Asian Journal of Chemistry; Vol. 26, No. 11 (2014), 3305-3310

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.17515

Study on CPS1: The Key Gene of Urea Cycle under the Stress of Aflatoxin B1

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Received: 7 February 2014; *Accepted*: 12 April 2014; *Published online*: 25 May 2014; AJC-15237

Proteomic analysis of AFB1-stressed mouse liver revealed that the level of carbamoyl-phosphate synthetase 1 (CPS1) protein decreased. The study further explored the role of CPS1 in AFB1-induced liver pathological change in mice. Under AFB1 stress, the amount of blood ammonia was found increased, the expression of CPS1, OTC and SIR5 was found decreased with the increased concentration of AFB1 and CPS1 in Chang liver cell was found down-regulated by fluorescence quantitative PCR. When CPS1 in BEL-7402 cell and Chang liver cell was interfered, respectively, both of the level of OTC and the AFB1 tolerance of cell was found decreased. This study demonstrates the depression of CPS1, OTC and SIR5 genes under AFB1 stress. It inferred that the urea cycle could be interrupted by AFB1 and CPS1 played an important role in the process and that CPS1 and OTC can serve as candidate markers for liver pathological change under AFB1 stress.

Keywords: Aflatoxin B1, CPS1, Urea cycle.

INTRODUCTION

Aflatoxins, the fungal secondary metabolites, were produced by toxigenic fungi (such as *Aspergillus flavus* and *Aspergillus parasiticus*) *via* polyketide biosynthetic pathway. They are hepatotoxic and hepatocarcinogenic compound to human and animals. Aflatoxin B1 (AFB1) is hepatotoxins and it has been showed that AFB1 contaminated food might positively correlate with the number of hepatocelluar carcinoma cases[1]. It was reported that the minimum concentration of AFB1 (10 μg per day) to induce cancer is 75 times as little as that of dimethylnitrosamine (750 μg per day)[2-6]. International Agency for Research on Cancer (IARC) of World Health Organization (WHO) has classified AFB1 as class I carcinogen as slow and daily ingestion of low concentration of AFB1 was considered the potential cause of hepatocarcinoma[7-9]. The function of urea cycle is to transform toxic ammonia into non-toxic urea. After secreted into the blood, urea was detoxic by kidney excretion, which avoid of the occurrence of hyperammonemia. The cycle was discovered by Hanks Kreds and it is the first metabolic cycle discovered[10-12]. Carbamoyl-phosphate synthetase 1 (CPS1) is the starting enzyme and the key enzyme of urea synthetases. As a rate-limiting enzyme in urea synthesis, CPS1 catalyzes the synthesis of carbamoyl phosphate from NH_3 and CO_2 in the presence of ATP, after which urea is produced. As a tissue-specific enzyme of mature hepatocyte, CPS1 plays a role in hepatocyte differentiation[13,14].

Carbamoyl-phosphate synthetase 1 was obviously down-regulated with the increase of AFB1 intake from our previous differential proteomics analysis [15]. The result suggested that CPS1 might play an important role in the process of liver pathological change induced by AFB1. To further elucidate the role of CPS1 played in this process, more experimental methods, including fluorescence quantitative PCR and RNAi, were implemented in the study, which would lay a solid foundation to clarify the function of CPS1 in the process of liver pathological change under AFB1 stimulation.

EXPERIMENTAL

Reverse transcription kit was purchased from Promega. Trizol extraction kit was purchased from Invitrogen. Real-time quantitative PCR kit, restriction enzymes, T4 DNA ligase and isopropyl-thiogalactopyranoside (IPTG) were purchased from TaKaRa. DNA gel extraction kit and endo-free plasmid mini kit were purchased from OMEGA. The other chemical reagents used were of analytical grade. Ni^{2+}-NTA resin was purchased from GE Healthcare. The primers synthesis and sequence analysis were carried out by Shanghai Sangon Co., Ltd.

Male Kunming mice (20-25 g per mouse) were purchased from Fujian Medical University and all animal studies complied

with Chinese law and regulation for animal protection and administration. Every two mice were fed in one cage at room temperature (about 25 ºC). *E. coli* BL21 (DE3) and DH5α were preserved by our lab. Plasmid vector pET28a (+) and PGPU6/GFP/Neo were also preserved by our lab.

