC and non-cirrhotic HCC than controls (49.1%, 65.7%, 66.7 vs. 39.3%).

Table 3 shows the univariate analyses of risk factors for cirrhosis, cirrhotic HCC and non-cirrhotic HCC. Elevated serum levels of ALT and HBV DNA were significantly associated with an increased risk of cirrhosis, cirrhotic HCC and non-cirrhotic HCC. While HBeAg seropositivity and serum HBsAg level were significantly associated with cirrhosis and
cirrhotic HCC, but not with non-cirrhotic HCC; alcohol drinking was significantly associated with non-cirrhotic HCC, but not with cirrhosis and cirrhotic HCC.

There was a significant dose-response relation of serum AFB1-albumin adduct level with the risk of cirrhosis ($p$-trend < 0.0001), cirrhotic HCC ($p$-trend < 0.0001), and non-cirrhotic HCC ($p$-trend = 0.0063). The crude ORs (95% CIs) for high serum levels compared to undetectable levels were 2.29 (1.44–3.64), 4.98 (2.07–11.96), and 4.75 (1.06–21.19) for cirrhosis, cirrhotic HCC and non-cirrhotic HCC, respectively.

In the comparison between cirrhosis and cirrhotic HCC cases, serum level of AFB1-albumin adducts was associated with an increased risk of developing cirrhotic HCC showing a dose-response relation [high vs. undetectable: crude OR (95% CI) = 3.12 (1.17–8.35); $p$-trend = 0.0072].

As serum AFB1-albumin adduct level was significantly associated with cigarette smoking, alcohol drinking, and serum ALT level in control participants (Supporting Information Table 1), they were considered as potential confounders and included in the multivariate analyses shown in Table 4. After adjusting for age, gender and other significant risk factors, the serum AFB1-albumin adduct level was significantly associated with an increased risk of cirrhosis, cirrhotic HCC and non-cirrhotic HCC in a dose-dependent manner ($p$-trend = 0.0001, < 0.0001 and 0.021, respectively). The multivariate-adjusted OR (95% CI) for high versus undetectable serum levels of AFB1-albumin adducts was 2.45 (1.51–3.98) for cirrhosis, 5.47 (2.20–13.63) for cirrhotic HCC, and 5.39 (1.11–26.18) for non-cirrhotic HCC. In the comparison between cirrhotic HCC

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Table 1. Associations between the serum aflatoxin B1 (AFB1)-albumin adducts level at study entry and the risk of hepatocellular carcinoma and cirrhosis in chronic hepatitis B virus carriers by the year of disease onset

