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Estradiol 17 beta-dehydrogenase 5 is involved in aflatoxin B1 transportation

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Abstract: Numerous studies have focused on the mechanism of aflatoxin B1 (AFB1) metabolism and its carcinogenic mechanism, but how AFB1 is transported into hepatocytes and how it is transferred inside hepatocytes remains unknown. In this study, the AFB1-interacting protein, estradiol 17 beta-dehydrogenase 5 (Akr1c6), was identified with an immobilized affinity chromatography technique and LC-MS/MS. The interaction between Akr1c6 and AFB1 was confirmed with ELISA, and the results showed that Akr1c6 could efficiently bind AFB1. Anti-Akr1c6 polyclonal antibody from rabbit was prepared, and the IC_{50} values of AFB1 to BRL (normal big rat liver cells) and NRK (normal rat kidney cells) were detected using the MTT assay. It was found that the Akr1c6 expression level in BRL was significantly affected under the IC_{50} value of AFB1 ($P < 0.05$), but no obvious expression difference of Akr1c6 was observed in NRK. This suggested that Akr1c6 in liver cells participates in the transportation and/or metabolism of AFB1, and though Akr1c6 was expressed in the kidneys, it did not play a role in AFB1 transportation or metabolism. The conclusions of this study lay a foundation for further exploring the role of AFB1 binding proteins in the toxicology of AFB1 to hepatocytes and the pathway through which AFB1 enters hepatocytes and their nuclei.

Key words: Aflatoxin B1-binding protein, estradiol 17 beta-dehydrogenase 5, toxicology of AFB1, hepatocytes

1. Introduction

The discovery of aflatoxins (AFs) was a result of the outbreak of turkey X disease in 1960 (1). AFs are metabolites produced by various *Aspergillus* spp. (including *Aspergillus flavus*). These are difuranocoumarin compounds, including B1, B2, G1, G2, M1, and M2 (2–5). The mycotoxins are known to have strong hepatotoxic and carcinogenic effects and are regulated by feed/food law in at least 100 countries (4). Among AFs, aflatoxin B1 (AFB1), the most common one with higher toxicity than any other aflatoxins, has been classified as a most important known carcinogenic compound (Group 1) by the International Agency for Research on Cancer (6). AFB1 toxicity is activated by biotransformation into the exo-AFB1-8,9-epoxide, which is mediated by cytochrome P450 located predominantly in hepatocytes, making the liver the primary target for AFB1 toxicity (7,8). Epidemiological studies have demonstrated that direct chronic exposure to AFB1 through diet was shown to cause liver cirrhosis, which led to a high rate of hepatocellular carcinoma (HCC) development, and AFB1 interacts synergistically with hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, which increases the risk of HCC (9,10).

For a long time, numerous studies have focused on the mechanism of AFB1 metabolism and the carcinogenic mechanism of AFB1. The DNA mutation mechanism induced by AFB1 and its interaction with HBV has been deeply explored (11–16). The knowledge on how AFB1 enters hepatocytes and whether there is a need for a protein vehicle to transport AFB1 into cells is still very limited (17,18). Iwaki et al. (19) found a cytosolic 25-kDa AFB1-binding protein and speculated that the 25-kDa protein is involved in the intracellular process of AFB1-mediated toxicity in 1983, and a 45-kDa protein was estimated by Dirr and Schabort (20) to be the major AFB1-binding component in 1987. Taggart et al. found that multiple proteins are capable of binding to AFB1 (21). In this study, AFB1-binding proteins were screened with the method of immobilized affinity chromatography technology (IAC) developed by Zhuang et al. in 2016 and identified by LC-MS/MS (22). The biofunctions of the AFB1-binding proteins were further explored in vitro

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(with ELISA) and in vivo (with liver and kidney cell lines). The findings in this research might pave the way for clarifying the biofunction of the AFB1-binding protein in the toxicity mechanism of AFB1 and reveal the pathway by which AFB1 enters into and is transported within hepatocytes.

2. Materials and methods

2.1. Detection and identification of the AFB1-binding protein

The AFB1-binding protein was pulled down by the method of IAC (22). The AFB1-binding proteins on BSA-AFB1-covered PVDF transfer membrane were eluted by precooling 2 M NaCl in phosphate-buffered saline (PBS) buffer. After dialysis in PBS at 4 °C for 2 days and condensation with PEG 20000, the elution solution was further analyzed by LC-MS/MS. Finally, the data were analyzed with UNIPRO (22).

