Chapter 10

The Serum MicroRNA Expression Modified the Genic Toxicity Caused by Aflatoxin B1

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Abstract

The serum microRNAs have been reported as potential biomarkers for hepatocellular carcinoma (HCC); however, their role in genic toxicity related to aflatoxin B1 (AFB1), such as TP53 mutation and DNA damage, has not yet been evaluated. Here, we conducted a hospital-based case-control study, including 558 patients with pathologically diagnosed HCC and positive AFB1 and healthy controls (n = 630) without any evidence of liver diseases. Genic toxicity related to AFB1 was evaluated using the hot-spot mutation at the codon 269 of TP53 gene (TP53M) and AFB1-DNA adducts. Through serum microRNA PCR microarray screening analysis, we observed 10 differentially expressed microRNAs (including miR-7-2-3p, miR-4651, miR-127-3p, miR-192-5p, miR-382-5p, miR-10b-5p, miR-532-3p, miR-16-5p, miR-106b-5p, and miR-4688) among HCC cases with positive AFB1 and controls with positive AFB1. The miR-4651 and miR-382-5p were further identified to be significantly higher in AFB1-positive HCC cases compared to controls. This kind of increasing serum levels was significantly and positively associated with frequency of TP53M and the levels of AFB1-DNA adduct. Furthermore, these microRNAs also modified the prognosis of HCC related to AFB1. These results suggest that the serum levels of microRNAs might be able to modify AFB1-induced genic toxicity, and microRNA-4651 and miR-382-5p, are such potential candidates.

Keywords: serum microRNA, hepatocellular carcinoma, aflatoxin B1, genic toxicity, DNA adduct, TP53M

1. Introduction

Aflatoxin B1 (AFB1) is an important mycotoxin mainly produced by the moulds *Aspergillus parasiticus* and *Aspergillus flavus.* Structurally, it is characterized by fusion of a cyclopentanone ring to the lactone ring of the coumarin moiety [1–3]. Because *A. parasiticus* and *A. flavus* usually multiply under hot and humid conditions, AFB1 is as a contaminant of human food (including core, peanuts, soya sauce, and fermented soy beans) in tropical areas [1–4]. Increasing evidence has shown that AFB1 has three toxicological effects: (a) the attraction of specific organs, especially liver; (b) genotoxicity, mainly inducing the formation of the hot-spot mutation of p53 gene (especially mutation at the codon 249) and AFB1-DNA adducts; and (c) carcinogenicity, primarily causing hepatocellular carcinoma (HCC) [4–11]. Studies have shown that DNA damage induced by AFB1 plays the central role of carcinogenesis of HCC related to AFB1 in the toxic studies [5–11]. Today, this toxin has been classified as a known human carcinogen by the International Agency for Research on Cancer [6, 12]. Therefore, early marker of genic toxicity of AFB1 before carcinogenesis induced by this toxin offers the best chance of prevention for individuals with AFB1 exposure.

Increasing evidence has shown that there is a link between dysregulation of microRNAs and HCC [13–19]. In particular, microRNAs are highly stable in circulation and expression patterns seem to be tissue specific, suggesting that circulating microRNAs may be potentially ideal biomarkers for some diseases including HCC [20–30]. However, information on whether serum microRNAs are correlated with AFB1-related genic toxicity is limited. In this study, we investigated the association between serum microRNAs and the toxicological effects of AFB1 exposure through the analysis of AFB1-DNA adduct amount and TP53 gene mutation frequency.

2. Materials and methods

2.1. Study design and participants

The protocol was approved by the Research Ethics Committee of the Affiliated Hospital of Youjiang Medical University for Nationalities. We conducted a hospital-based case-control study in the Guangxi area to elucidate the association between the serum microRNAs and genic toxicity of AFB1. All cases and controls were residents of the Guangxi Zhuang Autonomous Region from AFB1 exposure areas. All participants were recruited from affiliated hospitals of Guangxi Medical University and Youjiang Medical College for Nationalities and accepted enrollment in this study. All newly diagnosed HCC patients in hospitals affiliated with Youjiang Medical College for Nationalities and Guangxi Medical University from January 2006 to December 2015 were utilized. The inclusion criteria on cases are as follows: (1) cases with histopathology-confirmed HCC; (2) cases understanding the objective of the study and providing informed consent; (3) the ability to complete the necessary investigations and questionnaires; (4) cases with negative HBV markers (HBsAg, HBeAg, anti-HBe, anti-HBc, and HBV DNA) and negative anti-HCV; (5) cases with positive ln (AAA) (positive value: ≥1.00 ln fmol/mg); (6) cases without

preoperative chemotherapy, radiotherapy, transarterial chemoembolization, or ablation before collection of blood samples; (7) cases receiving resect treatment (curative or partial resection) or resect treatment plus postoperative adjuvant TACE as initial treatment according to Chinese Manage Criteria of HCC, but not treatment with radiotherapy or chemotherapy before surgical operative treatment; and (8) 5-year follow-up completed with available cancerous tissue specimens and clinical data. The exclusion criteria for cases consisted of: (1) cases with HCC but not confirmed by histopathological examination; (2) cases receiving chemotherapy or radiotherapy treatment before surgical operative treatment; (3) cases with positive HBV markers (HBsAg, HBeAg, anti-HBe, anti-HBc, or HBV DNA) or positive anti-HCV; (4) cases without positive ln (AAA) (positive value: ≥1.00 ln fmol/mg); and (5) cases rejected, dropped out, or lost information.

