Chapter

The Blood AFB1-DNA Adduct Acting as a Biomarker for Predicting the Risk and Prognosis of Primary Hepatocellular Carcinoma

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Abstract

Aflatoxin B1 (AFB1) is an important carcinogen for primary hepatocellular carcinoma (PHCC). However, the values of blood AFB1-DNA adducts predicting HCC risk and prognosis have not still been clear. We conducted a hospital-based case-control study, consisting of 380 patients with pathologically diagnosed PHCC and 588 controls without any evidence of liver diseases, to elucidate the associations between the amount of AFB1-DNA adducts in the peripheral blood and the risk and outcome of HCC. All subjects had not the history of hepatitis B and C virus infection. AFB1-DNA adducts were tested using enzyme-linked immunosorbent assay. Cases with PHCC featured an increasing blood amount of AFB1-DNA adducts compared with controls (2.01 ± 0.71 vs. 0.98 ± 0.63 μmol/DNA). Increasing adduct amount significantly grew the risk of PHCC [risk values, 1.82 (1.34–2.48) and 3.82 (2.71–5.40) for medium and high adduct level, respectively]. Furthermore, compared with patients with low adduct level, these with medium or high adduct level faced a higher death and tumor-recurrence risk. These results suggest that the blood AFB1-DNA adducts may act as a potential biomarker for predicting the risk and prognosis of PHCC.

Keywords: AFB1, DNA adduct, primary hepatocellular carcinoma, biomarker, risk, prognosis

1. Introduction

Aflatoxin B1 (AFB1) is a knowledge I-type chemical carcinogen for primary hepatocellular carcinoma (PHCC) [1–3]. This carcinogen is mainly produced by *Aspergillus parasiticus* and *Aspergillus flavus* and often found in crops and food (including maize, nuts, and beans), which are raised in the areas with humid and hot environment [1, 4, 5]. Once these AFB1-contaminated crops and food are ingested by human bodies, AFB1 will be metabolized through two stage reactions consisting of detoxification stage (such as reduction, oxidation, and hydrolytic reaction) and covalent stage (such as binding reaction and

conjugating reaction) [2, 3]. During the process of AFB1's metabolism, AFB1-DNA adducts, including AFB1-formamidopyrimidine adduct (AFB1-FAPa) and AFB1's 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy–adduct (AFB1-GA), are frequently formed [2, 3]. Growing evidence has shown that AFB1-DNA adducts are usually tested in the tissue samples (such as liver and placenta tissues) of these individuals from high AFB1 exposure areas [6–10]. Recent studies have displayed that they are also found in the peripheral blood white cells of peoples who are from high AFB1 exposure areas and are associated with the time of AFB1 exposure [11–17]. However, the potential of blood AFB1-DNA adducts predicting PHCC risk and prognosis is not clear. Here, we specifically conduced a hospital-based case-control study to investigate whether blood AFB1-DNA adducts were related to the risk and outcome of PHCC.

2. Materials and methods

2.1 Study population

A total of 380 patients were recruited from the affiliated hospitals of Youjiang Medical University for Nationalities and Guangxi Medical University (two main medical universities in the AFB1-exposure areas in China) between 2011 and 2013. All cases were newly diagnosed as patients using histopathological method and they had no history of radiation or chemotherapy treatment before enrollment. A total of 588 controls, who were randomly recruited from a pool of healthy individuals in the same hospitals during the same time, were all volunteers without any evidence with liver diseases. To control the effects of confounder factors such as age, gender, and race, controls were individually matched with the cases on these factors. In this study, all cases and controls had no history of hepatitis B virus (HBV) and/hepatitis C virus (HCV) infection, whereas these subjects with positive status of serum anti-HCV and/or hepatitis B surface antigen (HBsAg) were excluded. They all agreed to participate in this investigation and did not drop out. With informed consent, all clinicopathological data, including age, gender, race, hepatitis virus B and C infection information, survival follow-up information, were collected using healthy examination or medical records. Additionally, 10 ml of peripheral blood samples for all subjects were also collected for AFB1-DNA adduct analysis. In this study, the last following-up date was set on January 31, 2019. Overall survival (OS) and tumor recurrence-free survival (RFS) status were defined according to the previously described methods [11, 14, 18]. The study protocol was approved by the ethics committees of Youjiang Medical University for Nationalities and Guangxi Medical University.