2.2. Bioinformatics analysis

The Akr1c6 protein sequence from 8 species, *Mus musculus* (XP_006516573.1), *Peromyscus maniculatus* (XP_006987897.1), *Meriones unguiculatus* (BAF34659.1), *Colobus angolensis* (XP_011811374.1), *Homo sapiens* (XP_016871280.1), *Pongo abelii* (NP_001124803.1), *Macaca fascicularis* (NP_001270525.1), and *Microcebus murinus* (XP012596672.1), were downloaded from NCBI. A phylogenetic tree was constructed with MEGA 5.1. The protein domain was analyzed and constructed with IBS 1.0. The Akr1c6 proteins were further aligned with Clustal X.

2.3. Cloning, sequencing, and expression of Akr1c6

Polyadenylated (poly(A)) RNA was isolated from livers of Kunming mice using the TRIzol RNA extraction kit (23). First-strand cDNA synthesis was performed using an AMV First Strand cDNA Synthesis Kit (Promega, Beijing, China). The cDNA of Akr1c6 was amplified using the forward primer 5′-TACGAATTCATGGATTCTAAGCAGCAGAC-3′ (*EcoRI*) and reverse primer 5′-GCCAAGCTTCCG TTAGTATTCATCCC-3′ (*HindIII*) according to the sequence from NCBI GenBank (NM_030611). Both the amplified Akr1c6 sequence and expression vector pET-28a(+) were double-digested with *EcoRI* and *HindIII* and then ligated together. The recombinant plasmids were transformed into *E. coli* DH5a cells. After checking by PCR with the primers provided above, four randomly selected clones were sequenced and aligned against the published sequence (NM_030611). The positive recombinant plasmid (pET-28a(+)-akr1c6) was transformed into *E. coli* BL21 cells for expression with 0.2 mM isopropyl-β-D-thiogalactopyranoside.

2.4. Protein purification

Pellets of the induced BL21 with pET-28a(+)-akr1c6 were suspended in buffer D (20 mM Tris (pH 7.9), 5 mM imidazole, and 0.5 M NaCl). After ultrasonic treatment, the mixtures were centrifuged at 15,000 × *g* for 20 min, and the suspension was incubated in a 1-mL Ni-NTA column for 1 h at RT. The Ni-NTA columns were washed with 50 mL of buffer E (20 mM Tris (pH 7.9), 60 mM imidazole, and 0.5 M NaCl) and then incubated with elution buffer F (20 mM Tris (pH 7.9), 1 M imidazole, and 0.5 M NaCl) five times. The collected eluates were dialyzed overnight in PBS at 4 °C for 2 days and concentrated using PEG 8000 (24). Protein samples were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Detection of Akr1c6–AFB1 interaction by ELISA

Wells 1 and 2 of the microplate were coated with bovine serum albumin (BSA) and AFB1, wells 3 and 4 were coated with BSA, wells 5 and 6 were the negative controls (coated with BSA, with no first antibody applied), and wells 7 and 8 were the positive controls (coated with corresponding recombinant protein with His tag: Akr1c6, thioredoxin (TRX), and green fluorescent protein (GFP) protein). Recombinant Akr1c6 was then added to all wells and anti-His antibody was added, except for wells 5 and 6, followed by the addition of 1/8000 diluted goat antimouse IgG horseradish peroxidase antibody into all wells, and wells were held at 37 °C for 60 min. Next, 100 µL of 3,3′,5,5′-tetramethylbenzidine substrate solution was added. The reaction was terminated after 15 min by adding 50 µL of stopping solution (2 M H_2SO_4). Finally, the OD_{450} values of the wells were read with a microplate reader (25).

2.6. Immunization and titer detection of antiserum

Rabbits were immunized with purified Akr1c6 through dorsal subcutaneous injection. The mixture of Akr1c6 and complete Freund's adjuvant was used for the first injection. The later injections were administered with a mixture of Akr1c6 and incomplete Freund's adjuvant 3 times with an interval of 1 week between each injection. Serum titer was detected by enzyme-linked immune sorbent assay (ELISA) (24).