|                        | AFB1-albumin level (fmol/mg) | Control (%) (n = 577) | Case (%) | Crude OR (95% CI) | $p$-trend$^1$ |
|------------------------|-------------------------------|------------------------|----------|-------------------|--------------|
| **HCC**                |                               |                        |          |                   |              |
| Cases diagnosed between| Undetectable                  | 154 (26.7)             | 81 (30.9)| 1.00 (Referent)   | 0.13         |
| 1991–2011              | Low (<21.5)                   | 196 (34.0)             | 85 (32.4)| 0.83 (0.57–1.19)  |              |
| (n = 262)              | High (≥21.5)                  | 227 (39.3)             | 96 (36.6)| 0.80 (0.56–1.15)  |              |
| Cases diagnosed in 9   | Undetectable                  | 154 (26.7)             | 20 (17.9)| 1.00 (Referent)   | 0.0007       |
| Years of follow-up     | Low (<21.5)                   | 196 (34.0)             | 28 (25.0)| 1.10 (0.60–2.03)  |              |
| (n = 112)              | High (≥21.5)                  | 227 (39.3)             | 64 (57.1)| 2.17 (1.26–3.73)**|              |
| **Cirrhosis**          |                               |                        |          |                   |              |
| Cases diagnosed between| Undetectable                  | 154 (26.7)             | 48 (20.7)| 1.00 (Referent)   | 0.0068       |
| 1991–2004              | Low (<21.5)                   | 196 (34.0)             | 71 (30.6)| 1.16 (0.76–1.77)  |              |
| (n = 232)              | High (≥21.5)                  | 227 (39.3)             | 113 (48.7)| 1.60 (1.08–2.37)**|              |
| Cases diagnosed in 9   | Undetectable                  | 154 (26.7)             | 29 (16.4)| 1.00 (Referent)   | <0.0001      |
| Years of follow-up     | Low (<21.5)                   | 196 (34.0)             | 50 (28.3)| 1.36 (0.82–2.24)  |              |
| (n = 177)              | High (≥21.5)                  | 227 (39.3)             | 98 (55.4)| 2.29 (1.44–3.64)***|              |
| **Cirrhotic HCC**      |                               |                        |          |                   |              |
| Cases diagnosed between| Undetectable                  | 154 (26.7)             | 27 (23.7)| 1.00 (Referent)   | 0.0745       |
| 1991–2004              | Low (<21.5)                   | 196 (34.0)             | 32 (28.1)| 0.93 (0.54–1.62)  |              |
| (n = 114)              | High (≥21.5)                  | 227 (39.3)             | 55 (48.3)| 1.38 (0.84–2.29)  |              |
| Cases diagnosed in 9   | Undetectable                  | 154 (26.7)             | 6 (0.0)  | 1.00 (Referent)   | <0.0001      |
| Years of follow-up     | Low (<21.5)                   | 196 (34.0)             | 17 (25.4)| 2.23 (0.86–5.78)  |              |
| (n = 67)               | High (≥21.5)                  | 227 (39.3)             | 44 (65.7)| 4.98 (2.07–11.96)***|              |
| **Non-cirrhotic HCC**  |                               |                        |          |                   |              |
| Cases diagnosed between| Undetectable                  | 154 (26.7)             | 23 (34.9)| 1.00 (Referent)   | 0.0881       |
| 1991–2004              | Low (<21.5)                   | 196 (34.0)             | 21 (31.8)| 0.72 (0.38–1.34)  |              |
| (n = 66)               | High (≥21.5)                  | 227 (39.3)             | 22 (33.3)| 0.65 (0.35–1.21)  |              |
| Cases diagnosed in 4   | Undetectable                  | 154 (26.7)             | 2 (9.5)  | 1.00 (Referent)   | 0.0063       |
| Years of follow-up     | Low (<21.5)                   | 196 (34.0)             | 5 (23.8) | 1.96 (0.38–10.26) |              |
| (n = 21)               | High (≥21.5)                  | 227 (39.3)             | 14 (66.7)| 4.75 (1.06–21.19)*|              |

$^1$ One-sided $p$-value for Cochran-Armitage trend test.

*p < 0.05, **p < 0.01, ***p < 0.001.
cases and cirrhosis only cases, serum level of AFB1-albumin adducts was significantly associated with an increased cirrhotic HCC risk showing a dose-response relation ($p$-trend = 0.0291) with an adjusted OR (95% CI) of 3.04 (1.11–8.30) for high versus undetectable levels.

**Discussion**

In this nested case-control study on cirrhosis and HCC in chronic HBV carriers, the associations between the serum level of AFB1-albumin adducts at study entry and the newly developed cirrhosis and HCC were assessed. Cases and