Treatment of mice: After a week's feeding, the first 36 Kunming mice were divided into 6 groups (6 mice/group) for blood ammonia determination. The first to fifth group were injected intraperitoneally with 800 µL AFB1 solution (65 µg/mL AFB1 in 10 % methanol solution) every day for 2, 4, 6, 8 and 10 weeks, respectively; the mice in sixth group were injected intraperitoneally with 800 µL 10 % methanol solution every day for 10 weeks.

In the second set, 18 Kunming mice were divided into 3 groups evenly for fluorescence quantitative PCR analysis. The first and second group was injected intraperitonally with 800 µL AFB1 solution (65 µg/mL AFB1 in 10 % methanol solution) every day for 10 weeks, respectively and the third groups were control and they were injected intraperitonally with 800 µL 10 % methanol solution every day for 10 weeks.

Determination of ammonia in blood: The method of detection for ammonia concentration in blood using ultraviolet spectrophotometry was standardized. A series of standard ammonium sulfate solution (0, 10, 20, 50, 100, 150, 200 and 400 µmol/L) was prepared; 1 mL of each standard solution was mixed with 0.5 mL of 10 % sodium tungstate and 0.5 mol/L sulfuric acid solution, respectively, the mixtures were vortexed for 1 min and centrifuged at 4000 g/min for 5 min. After centrifugation, 1 mL phenol colour develop reagent and 1 mL alkaline sodium hypochlorite solution were added into 1 mL supernatant, respectively. Then, the mixtures were rotated with vortex for 1 min and kept in 37 ºC water bath for 20 min. The OD was measured at 630nmin (HITACHI U-1100 Spectrophotometer). The standard graph was prepared and samples were analyzed for the concentration of ammonia. Mice were bleeded from eye-pit at corresponding time point (at the 2nd, 4th, 6th, 8th and 10th week after AFB1 treatment). To check the concentration of ammonia in blood, 50 µL serum was mixed with 0.5 mL 10 % sodium tungstate solution and 0.5 mol/L sulfuric acid solution, the mixture was developed by phenol colour develop reagent and alkaline sodium hypochlorite solution. Finally, the concentration of blood ammonia was acquired.

RNA extraction and synthesis of the first strand of cDNA: The RNA from the mice liver was separated according to the protocol of trizol method and the extracted RNA was stored at -80 ºC[16]. Single-stranded cDNA was synthesized following the two-step protocol with the reverse transcription kit of Promega and the product was kept at -20 ºC[16].

Fluorescence quantitative PCR: According to the manual of real-time quantitative PCR kit, the primers of CPS1, OTC (ornithine transcarbamylase), SIRT5 (Sirtuin) and gapdh (control) were designed with the assistant of the software Premier 5.0 and they were shown in Table-1. Reagents preparation for quantitative PCR (total volume: 20 µL) was show as below: ddH_2O 7.2 µL, 2×EXTaq mix 10.0 µL, 0.4 µL of sense and antisense primers, respectively and 2 µL template which was 2-, 5- and 10-fold diluted, respectively by sterile water. The PCR reactions were performed at 95 ºC for 30 s, followed by 40 cycles of 5 s at 95 ºC and 34 s at 64 ºC, ended at 1 cycle of 15 s at 95 ºC, 60 s at 64 ºC and 15 s at 95 ºC. Melting curve from 64-95 ºC was read every 0.2 ºC for 2 s. The housekeeping gene, gapdh, was chosen as reference to normalize the test gene CPS1, OTC and SIRT5. ABI 7500 fluorescence ration PCR instrument used in the study carried inbuilt ΔΔCt algorithm and the expression differences of CPS1, OTC and SIRT5 were determined by calculation with relative quantitative, respectively.

Effect of AFB1 on CPS1 expression in Chang liver cell line: The total RNA of Chang liver cells were extracted at 12, 24, 36 and 48 h after cultured in a medium with 5 µg/mL AFB1 and were reverse transcribed into cDNA, respectively. The changing trend of CPS1 in transcription level was detected by fluorescence quantitative PCR (Sense: 52'-TTGGCAAGAAGACGGTG-32'; antisense: 52'-GCTTGTGCCCATGATCTT-32'), in which β-actin was chosen as internal reference and ΔΔCt algorithm was used to calculate the relative expression level of CPS1.

Construction of RNA interference vector: Three pairs of double-stranded oligonucleotides (DNA Oligo) which encoded short hairpin RNA (shRNA) was designed with shRNA design software provided by Invitrogn. The complementary sequences were two inverted repeat sequences, in the both ends of which the restriction enzyme sites, BamH I and Hind III, were designed to facilitate the ligation with PGPU6/GFP/Neo vector. Three pairs of double-stranded oligonucleotides were shown in Table-2. After the ligation reaction between double-digested pGPU6/GFP/Neo and phosphated Oligo DNA, the resulting recombinant plasmid was transferred into *E. coli* DH5α and checked by single-digestion (EcoR I or Hind III). Then, the confirmed recombinant was sequenced by Shanghai Sangon Co.