2.7. The expression level of Akr1c6 under the IC_{50} value of AFB1

MTT analysis was employed to detect the IC_{50} (half inhibitory concentration) of AFB1 to BRL (normal big rat liver) and NRK (normal rat kidney) cells. Both cell lines (at a concentration of 10^6/mL) were plated into 96-wells plates (100 µL/well) and exposed to AFB1 in DMSO at different concentrations (10, 20, 30, 40, 50, 60, 70, 80, and 90 µg/mL). Cell viabilities were determined at 490 nm with an MTT cell proliferation and cytotoxicity detection kit (KeyGEN Biotech, Nanjing, China). The IC_{50} values of AFB1 were extrapolated from relative cell viability vs. log [AFB1 concentration] curves (22). The BRL and NRK cell strains were incubated under the IC_{50} value of AFB1 for 24 h. In the control group, cells were inoculated with

DMSO (BRL: 0.61%; NRK: 0.67%). Cells were collected and centrifuged at 1000 × *g* for 5 min. Protein samples were prepared by adding 100 µL of RIPA lysis buffer/well (Beyotime, Haimen, China) into the cell pellet. Western blotting analysis was performed according to the protocol used by Zhuang et al. with minor adjustment, and β-actin was used as inner reference (24).

2.8. Statistical analysis

Data were presented as mean ± standard deviation (SD). The presence of statistical differences was determined by one-way ANOVA, and statistical significance was recognized at $P < 0.05$.

3. Results

3.1. Identification of AFB1-binding protein with LC-MS/MS

AFB1-binding proteins were pulled down by the method of IAC from the total liver proteins. Two different expression protein bands (data not shown) were collected and identified with LC-MS/MS. Estradiol 17 beta-dehydrogenase 5 (Akr1c6) was identified from band A (shown in the Table) and subjected to further analysis.

3.2. Bioinformatics analysis of Akr1c6

The results showed that Akr1c6 from *Peromyscus maniculatus* and *Meriones unguiculatus* had higher similarity with that from *M. musculus* (they are rodents), and the highest similarity was found between *M. musculus* and *M. unguiculatus* (80.8%) as shown in Figure 1a. Lower homology was found between *M. musculus* and the other five nonrodent species, and the lowest similarity was 60.1% between *M. musculus* and *Pongo abelii*. A phylogenetic tree of these species was constructed, in which three rodent species were classified into a cluster, and the other 5 primate species were grouped into another cluster (Figure 1b). The protein domain in Akr1c6 was analyzed with IBS 1.0 and an NADP-dependent oxidoreductase domain was found in all 8 species (Figure 1c).

3.3. Cloning, sequencing, and expression of Akr1c6

Poly(A) RNA was isolated, and cDNA was synthesized by reverse transcription. The target gene *akr1c6* was amplified with the primers shown in Section 2. The PCR product was analyzed by agarose gel electrophoresis and the results showed a 972-bp band (Figure 2a), which indicated that the gene had been amplified. The recombinant plasmid was transformed into competent *E. coli* DH5a cells. After confirmation by PCR analysis, five transcripts were sequenced by Sangon Biotech (Shanghai, China). Three verified pET-28a-*akr1c6* recombined vectors were transferred into *E. coli* BL21 for protein expression. Analysis of the induced *E. coli* BL21 cell pellet by SDS-PAGE electrophoresis indicated a band of ~37 kDa (Figure 2b, lane 3). No corresponding band was found in the control strain (Figure 2b, lane 2). The expression product was purified by Ni^{2+}-NTA, and the buffer E eluent was collected and analyzed by SDS-PAGE. It showed Akr1c6 (~37 kDa) had been purified (Figure 2b, lane 4).

3.4. Interactions between Akr1c6 protein and AFB1 in vitro

The results showed that Akr1c6 specifically bound to AFB1 in vitro (Figure 3a, Akr1c6, wells 1 and 2). The antibodies bound to the His tag on the recombinant Akr1c6 protein but did not bind to BSA (Figure 3a, Akr1c6, wells 3–6). Wells 7 and 8 showed that anti-His antibody efficiently bound to the His tag. The histogram from the ELISA results showed that protein Akr1c6 effectively bound to AFB1 compared to TRX and GFP ($P < 0.05$, Figure 3b).

3.5. Anti-Akr1c6 antiserum preparation

Several microliters of antiserum were collected from the ear vein of rabbits. The results from the ELISA assay showed that the titer of rabbit-1 was about 1/64,000 and that of rabbit-2 was about 1/128,000 (Figure 4). Both fully met the requirements for further Western blotting analysis.