All controls were recruited from the general health check-up center at the same hospitals during the same period for comparison. The inclusion criteria for controls included (1) controls individually matched to HCC cases based on gender, ethnicity (Han, Zhuang), age (±5 years), time when sampled, and hospital locations, to control the effects of confounders; (2) controls understanding the objective of the study and providing informed consent; (3) the ability to complete the necessary investigations and questionnaires; (4) controls with negative HBV markers (HBsAg, HBeAg, anti-HBe, anti-HBc, and HBV DNA) and negative anti-HCV; (5) controls with positive ln (AAA) (positive value: ≥1.00 ln fmol/mg); (6) controls without liver diseases and other systematic diseases; and (7) controls with persistently normal AST, ALT, and AFP levels. The exclusion criteria for controls consisted of (1) individuals with evidence of liver diseases; (2) individuals with positive HBV markers (HBsAg, HBeAg, anti-HBe, anti-HBc, or HBV DNA) or positive anti-HCV; (3) individuals without positive ln (AAA) (positive value: ≥1.00 ln fmol/mg); and (5) individuals rejected, dropped out, or lost information.

According to aforementioned criteria, a total of 558 cases with HCC and 630 controls, representing 97% of eligible cases and 94% of eligible controls, were interviewed and included in the present study. All patients and controls gave informed consent for participation and were interviewed uniformly before surgery by a well-trained interviewer. The questionnaire used in the interview sought detailed information on general demographic data (including sex, age, ethnicity, dietary and living history, medical history, and family disease history). Demographic information and therapeutic data were collected from medical records in the hospitals by a Youjiang Cancer Institution staff member. At the same time, 4 mL of peripheral blood was obtained for serum analysis of microRNAs. Surgically removed tumor samples of all cases were collected for genic toxicity assay of AFB1.

2.2. Serum preparation

For serum preparation, 5-mL peripheral whole blood was collected from each patient with HCC and control. Samples were centrifuged at 3000 r.p.m. for 10 min under the conditions of 4°C, followed by an additional centrifugation at 12,000 r.p.m. for 15 min to completely remove all remaining cells. The serum samples were aliquoted and stored at −80°C until analysis.

2.3. DNA detraction

Genomic DNA was extracted from HCC tumor tissues using *Genomic DNA Prep Kit* (cat#9K-6-0016, Bio Basic, Inc., Ontario, Canada) as described by standard procedures (Protocol #BS474, Bio Basic, Inc., Ontario, Canada). Briefly, about 15-mg fresh cancerous tissue was transferred to a 1.5-mL microcentrifuge tube, and 300 μL of *Cell Lysis Solution* and 1.5 μL of 20 mg/mL *proteinase K* were added for deparaffinization and digestion at 20°C overnight until the tissue had dissolved. After that, 100 μL of *Protein Precipitation Solution* was added for the cell lysate. The supernatant after centrifuge was transferred to another microcentrifuge tube. Then, DNA was extracted by phenol-chloroform extraction and ethanol precipitation and stored at −20°C until additional analysis.

2.4. Laboratory tests

Fasting venous blood samples were collected from all patients for routine workup, including complete blood picture, liver function tests, prothrombin concentration and prothrombin international normalized ratio, AFP, anti-HCV, HBsAg, and HBc-Ab using commercially available assays.

Because AAA is a stable AFB1 exposure biomarker, the levels of AAA were used to evaluate the AFB1 exposure levels of all subjects [1, 3]. AAA levels in the serum were tested using the comparative enzyme-linked immunosorbent assay as previously published. According to our previous reports with respect to AFB1 exposure, value more than 1.00 ln fmol/mg was considered as positive-AFB1 status [31–34].

2.5. Serum microRNAs expression profiling analysis

In this study, we screened the serum microRNAs using two methods: microRNA array analysis and TaqMan-PCR analysis. For microRNA array analysis, we collected sera from six HCC cases and six sex-, age-, and ethnicity-matched controls without any evidence of liver diseases. We sent sera to Shanghai Oe-Bio-Tech Medical Company (Shanghai, China) for microRNA array detection. Briefly, total RNA from 1-mL serum was extracted with the *PAXgene® Blood RNA Kit* (cat#762174, Qiagen, Duesseldorf, Germany), and RNA quality was evaluated using the analyses of RNA purity and concentration by NanoDrop spectrophotometer and RNA integrity (RIN) by BioAnalyzer 2100. RNA samples would be used for microRNA assay if RIN value was more than 7.2. *RT² First Strand Kit* (cat#330401, Qiagen) was used to synthesize all corresponding cDNA. After that, the amounts of human microRNAs in the serum samples were tested through the real-time PCR (on an Applied Biosystems 7900HT Real-Time PCR System) using RT² Profiler PCR Arrays (cat# PAHS-028ZF-2, Qiagen) in combination with RT² SYBR Green Mastermixes (cat# 330500, Qiagen). The cycle threshold (CT) values were analyzed using the PCR Array Data Analysis Web portal (at www.SABiosciences.com/pcrarraydataanalysis.php). In the present study, a total of 10 candidate microRNAs were chosen for TaqMan-PCR analysis according to the following criteria: more than two-times change between cases and controls, coefficient of variation for CT values <0.05, and high expression ($CT_{average}$ <29 cycles) in patients with HCC.