Cells transfection by RNA interference vector: The recombinant plasmid was extracted according to the instruction provided by the Endotoxin-free Mini Plasmid Prep Kit instruction. The recombinant plasmid and pGPU6/GFP/Neo (negative control) was transferred into the human normal liver cell line Chang liver and the carcinoma cell line BEL-7402 by liposome (Lipofectamine 2000), respectively[17,18]. After half month screening by G418 (selective antibiotic), only those

TABLE-1
PRIMERS USED IN THE STUDY OF FLUORESCENCE QUANTITATIVE PCR

Primers	Sequences	Temp. (°C)	Length (bp)	Genes
CPS1 sense	5'- TTGGCAAGAAGACAGTGGTG -3'	64	225	CPS1
Antisense	5'- GAGGGCTTGTACCCATGATC -3'	64		
OTC sense	5'- GCTCAACAATGCAGCTCTTAG -3'	64	267	OTC
Antisense	5'-GTGGACAGTCTTGTTCGAGTAC-3'	64		
SIRT5 sense	5'- TCATTTCCCAGTTGTGTTGTAG -3'	64	228	SIRT5
Antisense	5'- TGCCATTTTCTCCAGTAACCT -3'	64		

TABLE-2
SEQUENCE OF THREE PAIRS OF DNA OLIGO

Name of shRNA	Sequence of shRNA
Shcps447- sense	5'-gATCCgCTggCTACCAAgAgTTTAggCgAACCTAAACTCTTggTAgCCAgCA -3'
Shcps447-antisense	5'-AgCTTgCTggCTACCAAgAgTTTAggTTCgCCTAAACTCTTggTAgCCAgCg -3'
Shcps2122- sense	5'- gATCCgCCCTTCATCCTACCTCAATgCgAACATTgAggTAggATgAAgggCA -3'
Shcps2122-antisene	5'- AgCTTgCCCTTCATCCTACCTCAATgTTCgCATTgAggTAggATgAAgggCg -3'
Shcps3395-sense	5'-gATCCgCTTgTTgAggCCTTCCTATgCgAACATAggAAggCCTCAACAAgCA -3'
Shcps3395-antisene	5'- AgCTTgCTTgTTgAggCCTTCCTATgTTCgCATAggAAggCCTCAACAAgCg -3

Three pairs of double-stranded oligonucleotides (Shcps447- sense and Shcps447-antisense, Shcps2122- sense and Shcps2122-antisene, Shcps3395-sense and Shcps3395-antisene) in which short hairpin RNA (shRNA) was encoded.

cell with the recombinant plasmid could survive. The total RNA was extracted after Chang liver cell line with different kinds of RNA interference vector (empty vecter pGPU6/GFP/Neo, pGPU6/GFP/Neo-ShRNA (Shcps447), pGPU6/GFP/Neo-ShRNA (Shcps2122), pGPU6/GFP/Neo-ShRNA (Shcps 3395)) were digested by trypsin from 96-well microtiter plate, respectively. Single strand cDNA was amplified according to the method provided above. Primers of CPS1 for detection of RNAi interference were synthesized according to NCBI (sense: 52-TTGGCAAGAAGACGGTG-32, antisense: 52-GCTTGTGCCCATGATCTT-32'). The protocol and data analysis for quantitative PCR referred to the method provided above. β-Actin was chosen as reference to correct the RNA volume of CPS1.

Effect of RNAi to BEL-7402 and Chang liver cell lines by MTT assay: To detect the tolerance of cell against AFB1, the standard AFB1 (from Sigma) was dissolved into glycerol and diluted by 1640 incomplete medium to different concentration of 1, 2, 4, 8 and 16 μg/mL. The concentration of different cell lines (BEL7402 cell line, BEL7402 cell line with empty vector, BEL7402 cell line with CPS1 RNAi interference vector, Chang liver cell line, Chang liver cell line with empty vector and Chang liver cell line with CPS1 RNAi interference vector) in logarithmic growth phase was adjusted to 5×10^{-4} mL, respectively. The lg IC_{50} of different cell lines was detected by the instruction of MTT assay kit.