3.6. The expression level of Akr1c6 under the IC_{50} value of AFB1

The MTT assay was employed for the determination of cell viability of rat liver cell line BRL and kidney cell line NRK. IC_{50} values (BRL: 61.2 µg/mL; NRK: 67.1 µg/mL) were calculated from the exponential equations shown in Figures 5a and b. According to the results of Western blotting (Figure 6a), the Akr1c6 level in BRL was significantly downregulated (Figure 6c BRL, $P < 0.05$) under the IC_{50} value of AFB1. For NRK, no obvious difference was observed between the AFB1-treated group and the control group (DMSO-treated).

Table. Identification of AFB1-binding protein.

No.	Accession number	Sequence coverage	Peptide hits	Molecular weight	pI	Score	Protein summary
1	IPI: IPI00111950.1	27.86	5	37.0	8.28	85.07	Estradiol 17 beta-dehydrogenase 5
2	IPI: IPI00474446.4	10.16	2	36.1	5.08	18.79	Eukaryotic translation initiation factor 2 subunit 1

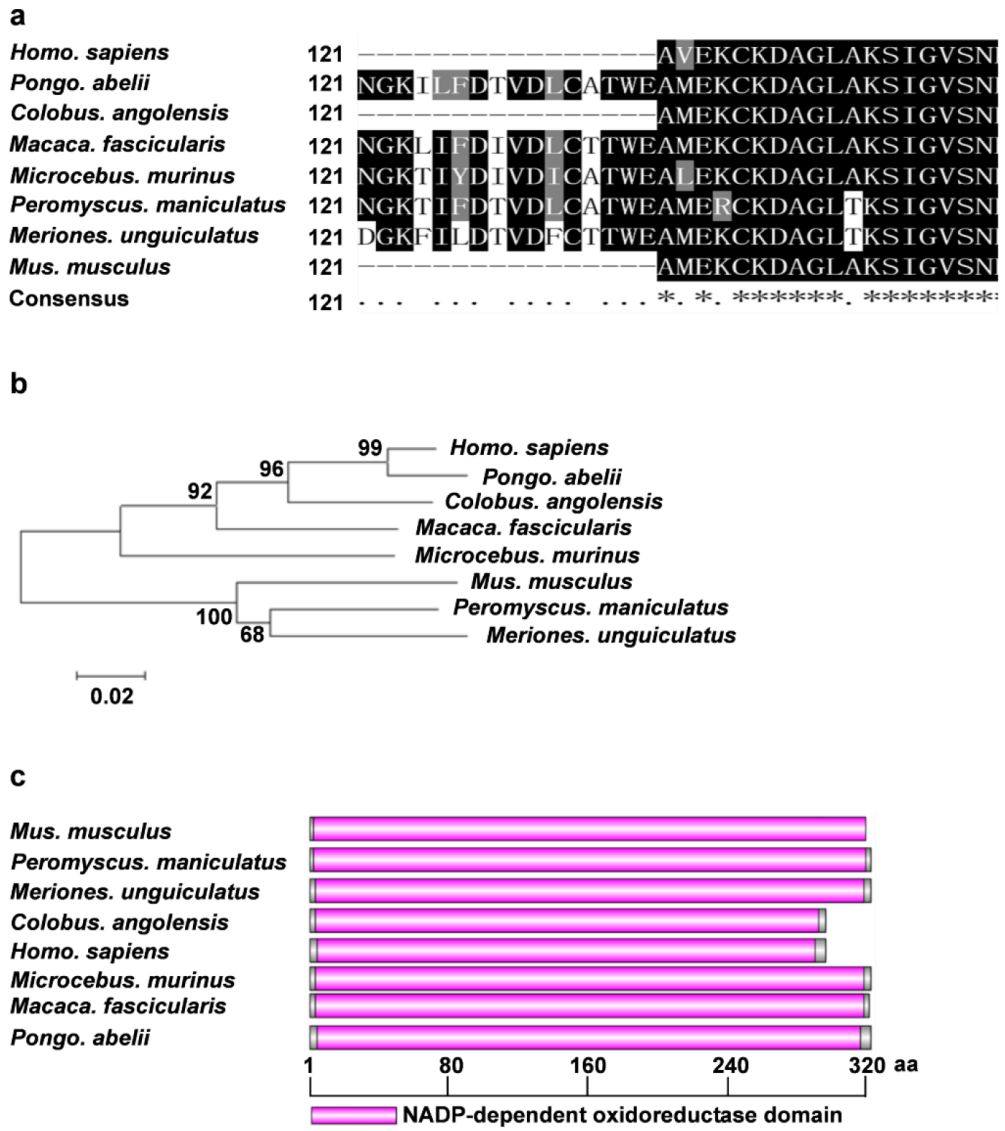

Figure 1. Bioinformatics analysis of Akr1c6. a) Amino acid alignment of *M. musculus* Akr1c6 and putative orthologs from *P. maniculatus*, *M. unguiculatus*, *C. angolensis*, *H. sapiens*, *P. abelii*, *M. fascicularis*, and *M. murinus*. Clustal X was utilized in this analysis. b) Diagram showing the phylogenetic tree of the 8 species above. c) Diagram showing the domains of Akr1c6 in the 8 species above. interPro (http://www.ebi.ac.uk/interpro/scan.html) and IBS 1.0 were used in the analysis.

4. Discussion

The method of IAC was adopted in this study to screen AFB1-binding proteins from mice livers (22). Akr1c6, a member of the aldo-keto reductase family, was identified by LC-MS/MS analysis (Table). The *akr1c6* gene was coded with 1359-bp nucleotide residues, and it encodes the Akr1c6 protein with 323 amino acid residues. Akr1c6 was reported to be mainly expressed in the liver (26). In this study, Akr1c6 was also found in the kidney by western blotting analysis. Bioinformatics analysis in this study showed that the *akr1c6* gene was commonly found among rodent and primate species with high similarity (at least 60.1%), suggesting that *akr1c6* plays a similar and important role in the biological activity of rodents and primates. Enzymes in the aldo-keto reductase family catalyze redox transformations involved in biosynthesis, intermediary metabolism, and detoxification. Substrates of these enzymes include glucose, steroids, glycosylation end products, lipid peroxidation products, and environmental pollutants. Enzymes in this family also play an important role in the phase II detoxification of a large number of pharmaceuticals, drugs, and xenobiotics (27). AKRs are thought to be key AF-detoxifying enzymes; *AKR-7A2* genes were found to be induced in HepG2 cells (28,29).