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**Table 2. Baseline characteristics of chronic hepatitis B virus carriers affected and unaffected with cirrhosis and/or hepatocellular carcinoma**

| Baseline characteristics | Control ($n = 577$) No. (%) | Cirrhosis only in 9 years of follow-up ($n = 110$) No. (%) | Cirrhotic HCC in 9 years of follow-up ($n = 67$) No. (%) | Non-cirrhotic HCC in 4 years of follow-up ($n = 21$) No. (%) |
|--------------------------|-----------------------------|-------------------------------------------------|-------------------------------------------------|--------------------------------------------------|
| Cigarette smoking¹       |                             |                                                 |                                                 |                                                  |
| No                       | 350 (60.9)                 | 66 (60.0)                                       | 37 (55.2)                                       | 10 (47.6)                                        |
| Yes                      | 225 (39.1)                 | 44 (40.0)                                       | 30 (44.8)                                       | 11 (52.4)                                        |
| Alcohol drinking²        |                             |                                                 |                                                 |                                                  |
| No                       | 491 (85.5)                 | 97 (88.2)                                       | 56 (83.6)                                       | 13 (65.0)                                        |
| Yes                      | 83 (14.5)                  | 13 (11.8)                                       | 11 (16.4)                                       | 7 (35.0)                                         |
| Body mass index (kg/m²)³ |                             |                                                 |                                                 |                                                  |
| <27                      | 456 (79.0)                 | 82 (75.2)                                       | 57 (85.1)                                       | 19 (90.5)                                        |
| ≥27                      | 121 (21.0)                 | 27 (24.8)                                       | 10 (14.9)                                       | 2 (9.5)                                          |
| Family history of HCC    |                             |                                                 |                                                 |                                                  |
| No                       | 548 (95.0)                 | 103 (93.6)                                      | 62 (92.5)                                       | 18 (85.7)                                        |
| Yes                      | 29 (5.0)                   | 7 (6.4)                                         | 5 (7.5)                                         | 3 (14.3)                                         |
| Serum ALT level (U/L)    |                             |                                                 |                                                 |                                                  |
| <45                      | 552 (95.7)                 | 87 (79.1)                                       | 57 (85.1)                                       | 16 (76.2)                                        |
| ≥45                      | 25 (4.3)                   | 23 (20.9)                                       | 10 (14.9)                                       | 5 (23.8)                                         |
| Anti-HCV⁴                |                             |                                                 |                                                 |                                                  |
| Negative                 | 532 (92.2)                 | 99 (90.0)                                       | 61 (92.4)                                       | 19 (95.0)                                        |
| Positive                 | 45 (7.8)                   | 11 (10.0)                                       | 5 (7.6)                                         | 1 (5.0)                                          |
| HBeAg seropositivity⁵    |                             |                                                 |                                                 |                                                  |
| No                       | 489 (86.9)                 | 63 (58.3)                                       | 35 (55.6)                                       | 14 (73.7)                                        |
| Yes                      | 74 (13.1)                  | 45 (41.7)                                       | 28 (44.4)                                       | 5 (26.3)                                         |
| HBsAg level (IU/mL)⁶     |                             |                                                 |                                                 |                                                  |
| <1,000                   | 323 (62.5)                 | 20 (21.1)                                       | 15 (40.5)                                       | 5 (62.5)                                         |
| ≥1,000                   | 194 (37.5)                 | 75 (79.0)                                       | 22 (59.5)                                       | 3 (37.5)                                         |
| HBV DNA level (copies/mL)⁷ |                             |                                                 |                                                 |                                                  |
| <10,000                  | 364 (66.8)                 | 16 (16.7)                                       | 7 (13.2)                                        | 4 (30.8)                                         |
| ≥10,000                  | 181 (33.2)                 | 80 (83.3)                                       | 46 (86.8)                                       | 9 (69.2)                                         |
| AFB1-albumin adducts (fmol/mg) |             |                                                 |                                                 |                                                  |
| Undetectable             | 154 (26.7)                 | 23 (20.9)                                       | 6 (9.0)                                         | 2 (9.5)                                          |
| Low (<21.5)              | 196 (34.0)                 | 33 (30.0)                                       | 17 (25.4)                                       | 5 (23.8)                                         |
| High (≥21.5)             | 227 (39.3)                 | 54 (49.1)                                       | 44 (65.7)                                       | 14 (66.7)                                        |