2.6. TaqMan-PCR assay for candidate microRNAs

The serum levels of 10 candidate microRNAs were tested using quantitative reverse transcription-PCR with TaqMan probe described in our previous reports [17–19]. Briefly, total RNA was extracted from 400-μL serum with 0.2 nM of cel-miR-67 using *PureLink® RNA Mini Kit* (cat#12183018A, Ambion, USA), and corresponding first-strand cDNAs were synthesized using *High Capacity cDNA Reverse Transcription Kit* (cat# 4368814, Invitrogen Grand Island, NY) and *TaqMan MicroRNA Reverse Transcription Kit* (cat#4366596, Applied Biosystems, Carlsbad, CA). After that, TaqMan-PCR analysis was performed using standard protocols on a Bio-Rad iCycler CFX Detection System. The serum levels of candidate microRNAs were assessed using *TaqMan microRNA assays* (cat#4427975, Applied Biosystems) with cel-miR-67 as the endogenous control. PCR reactions were run in a 5-μL final volume containing 1 × *TaqMAN Universal Master Mix II* (cat#4440041, Applied Biosystems), 1 × TaqMan microRNA probes and primers (cat#4427975, Applied Biosystems), and about 15 ng of cDNA. Cycling conditions were 30 s at 95°C for the initial denaturation, and 50 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for annealing. All reactions were conducted in triplicate, and controls (including negative and positive control) were performed for each gene. In this study, the relative amount of candidate microRNAs to cel-miR-67 was calculated as $2^{-\Delta CT}$ method, where $\Delta CT = (CT_{microRNA} - CT_{cel\text{-}miR\text{-}67})$.

2.7. Genic toxicity analysis of AFB1

In the present study, genic toxicity of AFB1 was evaluated using two markers: AFB1-DNA adducts and the hot-spot mutation at the codon 249 of TP53 gene (TP53M) in the cancerous tissues. The amount of AFB1-DNA adducts in HCC cancerous tissues was measured by competitive enzyme-linked immunosorbent assay as described by our previous report [33, 35–39]. Briefly, DNA samples were assayed at 50 ng/well and quantitated relative to AFB1-FAPy standard using monoclonal antibody *6A10*. The percent of inhibition was calculated by comparison with the nonmodified heat-denatured calf thymus DNA control. Each sample was measured in triplicate on the three different assay dates and had a variability of less than 10%.

For TP53M assay, TP53 codon 249 genotypes were genotyped using the TaqMan-PCR on iCycler iQ™ real-time PCR detection system (iQ5, Bio-Rad Laboratories Inc., Hercules, CA, USA). Primers and probes for TaqMan-PCR assay of TP53M are as follows: 5′-TTGGC TCTGA CTGTA CCACC AT-3′ (SY#NSO_533299_001, Applied Biosystems), 5′-TGGAG TCTTC CAGTG TGATG ATG-3′ (SY# NSO_533299_002, Applied Biosystems), 5′-FAM-ACCGG AGTCC CATC-MGB-3′ (SY#431603301-001, Applied Biosystems), and 5′- VIC-AACCG GAGGC CCAT-MGB-3′ (SY#431603301-002, Applied Biosystems) [33, 34]. Each PCR was performed in a total volume of 25 μL containing 1 × Premix Ex TaqTM (catalog # DRR039A, TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China), 0.2 μM of each primer, 0.2 μM of each probe, and 50–100 ng of genomic DNA using the running conditions: 95°C for 2 min for the initial denaturation and 50 cycles of 10 s at 95°C and 1 min at 60°C. For quality control, each PCR run included negative and positive controls. Additionally, a random 10% samples were analyzed using repeated genotyping and sequencing methods, and 100% identical genotype were yielded.

2.8. HCC patients following up

Patients with HCC were followed and underwent serial monitoring of chest radiograph, ultrasonography, AFP, and emission computed tomography every 2 months for the first 2 years and semiannually thereafter for detection of recurrence. Tumor recurrence was confirmed by imaging techniques (including chest radiograph, ultrasonography, and emission computed tomography), either intrahepatically or extrahepatically (distant metastases or lymph nodes). A new tumor with increasing AFP but without radiologic evidence was not regarded as recurrence until confirmed by imaging. The last follow-up day was set on August 31, 2015, and the survival status was confirmed via clinic records and patient or family contact. The data of two survival types, recurrence-free survival (RFS) and overall survival (OS), were collected in the present study. The duration of RFS was defined as the date of primary treatment to the date of tumor recurrence or last known date alive, whereas the duration of OS was defined as from the date of primary treatment to the date of death or last known date alive [17, 40, 41].

2.9. Statistical analysis

All statistical analyses were done using the statistical package for social science version 18 (SPSS Institute, Chicago, IL). The differences of age, race, gender, and liver function between groups were compared using Student *t* test and the χ^2 test. The nonparametric Mann-Whitney *U* test was used for comparison of microRNA data ($2^{-\Delta CT}$) from independent samples from two groups as this type data were not normally distributed. Unconditional logistical regression was conducted to estimate odds ratios (ORs) for the association between microRNAs and TP53M along with the 95% confidence intervals (CIs). Kaplan-Meier survival analysis with the log-rank test was used to evaluate the effects of the serum microRNAs levels on HCC prognosis. Risk factors for HCC prognosis were selected using the Cox multivariate regression model with stepwise forward selection based on a likelihood ratio test. Hazard ratios (HRs) and 95% CIs for risk factors were then calculated from a multivariate Cox regression model. All statistical tests were two tailed, and a *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. The characteristics of HCC cases and controls

According to eligibility criteria, we collected 1188 serum samples from patients with HCC and controls (**Table 1**). There were no differences between cases and controls in terms of the distribution of age, sex, race, and smoking and drinking status because these were individually matched. All participants had positive marker of AFB1 exposure but not history of HBV or HCV infection. About 60% of HCC cases featured abnormal liver function.