2.2 AFB1-DNA adducts data

The amount of AFB1-DNA adducts in the peripheral blood were tested using the previously published methods [8, 17]. Briefly, DNA samples were first extracted from the peripheral blood samples and adducts were next quantitated by the comparative enzyme-linked immunosorbent assay (ELISA). To investigate the association between different levels of AFB1-DNA adducts and the risk and prognosis of PHCC patients, the levels of AFB1-DNA adducts were divided into three subgroups according to the mean adduct amounts of cases and controls: low AFB1-DNA adduct level (LAL, <1.00 μmol/DNA), medium AFB1-DNA adduct level (MAL, 1.00–2.00 μmol/DNA), and high AFB1-DNA adduct level (HAL, >2.00 μmol/DNA).

2.3 Statistical analysis

All statistical analyses were accomplished with SPSS statistical package (Version 18, SPSS Institute, Chicago, IL, USA). Test for the distribution of age, gender, and race between patients with PHCC and controls was accomplished using chi-square test. The effects of blood AFB1-DNA adducts on PHCC risk were evaluated using odds ratio (OR) and 95% confidence interval (CI) in the conditional logistic regression model. For survival analyses, Kaplan-Meier survival model with *Log-Rank* test and Cox regression model (the selection of significant variates based on forward-step method with likelihood ratio test) was used to analyze the association between blood AFB1-DNA adducts and PHCC outcomes. Cumulative hazard value for the effects of adducts on the prognosis of patients with PHCC and corresponding 95% CI was calculated using hazard ratio (HR) from significant multivariate Cox regression model (including all significant variates). In this study, the P value <0.05 was defined as statistical significance.

3. Results

3.1 The features of study population

A total of 380 cases with PHCC and 588 controls were included in our final analyses. Baseline characteristics of all cases with PHCC and controls were summarized in **Table 1**, and results showed there were no significant distributions of age, gender, and race between cases and controls.

	Controls		PHCCs		*P*
	n	%	n	%	
Total	588	100.0	380	100.0	—
Gender					0.70
Male	426	72.4	271	71.3	
Female	162	27.6	109	28.7	
Age (years)					0.78
≤35	81	13.8	53	13.9	
36–40	62	10.5	38	10.0	
41–45	90	15.3	55	14.5	
46–50	83	14.1	43	11.3	
51–55	94	16.0	56	14.7	
56–60	56	9.5	42	11.1	
61–65	69	11.7	49	12.9	
≥66	53	9.0	44	11.6	
Race					0.88
Han	339	57.7	221	58.2	
Zhuang	249	42.3	159	41.8	

PHCCs, patients with primary hepatocellular carcinoma.

Table 1.
The characteristics of subjects.

AFB1-DNA adduct levels	Controls		PHCCs		OR (95% CI)[a]	*P*
	n	%	n	%		
Low	316	53.7	122	32.1	1	—
Medium	186	31.6	131	34.5	1.82 (1.34–2.48)	1.20×10^{-4}
High	86	14.6	127	33.4	3.82 (2.71–5.40)	2.35×10^{-14}

[a]*OR conditional on matched set.*
AFB1, aflatoxin B1; PHCC, primary hepatocellular carcinoma.

Table 2.
Associations between AFB1-DNA adduct levels and PHCC risk.

3.2 Blood AFB1-DNA adducts correlating with PHCC risk

The amount of AFB1-DNA adducts in the peripheral white blood cells were calculated using ELISA technique. Compared to controls, patients with PHCC featured a higher level of blood AFB1-DNA adducts (0.98 ± 0.63 vs. 2.01 ± 0.71 μmol/DNA), suggesting blood AFB1-DNA adducts may play an important role in the PHCC carcinogenesis. To investigate possible correlation between AFB1-DNA adducts and PHCC risk, the levels of blood AFB1-DNA adducts were divided into three groups. Results from multivariable logistic regression analyses showed that these individuals with medium AFB1-DNA adduct level (MAL) had an increasing risk of PHCC compared to those with low AFB1-DNA adduct level (LAL) (OR = 1.82 and 95% CI = 1.34–2.48), whereas risk value for high AFB1-DNA adduct level (HAL) was 3.82 (2.71–5.40) (**Table 2**). Altogether, these data were indicative of important potential risk role of blood AFB1-DNA adducts in the carcinogenesis of PHCC.