Western blot analysis: The method of cloning, expression, purification and polyclonal antibody preparation for OTC were referring to that of previous description[16,19]. The concentration of the protein solution was detected by the method of BCA (bicinchoninic acid). Western-blot was performed as previously described[20].

RESULTS AND DISCUSSION

Expression level of CPS1, OTC and SIR5 in mice liver: After 10 weeks treatment of AFB1, the livers from the mice were homogenized, respectively and the total RNA was abstracted by the method of Trizol. The first strand cDNA was synthesized according to previous discussion. After the solution for fluorescence quantitative PCR was prepared, CPS1, OTC, SIR5 and GAPDH were amplified. The amplification curve of CPS1, OTC, SIR5 showed that the specificity of primers and their amplification efficiency and the reaction system were in line with the requirement of fluorescence quantitative PCR. The in-build software of relative quantification in the ABI 7500 PCR instrument was used to calculate the expression volume of these genes after the treatment of AFB1 for 10 weeks and the volume of CPS1, SIR5 and OTC were corrected with the volume of GAPDH (the internal reference), respectively. The result showed in Fig. 1 (A, B and C) indicated that the expression volume of CPS1, SIR5 and OTC all had been down-regulated significantly after 10 weeks' treatment of AFB1 and it was consistent with the result of our previous differential proteomics analysis.

Determination of mice blood ammonia: According to materials and methods, the equation of linear regression (y = 0.0064x + 0.0181) was deduced by linear regression process through the emendated values of optical density 630 against the corresponding concentration of ammonium sulfate solution. The correlation coefficient for the equation was greater than 0.99 (R^2 = 0.9968). Mice were bleeded at 2, 4, 6, 8 and 10 week, respectively after AFB1 treatment, the concentration of blood ammonia was detected. After the value of optical density 630 was substituted into the equation of linear regression (y =

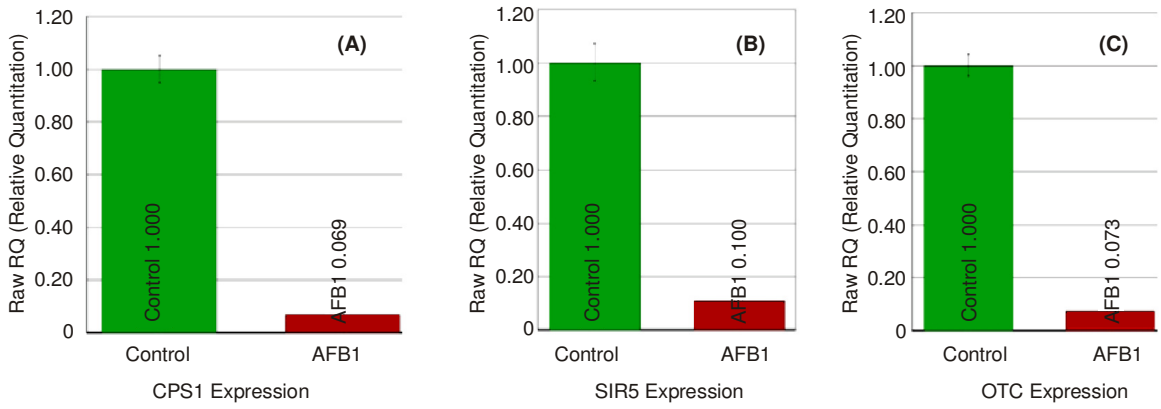

Fig. 1. Changing trend of CPS1, OTC and SIR5 gene under AFB1 stress. (a) The changing trend of CPS1. (b) The changing trend of SIR5. (c) The changing trend of OTC

0.0064x + 0.0181), the blood ammonia concentration was measured using the developed protocol. The histogram (Fig. 2) was established when the blood ammonia concentration of test groups divided by that of control group and the result showed that blood ammonia concentration was increased as the elongation of AFB1 treatment time.