3.2. Differential expression of serum microRNAs levels between AFB1-positive HCC cases and controls

In this study, we first examined the serum microRNA profiles in cases with AFB1-positive HCC compared to controls with positive AFB1 but without any evidence of liver diseases

Variable	Controls (n = 630)		HCCs (n = 558)		*P**
	n	%	n	%	
Age (y)					0.68
≤49	326	51.7	282	50.5	
>49	304	48.3	276	49.5	
Gender					0.90
Female	192	30.5	172	30.8	
Male	438	69.5	386	69.2	
Race					0.86
Han	331	52.5	296	53.0	
Minority	299	47.5	262	47.0	
Smoking					0.81
Negative	253	40.2	228	40.9	
Positive	377	59.8	330	59.1	
Drinking					0.29
Negative	242	38.4	231	41.4	
Positive	388	61.6	327	58.6	
AFP (ng/L)					-
≤20	630	100.0	216	38.7	
>20	0	0.0	342	61.3	
AST					-
Negative	630	100.0	236	42.3	
Positive	0	0.0	322	57.7	
ALT					-
Negative	630	100.0	328	58.8	
Positive	0	0.0	330	59.1	

*The *P* value indicates the statistical significance for the differences between HCC cases and controls.
Abbreviations: AST, aspartate transaminase; ALT, alanine transaminase; AFP, α-fetoprotein.

Table 1. Clinic characteristics of study subjects.

using microRNA PCR Array and identified 10 significantly different microRNAs (including miR-127-3p, miR-7-2-3p, miR-192-5p, miR-4651, miR-10b-5p, miR-382-5p, miR-16-5p, miR-532-3p, miR-4688, and miR-106b-5p) between cases and controls (**Figure 1**). Next, we further investigated the serum expression profiles of these microRNAs in all participants using TaqMan-PCR technique (**Figure 2**). Mann-Whitney *U* test showed that only miR-4651 and miR-382-5p were increased in HCCs compared to controls ($P < 0.05$).

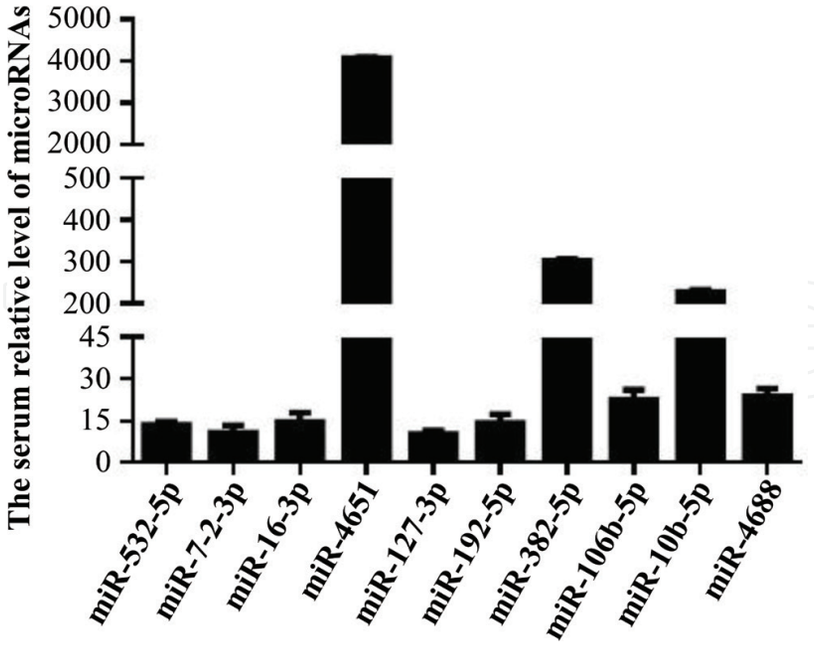

Figure 1. The screening of serum microRNAs for aflatoxin B1 (AFB1)-positive hepatocellular carcinoma (HCC). In this screening analysis, sera samples from six HCC cases and six age-, sex-, and race-matched controls with positive AFB1 exposure but without any evidence of liver tumors were collected, and serum microRNAs were tested using microRNA array analysis. Ten candidate microRNAs were chosen for further analysis according to the fitful criteria (see Section 2).

3.3. The serum miR-4651 and miR-382-5p positively correlated with AFB1-DNA adducts

To investigate whether serum levels of miR-4651 and miR-382-5p were associated with genic toxicity of AFB1, we first explored the correlation between the amount of AFB1-DNA adducts in the cancerous tissues and the serum levels of miR-4651 and miR-382-5p. The increasing serum levels of miR-4651 were found among HCC cases with higher amount of AFB1-DNA adducts in the cancerous tissues (**Figure 3A**). The correlation analysis showed serum miR-4651 level was linearly correlated with the levels of AFB1-DNA adducts, with a linear correlation formula:

$$y = 13.35 + 9.71\chi \quad (1)$$

Where y represents the serum miR-4651 level, and χ represents the amount of AFB1-DNA adducts in the cancerous tissues (mmoL/moL DNA). Similar results were also found in the correlative analysis of miR-382-5p and AFB1-DNA adducts (formula: $y = 2.2 + 0.17\chi$, where y represents the serum miR-382-5p level and χ represents the amount of AFB1-DNA adducts in the cancerous tissues) (**Figure 3B**). Taken together, our data suggested that serum miR-4651 and miR-382-5p expression might be correlated with AFB1-induced DNA damage.