3.3 Blood AFB1-DNA adducts correlating with PHCC outcome

To explore the effects of blood AFB1-DNA adducts on the prognosis of patients with PHCC, we accomplished two survival model analyses. Kaplan-Meier's

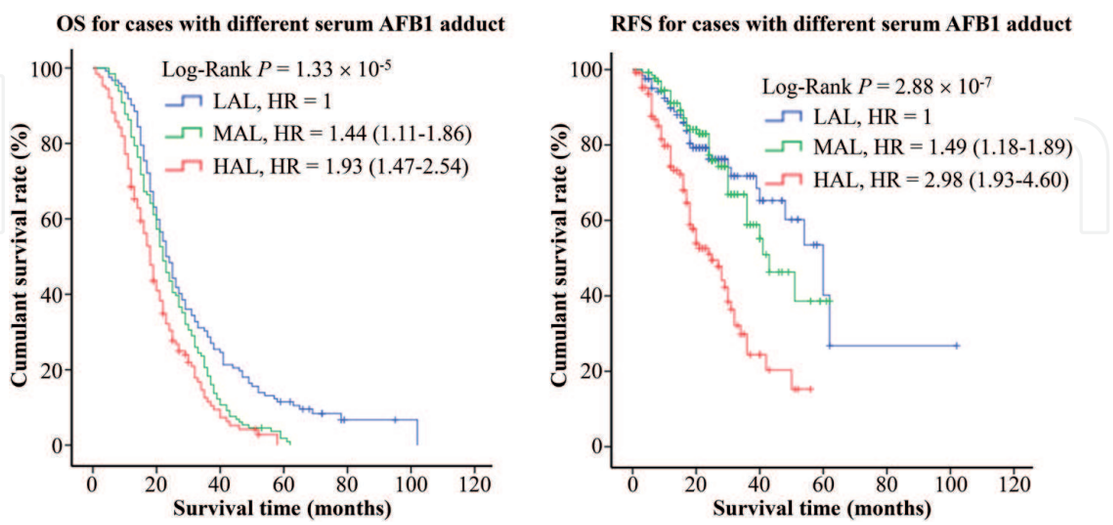

Figure 1.
The aflatoxin B1 (AFB1)-DNA adducts in peripheral blood white cells significantly correlating with the overall survival (OS) and tumor recurrence-free survival (RFS) of primary hepatocellular carcinoma (PHCC). Cumulative hazard function was plotted by Kaplan-Meier's methodology, and P value was calculated with two-sided log-rank tests. The relative hazard ratio (HR) values for genotypes were calculated using multivariable cox regression models (with all significant variables) based on forward-step method with likelihood ratio test. LAL, low AFB1-DNA adduct level; MAL, medium low AFB1-DNA adduct level; HAL, high low AFB1-DNA adduct level.

survival analyses first tested the association between blood AFB1-DNA adducts and patients' OS and results displayed that increasing level of adducts significantly shorten the OS time of patients ($P = 1.33 \times 10^{-5}$) (**Figure 1**, left). Similar effects were also found in the RFS analyses ($P = 2.88 \times 10^{-7}$) (**Figure 1**, right). Results from multivariate Cox's regression models further exhibited that these cases with an increasing level of blood AFB1-DNA adducts faced an increasing risk of death [HRs (95% CIs) = 1.44 (1.11–1.86) for MAL and 1.93 (1.47–2.54) for HAL, respectively] (**Figure 1**, left). For RFS, the corresponding tumor-recurrence risk was 1.49 (1.18–1.89) for MAL and 2.98 (1.93–4.60) for HAL, respectively (**Figure 1**, right).

4. Discussion

In this study, we explored the relationship between the blood AFB1-DNA adducts and the risk and prognosis of PPHCC. We found that individuals with an increasing level of AFB1-DNA adducts in peripheral blood white cells would feature higher PHCC risk (OR = 1.82 for MAL and 3.82 for HAL, respectively). Furthermore, the blood AFB1-DNA adduct levels were significantly associated with poor OS and RFS of patients with PHCC.

AFB1 acts as a major cause of PHCC in the southeast areas of China and is taken into human bodies through its contaminating staple foods [2]. AFB1 is transferred into AFB1-DNA adducts and displays its genic toxicity and hepato carcinogenicity [3, 19]. Mechanically, PHCC induced by AFB1 is mainly concerned with DNA damage (including DNA single-/double-strand breaks, base damage, adduct formation, genic mutation), the dysregulation of DNA repair, the activation of cancer genes (such as ras and myc), the inactivation of cancer suppressor genes (such as TP53, BP1, H2AX, bcl2, p21, and p27), inheritance alterations, and/or abnormal immunoreaction [1, 20–25]. Among these knowledge mechanisms and pathways, AFB1-DNA adducts and mutations at codon 249 of TP53 gene (also termed as hot-spot mutation induced by AFB1) have been especially concerned in the past decades [26–29]. This is mainly because AFB1-DNA adducts are the key central forms in the metabolism of AFB1 in human bodies [19, 26, 30], whereas spot mutations at codon 249 of TP53 gene are highly frequent in HCC patients with AFB1 exposure [31–36]. Evidence from clinical epidemiology and experimental animal models has exhibited that they are constantly tested in biopsy samples, such as liver tissues, tumor tissues, placenta tissues, and blood cells, of individuals from AFB1 exposure areas [6, 8, 11, 12, 15–17].