¹Two controls were missing for baseline smoking status.
²Three controls and one non-cirrhotic HCC case were missing for baseline drinking status.
³One cirrhosis only case was missing for baseline body mass index data.
⁴One cirrhotic HCC case and one non-cirrhotic HCC case were missing for baseline anti-HCV serostatus.
⁵14 controls, two cirrhosis only cases, four cirrhotic HCC cases and two non-cirrhotic HCC cases were missing for baseline serostatus of HBeAg.
⁶60 controls, 15 cirrhosis only cases, 30 cirrhotic HCC cases and 13 non-cirrhotic HCC cases were missing for baseline serum HBsAg level.
⁷32 controls, 14 cirrhosis cases, 14 cirrhotic HCC cases and eight non-cirrhotic HCC cases were missing for baseline serum HBV DNA level.
unaffected controls were selected from the CBCSP cohort which had been followed for 20 years from 1991 to 2011. In Taiwan, people had been exposed to aflatoxin through consumption of aflatoxin-contaminated foods, particularly peanut products.25,26 Although the dietary intake of AFB1 of study participants was not measured, serum AFB1-albumin adduct levels were measured to reflect the biologically effective dose of AFB1 exposure in liver over past months.27 With a half-life of 21 days, AFB1-albumin adducts are the most reliable and feasible long-term marker for AFB1 exposure in humans.27,28 While no significant associations between the serum AFB1-albumin adduct level at study entry and the risk of HCC newly developed in the entire follow-up period of 20 years, a significant dose-response relation with the baseline serum AFB1-albumin adduct level was observed for HCC cases diagnosed within 9 years after study entry. There

Table 3. Univariate analysis of risk factors for cirrhosis, cirrhotic hepatocellular carcinoma (HCC) and non-cirrhotic HCC among chronic hepatitis B virus carriers