3.4. The serum miR-4651 and miR-382-5p increased risk of TP53M

Because TP53M is the most important molecular signature of AFB1-induced DNA damage [1, 42], we next investigated whether the serum levels of miR-4651 and miR-382-5p modified this mutation in the 558 cancer cases. To analyze, the serum levels of miR-4651 and miR-382-5p

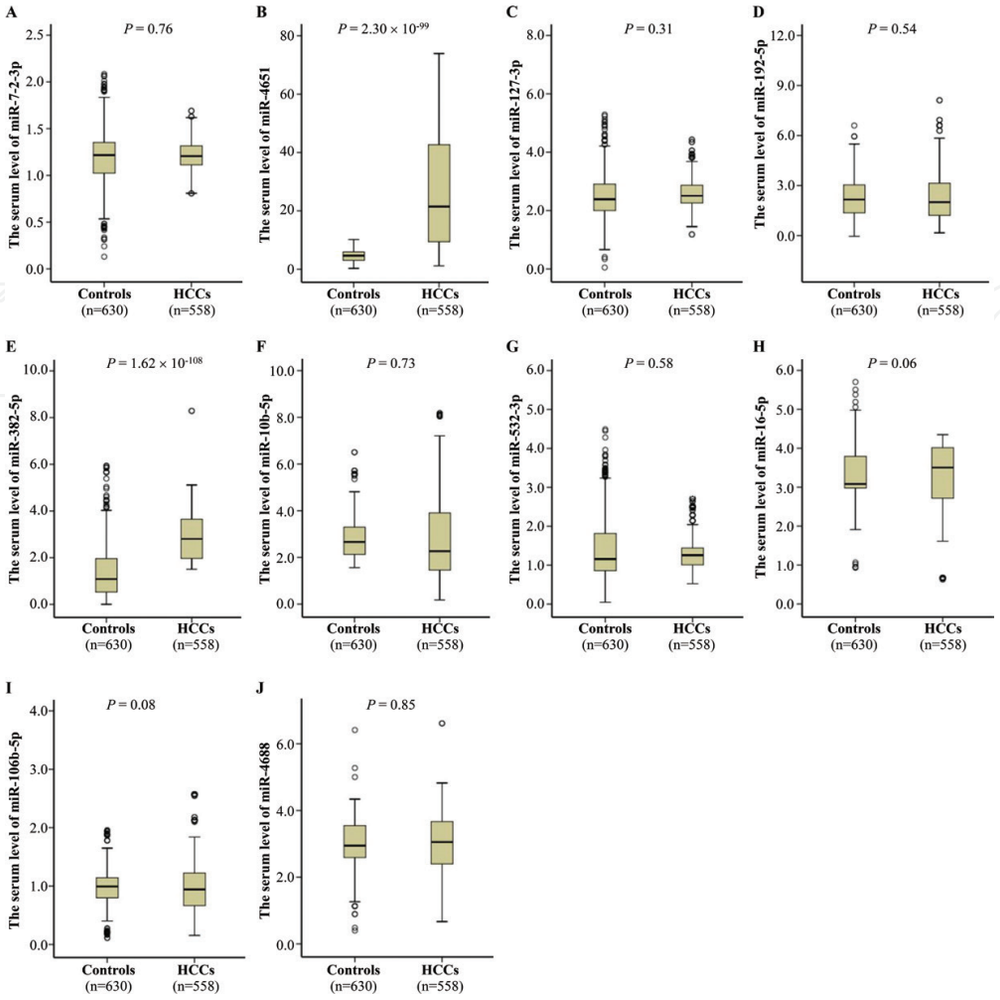

Figure 2. Differentially expressed microRNAs in the serum samples. The differentially expressed microRNAs in the screening analysis, including miR-7-2-3p (**A**), miR-4651 (**B**), miR-127-3p (**C**), miR-192-5p (**D**), miR-382-5p (**E**), miR-10b-5p (**F**), miR-532-3p (**G**), miR-16-5p (**H**), miR-106b-5p (**I**), and miR-4688 (**J**), were further analyzed using TaqMan-PCR method in 558 cases with aflatoxin B1 (AFB1)-positive hepatocellular carcinoma (HCC) and 630 controls with positive AFB1 exposure but without any evidence of liver tumors. The relative levels of microRNA expression were calculated according to $2^{-\Delta CT}$ method (see Section 2). The microRNA data are shown as box plots, with horizontal lines representing the median, the bottom and the top of the boxes representing the 25th and 75th percentiles, respectively. We compared expression data between groups using the Mann-Whitney *U* test.

were divided into two classifications: low (relative expression value ≤20) and high (relative expression value >20) for miR-4651 levels and low (relative expression value ≤3) and high (relative expression value >3) for miR-382-5p, respectively, according to their median relative expression levels. Increasing serum levels of miR-4651 and miR-382-5p increased the frequency of TP53M (**Table 2**); the corresponding risk values were 2.52 (1.65–3.84) and 4.06 (2.72–6.07) for miR-4651 and miR-382-5p, respectively (**Table 2**).