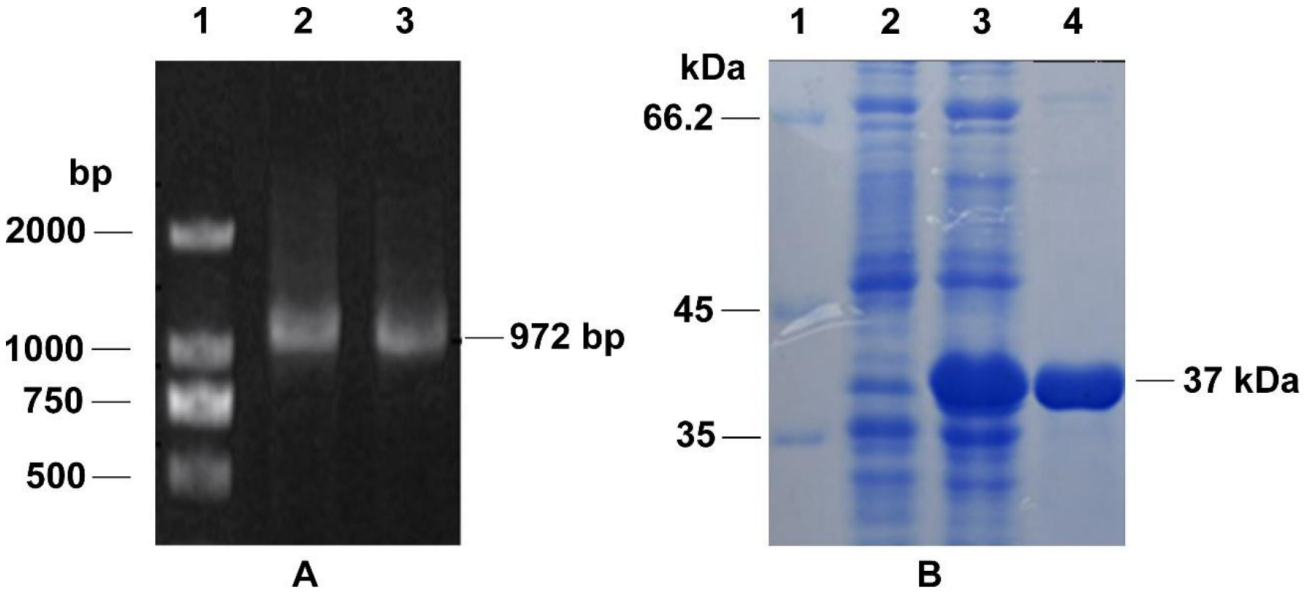

Figure 2. Cloning, sequencing, and expression of Akr1c6. a) Amplification of the *akr1c6* gene fragment from cDNA (1: DL2000 marker; 2 and 3: *akr1c6* gene fragment). b) The SDS-PAGE analysis of Akr1c6 expression and purification (1: Mid-range Protein molecular weight markers; 2: control (empty vector pET-28a); 3: SDS-PAGE of total proteins of recombinant plasmid (pET-28a-*akr1c6*); 4: purified Akr1c6 protein by Ni^{2+}-NTA).

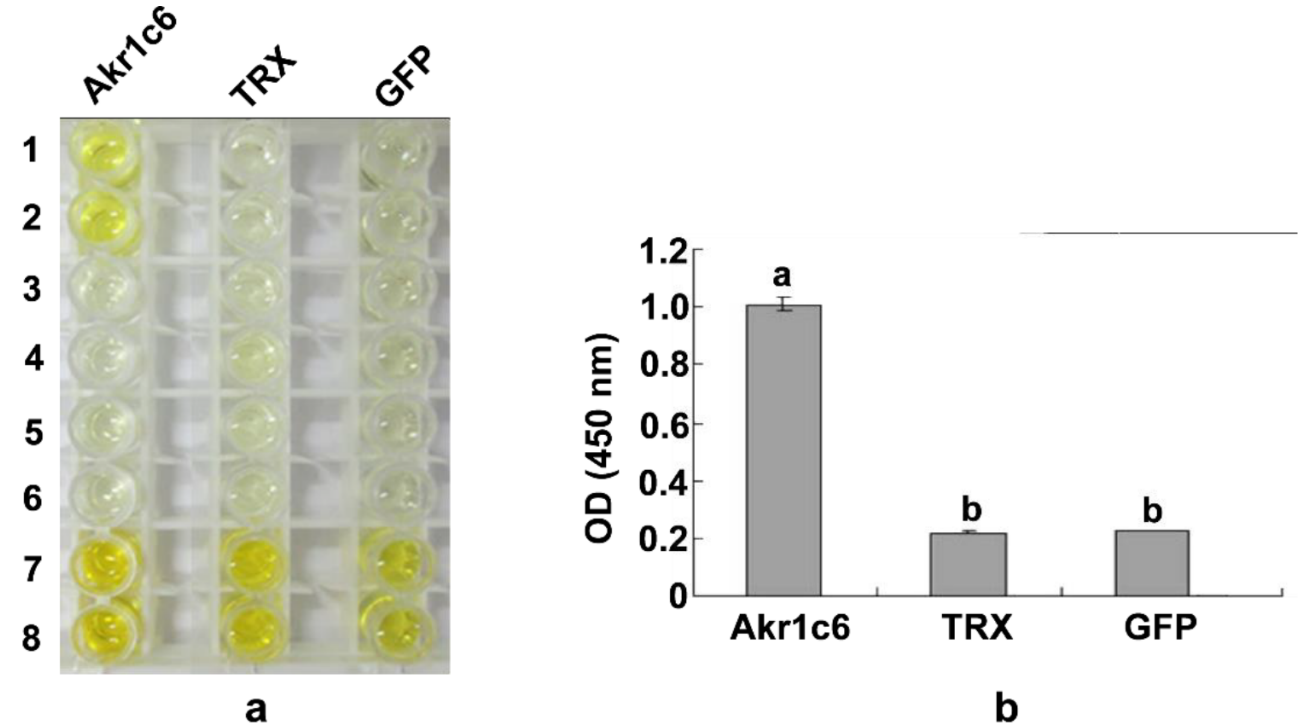

Figure 3. Verification of the interaction between Akr1c6 protein and AFB1 in vitro. a) ELISA analysis of the interaction between AFB1 and Akr1c6. b) Relative levels of the binding interaction. Data are presented as mean ± SD (n = 3, $P < 0.05$).