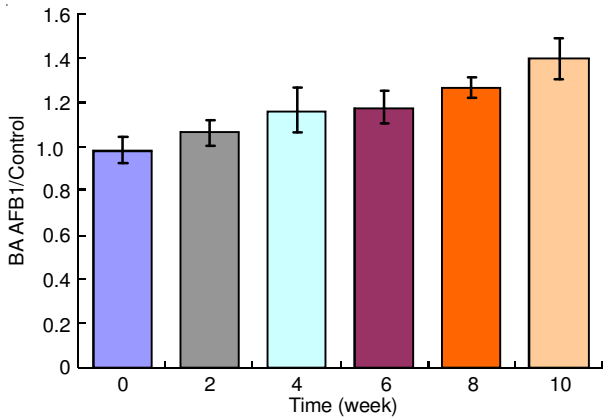

Fig. 2. Relative concentration of blood ammonia after AFB1 treatment

Restraining effect of AFB1 on the expression level of CPS1 in Chang liver cell line: After cultivated in 5 μg/mL AFB1 contained medium for 12, 24, 36 and 48 h, the Chang liver cells were used to extract total RNA and then reverse PCR was carried out. The changing trend of CPS1 transcription level was tracked by fluorescence quantitative PCR with ΔΔCt algorithm. The result showed that the transcription level of CPS1 was also reducing over time under the stress of AFB1 (Fig. 3). Combined to the result above, it was indicated that transcription level of CPS1 was decreased under the stress of AFB1.

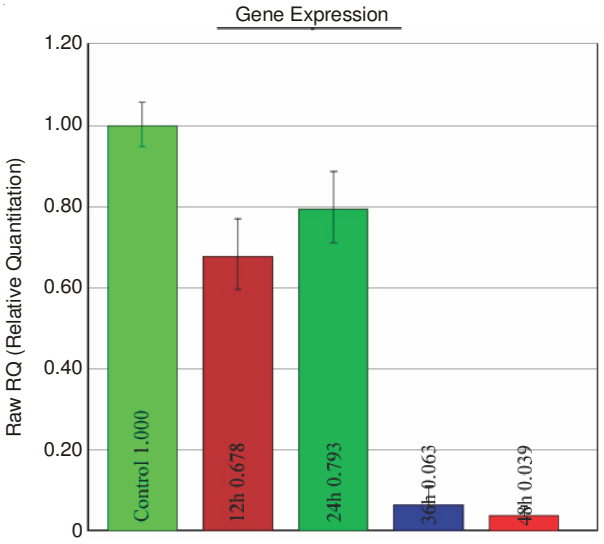

Fig. 3. Relative quantification analysis for CPS1 transcription level in Chang liver cell

Role of CPS1 played in the tolerance of cell against AFB1: The interference plasmid pGPU6/GFP/Neo-ShRNA was successfully constructed and the inbuilt ΔΔCt algorithm in ABI 7500 fluorescence ration PCR instrument was used to analyze the expression volume of CPS1 under the influence of three pairs of ShRNA, respectively. The result was shown in Fig. 4, suggesting that the 2nd DNA Oligo had a strong interference effect on expression level of CPS1. So the 2nd DNA Oligo (Shcps2122) was selected to be used in the further study.

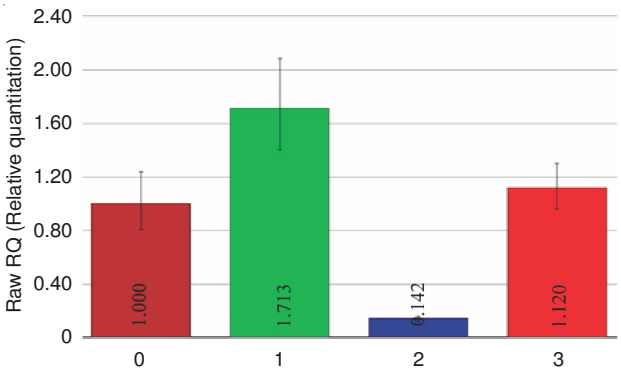

Fig. 4. RNAi interference effect analysis for three pair of DNA Oligo. 0: The empty vecter pGPU6/GFP/Neo; 1: The recombinant plasmid-pGPU6/GFP/Neo-ShRNA (Shcps447); 2: The recombinant plasmid-pGPU6/GFP/Neo-ShRNA(Shcps2122); 3: The recombinant plasmid -pGPU6/GFP/Neo-ShRNA(Shcps3395)

Chang liver and BEL-7402 cell lines were transfected by the RNAi interference vector, PGPU6/GFP/Neo-shRNA (Shcps2122) and the cells without the RNAi interference vector and with empty vector (PGPU6/GFP/Neo-shRNA) were set as controls. The proliferation activity of CPS1 interfered cells under AFB1 stress was detected by colourimetric MTT assay and the results in Fig. 5 (A and B) showed that the mortality rate of CPS1 interfered BEL-7402 and Chang liver cell line reached about 60 and 69 % under AFB1 stress, respectively, but there was no obvious changes observed in the mortality rate of cells without the RNAi interference vector and with empty vector. These results manifested that cell tolerance against AFB1 reduced when the gene CPS1 gene was interfered. Study on the changing trend of cps1-associated proteins in CPS1 interfered Chang liver cells by Western blotting analysis.