3.5. The serum miR-4651 and miR-382-5p modified the prognosis of AFB1-positive HCC

To study the effects of the serum miR-4651 and miR-382-5p on outcome of patients with AFB1-positive HCC, we analyzed the survival follow-up information of all HCC patients. Results from the Kaplan-Meier survival analysis showed that increasing serum miR-4651

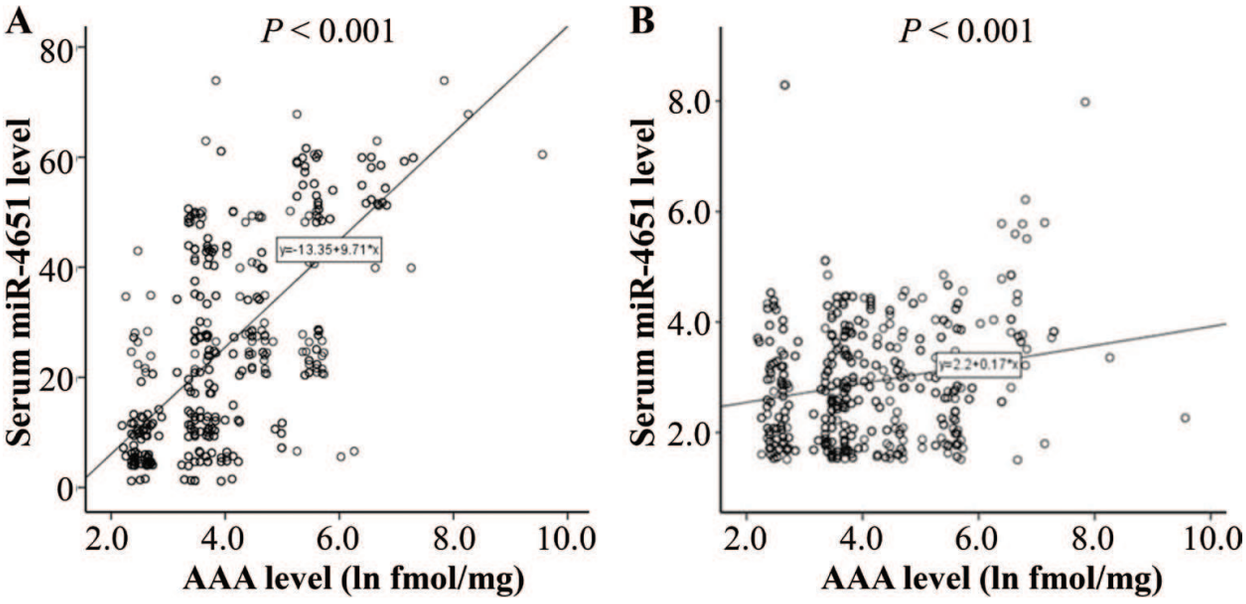

Figure 3. The correlation between the serum levels of miR-4651 and miR-382-5p and the amount of aflatoxin B1 (AFB1)-DNA adducts in cancerous tissues among patients with AFB1-positive hepatocellular carcinoma (HCC) (n = 558). AFB1-DNA adducts and microRNAs were tested using the comparative enzyme-linked immunosorbent assay and TaqMan-PCR techniques, respectively. The serum levels of miR-4651 (**A**) and miR-382-5p (**B**) were linearly associated with the amount of AFB1-DNA adducts.

level significantly correlated with shorter OS and RFS of HCC cases (**Figure 4A and B**). From Cox regression analysis (**Figure 4A and B**), we showed that the miR-4651 is correlated with poor prognosis of HCC (high miR-4651-level risk value, HR = 1.86 and $P = 2.42 \times 10^{-8}$ for OS and 2.28 and 4.32×10^{-9} for RFS, respectively). Survival analysis also exhibited that increasing serum level of miR-382-5p increased death risk (HR = 2.46 and 95% CI = 1.99–3.03) and tumor-recurrence risk (HR = 2.64 and 95% CI = 1.89–3.69) of HCC (**Figure 4C and D**). Taken together, these results indicated that the serum miR-4651 and miR-382-5p are independent of other clinical covariates and suggested its potential as an independent prognostic factor for HCC related to AFB1.

Serum level	TP53M (–) (n = 174)		TP53M (+) (n = 384)		OR (95% CI)[a]	*P*
	n	%	n	%		
miR-4651						
Low	58	33.3	64	16.7	Reference	
High	116	66.7	320	83.3	2.52 (1.65–3.84)	1.70×10^{-4}
miR-382-5p						
Low	86	49.4	75	19.5	Reference	
High	90	51.7	309	80.5	4.06 (2.72–6.07)	8.54×10^{-12}

[a]Adjusted by age, race, and gender.

Table 2. The serum miR-4651 and miR-382-5p levels and TP53M risk.