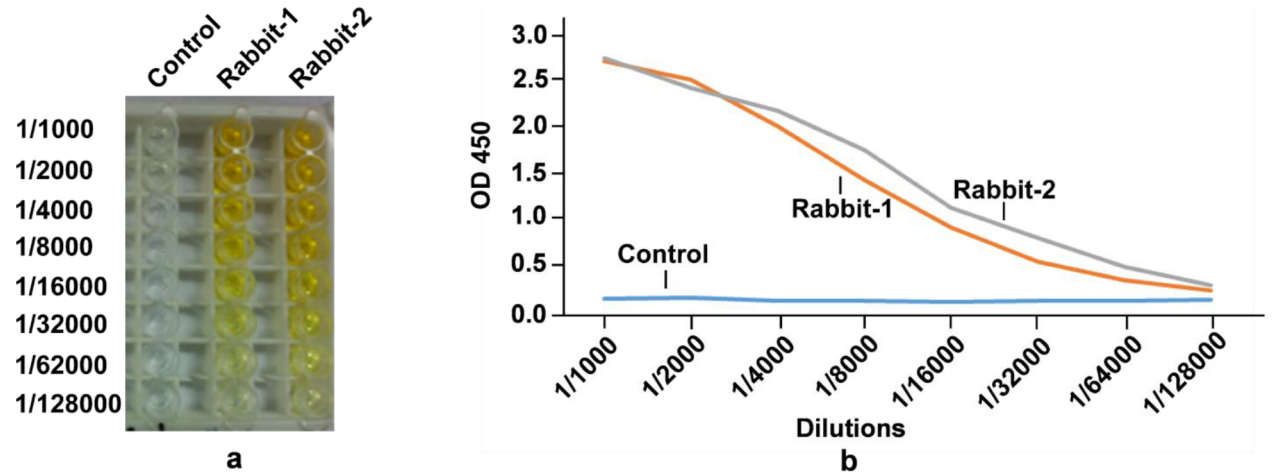

Figure 4. Titer of antiserum from rabbit-1 and rabbit-2. a) The ELISA results for serum from rabbit-1 and 2. b) The curve graph according to the OD_{450} volume of each antiserum dilution (1/1000, 1/2000, 1/4000, 1/8000, 1/16,000, 1/32,000, 1/64,000, and 1/128,000).

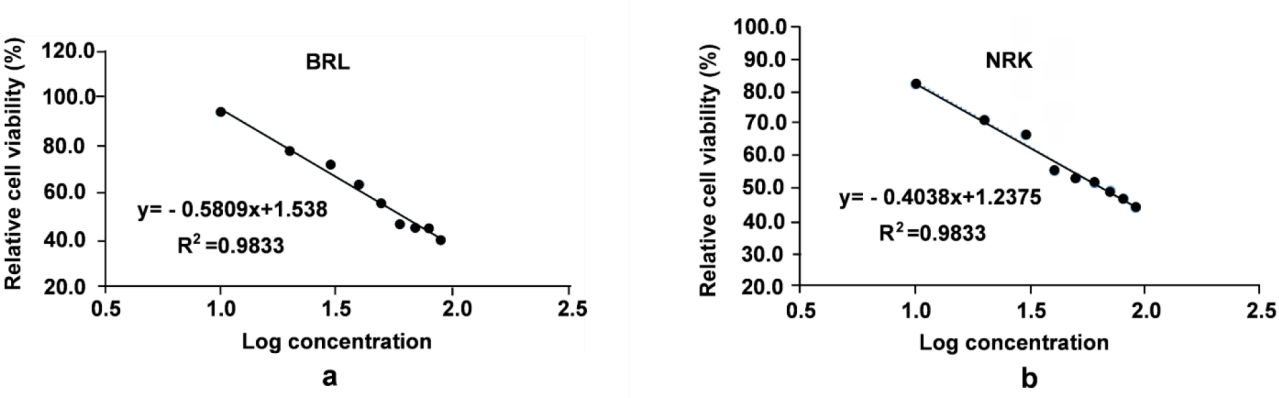

Figure 5. The relative cell viability of cells treated with AFB1. a) The exponential equations according to the results from the MTT assay for the BRL cell strain (4 repetitions). b) The exponential equations according to the results from the MTT assay for the NRK cell strain (4 repetitions).