As the second critical enzyme in urea cycle, ornithine carbamoyltransferase (OTC) is a crucial important enzyme for cellular ammonium secretion (in the form of urea) and amino acid catabolism[21]. So it is crucial to monitor the expression level of OTC when CPS1 was interfered. For protein expression and purification, the positive recombinant plasmids (pET-28a-OTC) and empty vector (pET-28a) were transformed into *E. coli* BL21 and induced by 0.2 mM IPTG (isopropyl-b-d-thiogalactopyranoside) at 37 ºC for 3 h when the OD600 of the bacterial culture reached 0.5. The protein product was purified by Ni-NTA resin column, respectively. OTC (42 kDa) was over expressed and successfully purified (Data not shown). Then, the antiserum of OTC from Bal/c mice was prepared and the titers of antisera were obtained (1:64000). The pooled antiserum was stored at -80 ºC for further use.

The total protein of three kinds of Chang liver cell lines, including cells without interference vector, cells with empty vector (PGPU6/GFP/Neo) and cells with interference vector (PGPU6/GFP/Neo-shRNA (Shcps2122)), was extracted, respectively. Then, the expression trend of OTC gene was detected by Western-blotting with prepared anti OTC polyclonal antibody. The result showed that the expression level of OTC gene were down-regulated when CPS1 gene was interfered (Fig. 6).

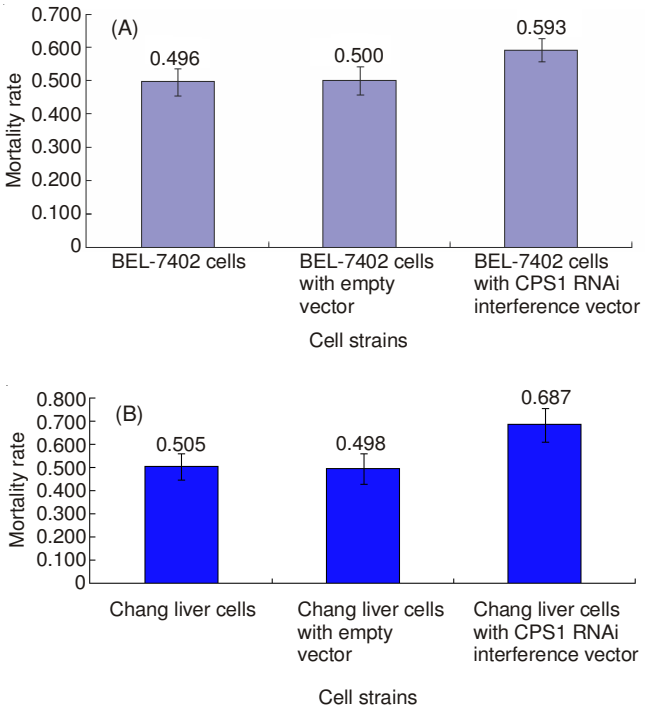

Fig. 5. Mortality of cell strains under the IC_{50} dose of AFB1 in 24 h. (A) The mortality of BEL7402, BEL7402 cells with empty vector and BEL7402 cells with CPS1 RNAi interference vector under the IC50 (4.26 μg/mL) of AFB1 in 24 h. (B) The mortality of Chang liver, Chang liver with empty vector and Chang liver cells with CPS1 RNAi interference vector under the IC50 (9.54 μg/mL) of AFB1 in 24 h. The mortality rate of CPS1 interfered BEL-7402 cells and Chang liver cells were about 60 % (59.8 %) and 69 % (68.7 %) under the IC50 of AFB1, respectively

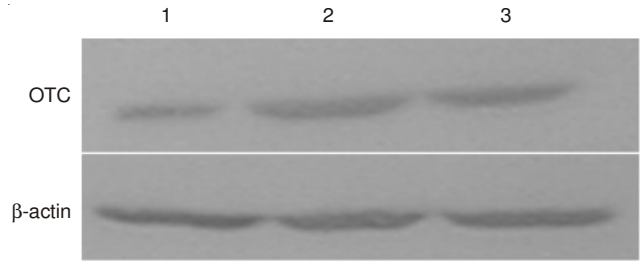

Fig. 6. Western blot analysis of OTC expression when CPS1 gene was interfered. 1: the total proteins of Chang liver cells transfected by interference vector (PGPU6/GFP/Neo-shRNA); 2: the total proteins of Chang liver cells transfected by empty vector (PGPU6/GFP/Neo); 3: Negative control (the total proteins of Chang liver without RNAi interference vector)