| Baseline characteristics | Cases developed cirrhosis/cirrhotic-HCC in 9 years of follow-up | Cases developed non-cirrhotic-HCC in 4 years of follow-up |
|--------------------------|---------------------------------------------------------------|----------------------------------------------------------|
|                          | Cirrhosis vs. control | Cirrhotic HCC vs. cirrhosis only | Cirrhotic HCC vs. control | Non-cirrhotic HCC vs. control |
| Cigarette smoking        |                  |                               |                           |                           |
| No                       | 1.00 (Referent)   | 1.00 (Referent)               | 1.00 (Referent)           | 1.00 (Referent)           |
| Yes                      | 1.12 (0.79–1.57)  | 1.22 (0.66–2.25)              | 1.26 (0.76–2.10)          | 1.71 (0.72–4.10)          |
| Alcohol drinking         |                  |                               |                           |                           |
| No                       | 1.00 (Referent)   | 1.00 (Referent)               | 1.00 (Referent)           | 1.00 (Referent)           |
| Yes                      | 0.93 (0.57–1.51)  | 1.47 (0.62–3.49)              | 1.16 (0.59–2.31)          | 3.19 (1.24–8.22)*         |
| Body mass index (kg/m²)  |                  |                               |                           |                           |
| <27                      | 1.00 (Referent)   | 1.00 (Referent)               | 1.00 (Referent)           | 1.00 (Referent)           |
| ≥27                      | 1.03 (0.68–1.55)  | 0.51 (0.23–1.14)              | 0.66 (0.33–1.33)          | 0.39 (0.09–1.73)          |
| Family history of HCC    |                  |                               |                           |                           |
| No                       | 1.00 (Referent)   | 1.00 (Referent)               | 1.00 (Referent)           | 1.00 (Referent)           |
| Yes                      | 1.37 (0.69–2.75)  | 1.19 (0.36–3.90)              | 1.52 (0.57–4.08)          | 3.15 (0.88–11.31)         |
| Serum alanine ALT level (U/L) |     |                               |                           |                           |
| <45                      | 1.00 (Referent)   | 1.00 (Referent)               | 1.00 (Referent)           | 1.00 (Referent)           |
| ≥45                      | 5.06 (2.92–8.78)*** | 0.66 (0.29–1.50)             | 3.87 (1.77–8.47)**        | 6.90 (2.34–20.34)***      |
| Anti-HCV                 |                  |                               |                           |                           |
| Negative                 | 1.00 (Referent)   | 1.00 (Referent)               | 1.00 (Referent)           | 1.00 (Referent)           |
| Positive                 | 1.18 (0.65–2.15)  | 0.74 (0.25–2.23)              | 0.97 (0.37–2.53)          | 0.62 (0.08–4.76)          |
| HBeAg seropositivity     |                  |                               |                           |                           |
| No                       | 1.00 (Referent)   | 1.00 (Referent)               | 1.00 (Referent)           | 1.00 (Referent)           |
| Yes                      | 4.92 (3.34–7.27)*** | 1.12 (0.60–2.10)             | 5.29 (3.04–9.20)***       | 2.36 (0.83–6.75)          |
| HBSAg level (IU/mL)      |                  |                               |                           |                           |
| <1,000                   | 1.00 (Referent)   | 1.00 (Referent)               | 1.00 (Referent)           | 1.00 (Referent)           |
| ≥1,000                   | 4.61 (3.02–7.06)*** | 0.39 (0.17–0.89)*             | 2.44 (1.24–4.82)**        | 1.00 (0.24–4.23)          |
| HBV DNA level (copies/mL)|                  |                               |                           |                           |
| <10,000                  | 1.00 (Referent)   | 1.00 (Referent)               | 1.00 (Referent)           | 1.00 (Referent)           |
| ≥10,000                  | 11.02 (6.83–17.78)*** | 1.31 (0.50–3.43)             | 13.21 (5.85–29.85)***     | 4.53 (1.38–14.89)*        |
| AFB1-albumin adducts (fmol/mg) |         |                               |                           |                           |
| Undetectable             | 1.00 (Referent)   | 1.00 (Referent)               | 1.00 (Referent)           | 1.00 (Referent)           |
| Low (<21.5)              | 1.36 (0.82–2.24)  | 1.98 (0.68–5.77)              | 2.23 (0.86–5.78)          | 1.96 (0.38–10.26)         |
| High (≥21.5)             | 2.29 (1.44–3.64)** | 3.12 (1.17–8.35)*            | 4.98 (2.07–11.96)**       | 4.75 (1.06–21.19)*        |
| (p-value for trend)      | <0.0001          | 0.0072                      | <0.0001                   | 0.0063                    |

*p < 0.05, **p < 0.01, ***p < 0.001.
seemed to be a time-dependent effect of AFB1 on the development of HCC. In other words, AFB1 exposure in recent 9 years may predict the development of HCC in chronic HBV carriers, but not the AFB1 exposure 10 or more years ago. The failure for AFB1 exposure to predict the risk of HCC developed 10 or more years later may be explained by (i) A single measurement of serum AFB1-albumin adduct level may not accurately reflect the cumulative AFB1 exposure over a long follow-up period due to the change in diet intake or other environmental exposure of AFB1. Repeated measurement of AFB1-albumin adduct levels in serial serum samples collected at different follow-up time points will be needed to clarify this limitation. (ii) The hepatocarcinogenic effect of AFB1 may be more potent at the late stage of disease progression of chronic hepatitis B, that is, the existence of cirrhosis. In other words, AFB1 exposure at the early stage of chronic hepatitis B progression may have less impact on the HCC development due to the normal liver function to repair genetic damages induced by AFB1. (iii) AFB1 exposure may accelerate the progression of (chronic) hepatitis B to cirrhosis and lead to the development of cirrhotic HCC.