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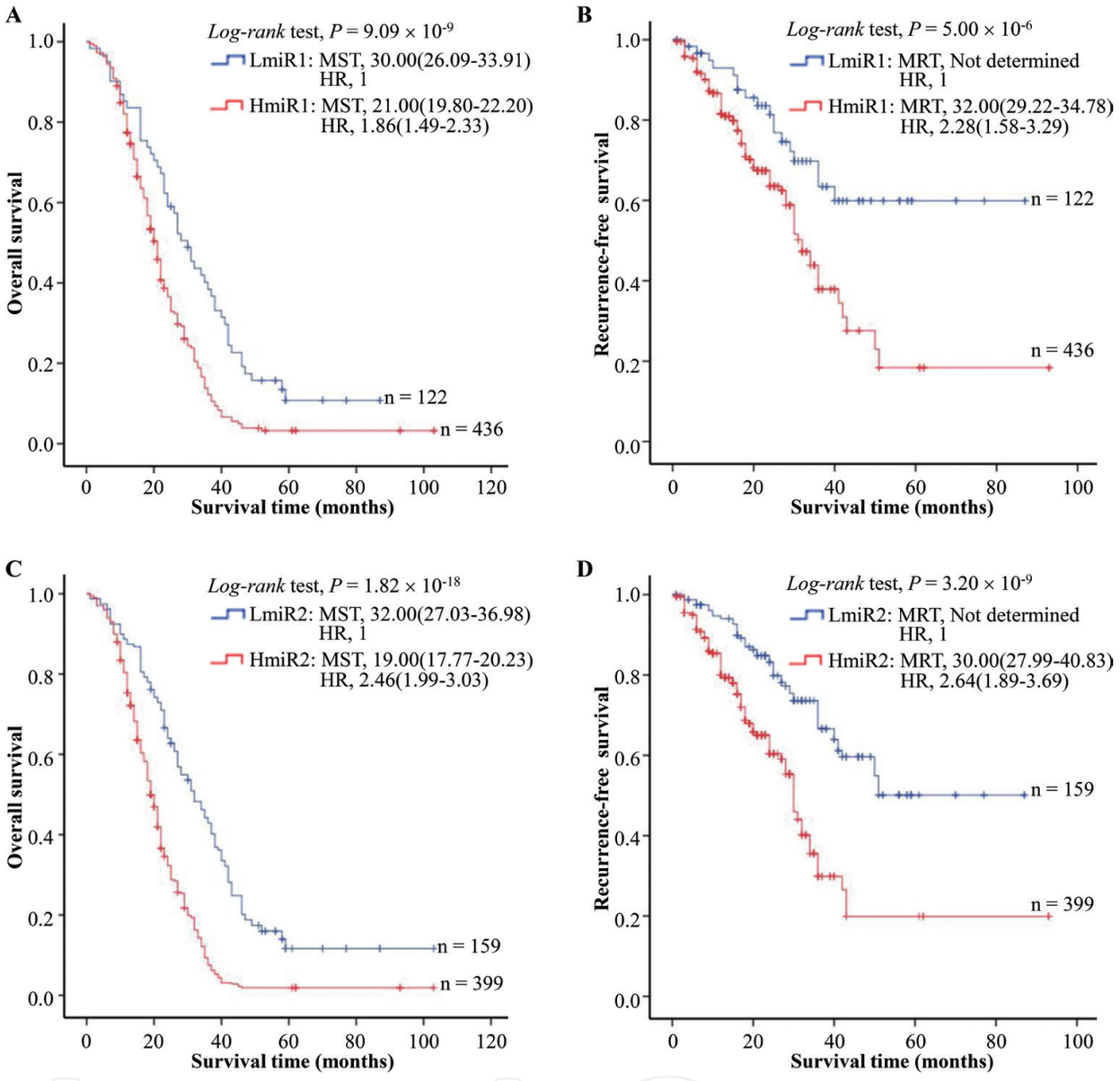

Figure 4. The association between the serum miR-4651 and miR-382-5p and HCC prognosis in 558 aflatoxin B1 (AFB1)-positive cases with hepatocellular carcinoma (HCC). The serum levels of miR-4651 (A and B) and miR-382-5p (C and D) were found to correlate with the overall survival (A and C) or tumor recurrence-free survival (B and D) of HCC. Cumulative hazard function was plotted by Kaplan-Meier's methodology, and *P* value was calculated with two-sided log-rank tests. *Abbreviations*: MST, the median overall survival time; MRT, the median tumor recurrence-free survival time; LmiRN1, low miR-4651 expression; HmiRN1, high miR-4651 expression; LmiRN2, low miR-382-5p expression; HmiRN2, high miR-382-5p; and HR, hazard ratio.

4. Discussion

4.1. The evaluation of toxicological effects of AFB1

A main genic toxicological effect of AFB1 is to induce DNA damage, consisting of AFB1-DNA adducts and the hot-spot mutation of tumor suppressor gene p53 at codon 249 (TP53M) [1, 12].

AFB1 can produce several DNA adducts formation, including 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl formamide)-9-hydroxy-AFB1 (AFB1-FAPy) adduct, 8,9-dihydro-8-N^7-guanyl-9-hydroxy-AFB1 (AFB1-N^7-Gua) adduct, and so on. Among these adducts, AFB1-FAPy adduct is a kind of stable imidazole ring-opened form originating from AFB1-N^7-Gua adduct and may display an important role in HCC progress [12]. Furthermore, the accumulation of AFB1-FAPy is nonenzymatic and time-dependent and displays apparent persistence in DNA and potential biological importance in AFB1-related research field [1]. Thus, many researchers in the relative fields regard AFB1-FAPy adduct as a validated biomarker of AFB1 exposure [1]. Increasing evidences have exhibited that the amount of AFB1-FAPy adduct in the liver or placenta tissues are lineally correlated with AFB1 exposure levels and HCC risk, suggesting this adduct should be regarded as a toxicological elucidation biomarker of AFB1 [32]. Our previous studies have shown that the amount of AFB1-DNA adducts in the peripheral blood leukocytes were positively and linearly related to HCC cancerous tissue [3]. These data implied that AFB1-DNA adducts in the peripheral blood leukocytes could be regard as a biomarker for AFB1 exposure as well as adducts in the cancerous tissues. Our following studies exhibited more amount of AFB1-DNA adducts in cancerous tissues than in the peripheral blood. Thus, AFB1-DNA adducts in tumor tissues were furthermore analyzed in the present study.