For example, Hsieh and Hsieh [8] examined the amounts of AFB1-DNA adducts in the 120 placenta tissue samples from women in Taipei, a high AFB1 exposure area, and observed that 57.5% (69/120) of samples were positive AFB1-DNA adducts with the range of 0.6 and 6.3 μmol/mol DNA. Furthermore, they found higher amount of AFB1-DNA adducts in samples collected in the summer than in the winter. Shirabe et al. [37] investigated the association between AFB1-DNA adducts in hepatocyte nuclei and TP53 mutation in PHCC among Japanese population. They found that 6% (118/279) patients with PHCC and 16% (13/83) patients with HBV- and HCV-negative PHCC were positive for AFB1-DNA adducts. Higher hot-spot mutations in the TP53 gene were also found in these with positive AFB1-DNA adduct status [37]. A relatively large-size sample clinical study, including 501 PHCC cases with different AFB1 exposure, also shows that positive status of AFB1-DNA adducts in the tumor tissues significantly increases the risk of TP53 mutations (OR = 3.38 and 95% CI = 2.23–5.11) [7].

Following epidemiological studies on based clinical samples further prove that the amount of AFB1-DNA adducts is higher in the tumor tissues than in the peri-tumor tissues [6]. This increasing tissular AFB1-DNA adducts are significantly associated with poor OS and RFS of patients with PHCC [6].

In this study, we designed and finished a hospital-based case-control study in the southwestern of Guangxi, a knowledge-high AFB1 exposure area. Our data exhibited that increasing the amount of AFB1-DNA adducts in peripheral white blood cells not only increased PHCC risk, but also modified the OS and RFS of patients with PHCC. Supporting our findings through several studies from high AFB1 exposure areas, the amount of blood AFB1-DNA adducts can reflect the levels of AFB1 exposure information and may be related to PHCC risk and prognosis [11, 12, 14, 15, 17, 38]. Taken together, these results suggest that AFB1-DNA adducts in the blood as well as in the tumor tissues may be potential biomarkers for PHCC risk and outcome.

This study has several strengthens. We accomplished the predictive value analyses using these individuals only with AFB1 exposure but without HBV or HCV. This is done mainly because both HBV and HCV infection will alter effects of AFB1-DNA adducts predicting the risk and outcome of PHCC. Additionally, to control potential confounders such as age, gender, and race, the individually matched design was finished in this study. Therefore, our study may represent a relatively more actual predictive role of blood AFB1-DNA adducts.

To conclude, this study explored the association between blood AFB1-DNA adducts and the risk and prognosis of PHCC using a retrospective clinic-sample research approach and displayed that blood AFB1-DNA adduct may be a potential biomarker for HCC risk and outcome. Several limitations should be focused for our study. First, relatively small-size samples may underestimate the effects of blood AFB1-DNA adducts on PHCC risk and outcome. Second, selective bias may happen because of this hospital-based retrospective investigative design. Finally, the mechanical analyses for AFB1-DNA adducts predicting PHCC risk and prognosis were not finished. Thus, the blood AFB1-DNA adducts may be valuable biomarkers for predicting the risk and prognosis of PHCC once the present findings were proved by larger clinic samples and functional analyses.

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Conflict of interests and source of funding

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Abbreviations

AFB1	Aflatoxin B1
AFB1-FAPa	AFB1-formamidopyrimidine adduct
AFB1-GA	AFB1's 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy–adduct
HAL	high AFB1-DNA adduct level
HBV	hepatitis B virus
HBsAg	hepatitis B surface antigen
HCV	hepatitis C virus
CI	confidence interval
HR	hazard ratio
LAL	low AFB1-DNA adduct level
MAL	medium AFB1-DNA adduct level
OR	odds ratio
PHCC	primary hepatocellular carcinoma
OS	overall survival
RFS	tumor recurrence-free survival

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