AFB1 was reported as a hepatotoxic secondary metabolic product (30), and the liver cell strain BRL was chosen in this study to detect if Akr1c6 plays a role in the toxicology of AFB1 to hepatocytes, while kidney cell line NRK was set as a negative control. By preparation of the anti-Akr1c6 polyclonal antibody, the Akr1c6 expression level was found significantly downregulated under the IC_{50} value of AFB1 in the liver cell strain (BRL), but no obvious expression difference of Akr1c6 was found between control and AFB1-treated kidney cells (NRK). The results suggested that Akr1c6 directly participated in the transport and/or metabolism of AFB1 in liver cells, but not in kidney cells (even though some degree of expression was observed in the kidneys). In view of the result from bioinformatics that the human Akr1c6 protein sequence was not in the same cluster of the protein sequence from rats (Figure 1b), it would be worthwhile to repeat our work in human cell lines to confirm the biofunction of the Akr1c6 protein in the process of AFB1 transportation into or between human cells. The results of this study laid a foundation for further exploration of the role of AFB1-binding proteins in the toxicology of AFB1 to hepatocytes and the pathway through which AFB1 enters hepatocytes.

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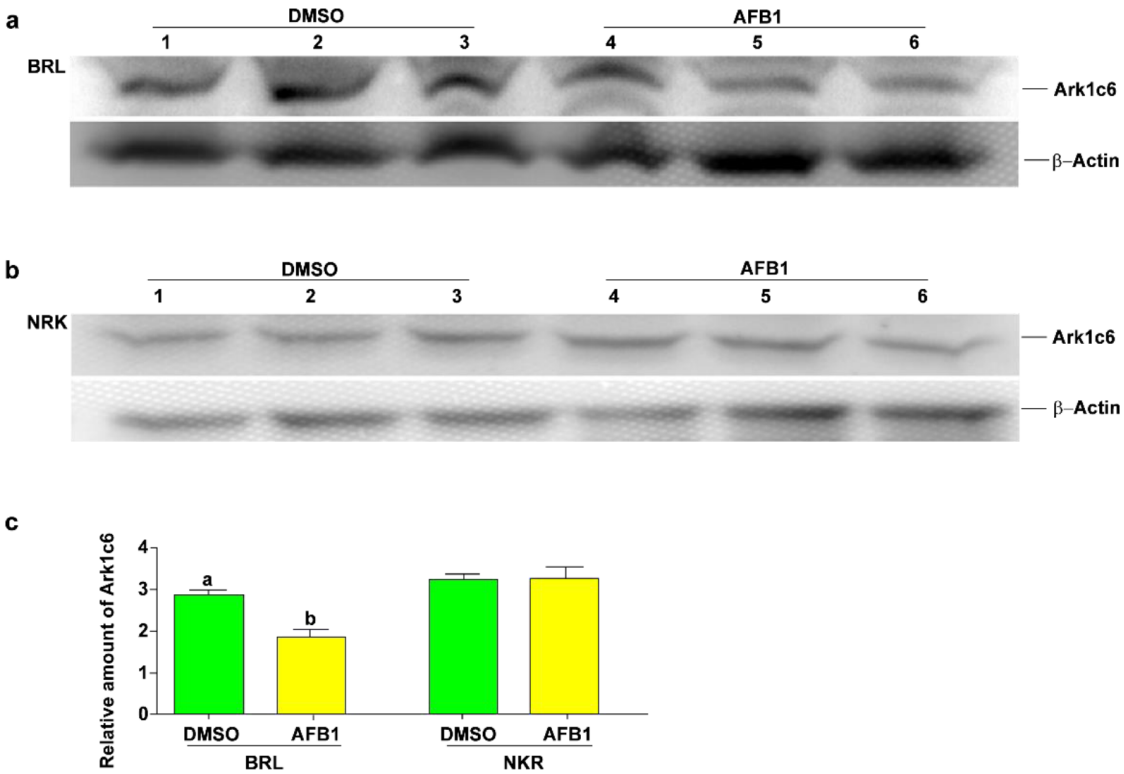

Figure 6. The expression level of Akr1c6 under IC50 concentration of AFB1. a) Detection of Akr1c6 expression level in BRL cell strain by western blotting analysis (3 repetitions). b) Akr1c6 expression level detection in NRK cell strain by western blotting analysis (3 repetitions). c) The relative expression amount of Akr1c6 from BRL and NRK cell strains ($P < 0.05$).

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