Conclusion

The protein CPS1, a critical enzyme in urea cycle, was found to be down-regulated under AFB1 stress in previous differential proteomics analysis[15]. To verify this result of differential proteomics analysis, fluorescence quantitative PCR was implemented in individual level (RNA extracted from liver of mice) and cell level (RNA extracted from Chang liver cells) and the results showed that the transcription level of CPS1 was down-regulated when treated with AFB1, which coincided with our previous result (Figs. 1,A and 3). It meant that CPS1 was down-regulated in both protein and mRNA level under the stress of AFB1 in individual level (mice liver) and cell level (Chang liver cell line).

In the study, it was found that mice blood ammonia concentration was increasing over time under the AFB1 stress, which suggested that mice urea cycle was interfered by AFB1 and the metabolism of ammonia was impacted (Fig. 2) To further clarify the hypothesis, the transcription level of OTC, an important gene in urea cycle[22], was also tracked by fluorescence quantitative PCR, the result indicated that the mRNA volume of OTC was significantly reduced (Fig. 1,C), which meant that the transcription level of two important genes in urea cycle, CPS1 and OTC, were obviously down-regulated under AFB1 stress. So mice urea cycle was interfered by AFB1, which played an important role in ammonia retention in mice blood. From the fact that CPS1 was down-regulated in gene transcription level, protein translation level, it was concluded that CPS1 might play a critical role in the increase of mice blood ammonia concentration under AFB1 stress.

It was found by Western-blotting analysis that when CPS1 was interfered, the expression level of OTC gene was down-regulated (Fig. 6). OTC gene is the second critical enzyme in urea cycle[21]. In the study, it had been found that under the stress of AFB1 the transcriptional level of OTC reduced (Fig. 1,C), so it could be concluded that OTC was down-regulated in transcriptional level by AFB1 and that it was down-regulated when CPS1 was interfered. It was also reported that the activity of CPS1 was up-regulated only when it was modified by SIR5 through deacetylation, so mice blood ammonia concentration would increase when SIR5 was interfered[23]. In the study, Gene SIR5 was detected down-regulated in the study by fluorescence quantitative PCR under the stimulation of AFB1 (Fig. 1, B). It was found in the study that AFB1 could disturb the urea cycle through CPS1, OTC and SIR5 in all levels and there were complicated interaction among these genes and their production under AFB1 stress. So the relationship between the toxic mechanism of AFB1 and urea cycle need to be further exploration.

AFB1 is a strong inducer of liver cancer, but urea (the production of urea cycle) had ever been used as one of the drugs for cancer treatment[24-27]. The relationship between down-regulation of CPS1 or, more exactly, urea cycle disorder by AFB1 and the mechanism of liver pathological change occurrence under AFB1 stress is worthy of further study. The IC_{50} of Chang liver cell line (normal liver cell) and BEL-7402 cell line (cancer cells) was detected under the stress of AFB1. When CPS1 in Chang liver and BEL-7402 cell lines was interfered, the mortality rates of both kinds of cells under IC_{50} doses of AFB1 (Chang liver cell line, 9.54 μg/mL; BEL-7402 cell line, 4.26 μg/mL) obviously increased (Chang liver cell line, about 69 %; BEL-7402 cell line, about 60 %) (Fig. 5). It was inferred from the results that the tolerance of cells against AFB1 reduced when urea cycle was interrupted.

As reported by Butler *et al.*[28], CPS1 may serve as a marker of liver cancer differentiation and it was associated with initiation of liver cancer and the function of urea cycle. The study explored the biological function of CPS1, which laid a solid foundation to clarify the role of CPS1 played in the urea cycle dysfunction under AFB1 stimulation. It would be worthy to elucidate the role of CPS1 played in the development of hepatocellular carcinoma under AFB1 stress.

ACKNOWLEDGEMENTS

The authors sincerely appreciated the support from the National Natural Science Fund Project (31000961, 31172297), the Nature Science Foundation of Fujian Province (2010J01068, 2009J06008), Program for New Century Excellent Talents in University (NCET-10-0010).