For the mutations of p53 gene, AFB1 mainly induces the transversion of G → T in the third position at codon 249 of this gene, also called hot-spot mutation at the codon 249 (TP53M). The frequency of TP53M is more persistent biomarker and more directly represents genic toxic effects compared with AFB1-DNA adducts [1, 12]. Our study also showed more than 68.8% (384/558) of patients with AFB1-positive HCC had TP53M in the cancerous tissues. Because of the aforementioned reasons, the genic toxic effects of AFB1 exposure were evaluated through the following two biomarkers: AFB1-DNA adducts amount in HCC cancerous tissues and the frequency of TP53M in this study. Our results also show that these two biomarkers reflected AFB1 exposure information and represented the toxicological capacity of AFB1.

4.2. The serum microRNAs and AFB1-induced genic toxicity

MicroRNAs are a type of small noncoding RNAs and can regulate the translation of protein-coding genes through enhancing protein-coding mRNA degradation or repressing translation of protein-coding mRNA. Their dysregulation affects cell proliferation and differentiation, ultimately resulting to a variety of disorders [43–45]. To date, more than 2000 mature microRNAs have been annotated in the official registry (the MicroRNA Registry). Increasing studies have proved that microRNAs have a crucial role in human carcinogenesis, including hepatocarcinogenesis, via acting as oncogenes or tumor suppressor genes [43–45]. Recent evidence has shown that serum microRNAs are remarkably stable and expression patterns may be tissue specific [20, 21, 24, 27, 28, 30, 46, 47]; thus, they may be important potential candidates for carcinogens and corresponding non-invasive cancer testing. It has been hypothesized that serum microRNAs might correlate with toxicity of carcinogen such as AFB1. Therefore, in the present study, we conducted a case-control study to screen and analyze potential serum microRNAs for genic toxicity testing of AFB1 in a high-AFB1 exposure area, Guangxi area of China [4, 48]. Our results proved that serum microRNAs such as miR-4651 and miR-382-5p were significant and linearly associated with the amounts of AFB1-DNA

adducts in the cancerous tissues; moreover, increasing serum levels of these two microRNAs modified the risk of TP53M. Collectively, these results suggest that serum microRNAs might be important biomarkers for predicting genic toxicity of AFB1 and ultimately for preventing HCC induced by AFB1.

In this study, miR-4651 and miR-382-5p were particularly concerned because of its different expression between patients with positive-AFB1 HCC and nontumor controls with positive AFB1. They are encoded by *miR-4651* gene (located at chr7: 75915197–75915269) and miR-382-5p gene (located at chr14: 101520643–101520718), respectively. Until now, it has been not clear whether they act as tumor suppressors or oncogenes. However, we observed that miR-4651 and miR-382-5p had higher expression in the AFB1-positive HCC cases than in non-HCC-harboring individuals, and this increasing expression was further positively associated with poor prognosis of HCC related to AFB1. This implies that they might act as oncogenes or have a similar role of oncogenes through decreasing detoxication and genomic DNA damage repair capacity because the amounts of AFB1-DNA adducts and the frequency of TP53M correlate with HCC risk and prognosis and can reflect the deficiency of detoxication and genomic DNA damage repair capacity. Taken together, these results implied that miR-4651 and miR-382-5p might be useful biomarkers for HCC induced by AFB1 exposure.

This study has several strengths. First, we finished a high-throughput screening analysis for serum microRNAs that exhibited differential levels between cases with positive-AFB1 HCC and healthy controls with positive AFB1. Through this methodology, we not only improved the chance to identify serum biomarkers but also obtained 10 possible AFB1-related microRNAs. Second, only HBV- and HCV-negative cases were included in this study, whereas HBV- or HCV-positive individuals were excluded. This efficiently controlled the effects of other carcinogenetic factors, such as HBV and HCV, and improved correlation analysis of serum microRNAs and genic toxicity of AFB1.

4.3. Limitation

This study had several limitations. First, the increased risk with AFB1 exposure status noted in this study was probably underestimated, because the liver disease itself may affect the metabolism of AFB1 and modify the levels of AFB1-DNA adducts. Second, because the present study is a hospital-based study, potential selection bias might have occurred. Third, in spite of the fact that the status of TP53M was investigated in cases of HCC, other AFB1-related mutations of the TP53 gene were not evaluated. Finally, we did not examine additional functional analysis. Therefore, more functional analyses should be performed based on large samples and a combination of biomarkers and AFB1 exposure.

5. Conclusions

In conclusion, to the best of our knowledge, this is the first report to investigate association between the serum microRNAs and the toxicological effects of AFB1 among Guangxi population from a high AFB1-exposure area. We find that serum levels of miR-4651 and miR-382-5p

might increase the amount of AFB1-DNA adducts and the frequency of TP53M, and their dysregulation should contribute to the toxicological effects of AFB1. Given that AFB1 is an important genic agent and a kind of I type carcinogen, our findings might have prevention implications through identifying population with high serum levels of these two microRNAs, once these findings are replicated by other studies based on a larger scale or prospective studies.

Conflicts of interest and source of funding

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Abbreviations

AFB1	Aflatoxin B1
AFB1-FAPy	8,9-Dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl formamide)-9-hydroxy-AFB1
AFB1-N^7-Gua	(8,9-dihydro-8-N^7-guanyl-9-hydroxy-AFB1)
HCC	Hepatocellular carcinoma
HR	Hazard ratio
miR-4651	MicroRNA-4651
miR-382-5p	MicroRNA-382-5p
OR	Odds ratio
PCR	Polymerase chain reaction
TP53M	The hot-spot mutation at codon 249 of TP53 gene

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