The hepatocarcinogenic effect of AFB1 is well-documented, but whether AFB1 may contribute to the development of cirrhosis in humans still awaits elucidation. Owing to the difficulties in the recruitment of cirrhosis cases, the research on the effect of AFB1 on cirrhosis is very limited. In this study, we found elevated serum AFB1-albumin adduct levels at study entry were significantly associated with an increased risk of developing cirrhosis and cirrhotic HCC; high serum levels of AFB1-albumin adducts were associated with a greater odds ratio of developing cirrhotic HCC than that of developing cirrhosis; and the serum AFB1-albumin adduct level was associated with the risk of developing HCC in a dose-dependent manner in cirrhosis patients. In addition, the dose-response relation between the serum AFB1-albumin adduct level and the risk of cirrhosis and cirrhotic HCC remained statistically significant in the stratification analyses by other major risk factors including age, gender, HBeAg serostatus and serum levels of ALT, HBsAg and HBV DNA. These findings together suggest that AFB1 exposure may contribute not only to the development of cirrhosis, but also to the development of HCC even after cirrhosis is established.

Until now, only one epidemiological study reported the detrimental impact of aflatoxin on cirrhosis. A case-control study in Gambia found a significant association between cirrhosis and aflatoxin exposure assessed by lifetime groundnut intake and plasma TP53 R249S mutations. Although different biomarkers of aflatoxin exposure were used, our findings are in consistence with this previous report on the effect of aflatoxin on the development of cirrhosis. The consumption of aflatoxin-contaminated peanut meal was reported to induce different degrees of hepatic lesions including formation of fatty cysts, fibrosis, and cirrhosis among children in a clinical study. In animal studies, administration of aflatoxin or aflatoxin poisoning lead to liver cell damage, mononuclear cell infiltration, and perportal fibrosis in the liver. The underlying mechanism of aflatoxin-induced cirrhosis remains unclear. However, a recent study proposed that myofibroblasts may be involved in the fibrosis-associated liver damage induced by AFB1 exposure. Molecular mechanisms involved in aflatoxin-induced carcinogenesis including formation of DNA and protein adducts, and also lipid peroxidation have been proposed.

Furthermore, the baseline serum level of AFB1-albumin adducts was also significantly associated with non-cirrhotic HCC developed within 4 years after study entry. In other words, AFB1 may cause HCC directly without inducing cirrhosis. Based on our findings and previous evidence, it is possible that AFB1 plays a dual role in hepatocarcinogenesis: (i) AFB1 acts as a procarcinogen to induce DNA damages after being activated into AFB1-8,9 epoxide; (ii) AFB1 acts as a liver damag-
study examining the immunological parameters of Ghanaians in relation to the AFB1 exposure level demonstrated that high AFB1-albumin adduct levels were associated with impairment in cellular immunity. AFB1 exposure could probably cause immune suppression and render the exposed chronic HBV carriers more susceptible to progression to cirrhosis and HCC. However, it would be arbitrary to draw the conclusion by our current data with a single measurement. It is to be noted that in cirrhotic HCC patients, higher AFB1 exposure resulted in a shortened induction period of cirrhotic-HCC due to a more rapid progression to cirrhosis rather than to HCC. It may be possible that AFB1 exposure may not only accelerate the progression of chronic hepatitis B into cirrhosis, but also increase the risk of developing HCC in cirrhosis patients.

To the best of our knowledge, this study is the first follow-up study on the association between the risk of both cirrhosis and HCC and the AFB1 exposure with the adjustment of potential confounders. Among chronic HBV carriers, the serum level of AFB1-albumin adducts was found to be an independent predictor of cirrhosis and HCC developed within 9 years after study entry. In addition, the onset of cirrhosis and HCC was more rapid in chronic HBV carriers with high levels of AFB1 exposure. These findings together not only suggest that AFB1 may increase the risk of cirrhosis but also reveal a complicated role of AFB1 in the etiology of hepatocarcinogenesis.

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