REFERENCES

1. P. Tao, L. Zhi-Ming, L. Tang-Wei, L. Le-Qun, P. Min-Hao, Q. Xue, Y. Lu-Nam, L. Ren-Xiang, W. Zong-Liang, W. Lian-Wen, W. Qiao, S. Han-Ming, O. Choon-Nam and R.M. Santella, *Dig. Dis. Sci*., **50**, 525 (2005).
2. A. Chanda, L. Roze, A. Pastor, M. Frame and J.E. Linz, *J. Microbiol. Methods*, **78**, 28 (2009).
3. G. Leema, J. Kaliamurthy, P. Geraldine and P.A. Thomas, *Mol. Vis*., **16**, 843 (2010).
4. C.P. Wild and Y.Y. Gong, *Carcinogenesis*, **31**, 71 (2010).
5. G.N. Wogan, *Bacteriol. Rev*., **30**, 460 (1966).
6. Y. Yin, L. Yan, J. Jiang and Z. Ma, *J. Zhejiang Univ. Sci. B*, **9**, 787 (2008).
7. IARC, *Carcinogen Risk Hum*., **82**, 1 (2002).
8. C.P. Wild and R.A. Montesano, *Cancer Lett*., **286**, 22 (2009).
9. J.H. Williams, T.D. Phillips, P.E. Jolly, J.K. Stiles, C.M. Jolly and D. Aggarwal, *Am. J. Clin. Nutr*., **80**, 1106 (2004).
10. J.L. Deignan, S.D. Cederbaum and W.W. Grody, *Mol. Genet. Metab*., **93**, 7 (2008).
11. F.L. Holmes, *Federation Proc.*, **39**, 216 (1980).
12. M. Takiguchi, T. Matsubasa, Y. Amaya and M. Mori, *Bioessays*, **10**, 163 (1989).
13. V. Gouon-Evans, L. Boussemart, P. Gadue, D. Nierhoff, C.I. Koehler, A. Kubo, D.A. Shafritz and G. Keller, *Nat. Biotechnol*., **24**, 1402 (2006).
14. S.J. Wu and S.E. Li, *Sci. Sin. [B]*, **31**, 590 (1988).
15. Z.H. Zhuang, Y.Y. Huang, F. Zhang, Y.Y. Li, J. Yuan, Y.L. Yang, L. Lin and S.H. Wang, *Chin. J. Trop. Crops*, **31**, 2072 (2010).
16. R. Zhang, W.X. Ma and X.Q. Lu, *Adv. Mater. Res.*, **346**, 236 (2011).
17. B.I. Okeleye, N.T. Mkwetshana and R.N. Ndip, *Scientific World J.*, **1** (2013).
18. X. Zhong, M. Xiong, X. Meng and R. Gong, *J. Exp. Clin. Cancer Res*., **29**, 115 (2010).
19. Z.H. Zhuang, X.L. Zhao, H. Li, S.Y. Wang and X.X. Peng, *Fish Shellfish Immunol*., **31**, 217 (2011a).
20. Z.H. Zhuang, H. Li, J.N. Yang, X. Liu, Y.-Y. Gao, Q.-F. Li, S.-Y. Wang and X.-X. Peng, *Fish Shellfish Immunol*., **30**, 700 (2011).
21. S.C. Cunningham, A. Spinoulas, K.H. Carpenter, B. Wilcken, P.W. Kuchel and I.E. Alexander, *Mol. Ther*., **17**, 1340 (2009).
22. Z. Ben-Ari, A. Dalal, A. Morry, S. Pitlik, P. Zinger, J. Cohen, I. Fattal, R. Galili-Mosberg, D. Tessler, R.G. Baruch, J.M. Nuoffer, C.R. Largiader and H. Mandel, *J. Hepatol*., **52**, 292 (2010).
23. T. Nakagawa, D.J. Lomb, M.C. Haigis and L. Guarente, *Cell*, **137**, 560 (2009).
24. P.I. Clark, M.L. Slevin, J.A. Webb, R.J. Osborne, S. Jones and P.F. Wrigley, *Br. J. Cancer*, **57**, 317 (1988).
25. E.D. Danopoulos and I.E. Danopoulou, *Clin. Oncol*., **7**, 281 (1981).
26. G.M. Gandhi, S.R. Anasuya, P. Kawathekar, Bhaskarmall and K.R. Krishnamurthy, *J. Surg. Oncol*., **9**, 139 (1977).
27. T.L. Hooper, M. Rahman and J. Magell, *Clin. Oncol*., **10**, 341 (1984).
28. S.L. Butler, H. Dong, D. Cardona, M. Jia, R. Zheng, H. Zhu, J.M. Crawford and C. Liu, *Lab. Invest*., **88**, 78 (2008).