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Evaluation of hepatitis B virus replication and proteomic analysis of HepG2.2.15 cell line after cyclosporine A treatment[1]

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Key words

hepatitis B virus; cyclosporine A; replication; proteomics

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Abstract

Aim: The effect of cyclosporine A (CsA) on hepatitis B virus (HBV) replication was investigated, and proteomics expression differentiation after CsA treatment was studied in order to provide clues to explore the effect of CsA on HBV replication. **Methods:** Methyl thiazolyl tetrazolium (MTT) assay was used to evaluate the cytotoxicity of CsA. The HBV replication level in the HBV genomic DNA transfected HepG2.2.15 cell line was determined by an ELISA analysis of hepatitis B surface antigens (HBsAg) and Hepatitis B e antigens (HBeAg) in culture supernatant, while the intracellular HBV DNA replication level was analyzed by slot blot hybridization. Two-dimensional electrophoresis was used to investigate the alteration of protein expression in HepG2.2.15 after CsA treatment *in vitro*. The differentially-expressed proteins were identified by Matrix-assisted laser desorption/ionization-time of flight mass spectrometry combined with an online database search. **Results:** CsA was able to inhibit the expression of HBsAg, HBeAg, and HBV DNA replication *in vitro* in a dose-dependent manner. A proteomics analysis indicated that the expression of 17 proteins changed significantly in the CsA treatment group compared to the control group. Eleven of the 17 proteins were identified, including the overexpression of eukaryotic translation initiation factors (eIF) 3k, otubain 1, 14.3.3 protein, eIF2-1α, eIF5A, and the tyrosine 3/tryptophan 5-mono-oxygenase activation protein in CsA-treated HepG2.2.15 cells. The downregulation of the ferritin light subunit, erythrocyte cytosolic protein of 51 kDa (ECP-51), stathmin 1/oncoprotein, adenine phosphoribosyl-transferase, and the position of a tumor protein, translationally-controlled 1, was shifted, suggesting it had undergone posttranslational modifications. **Conclusion:** Our study identified the inhibitory effect of CsA on HBV replication, and found that a group of proteins may be responsible for this inhibitory effect.

Introduction

The hepatitis B virus (HBV) is regarded as one of the most fatal human pathogens, with an estimated 350 million individuals chronically infected worldwide and approximately one million deaths annually. Many chronically-infected patients gradually develop severe liver cirrhosis that may eventually progress to hepatocellular carcinoma (HCC). It has been reported that chronic HBV infection is associated with a 100-fold-higher risk of developing HCC[1], thus the HBV has been classified by the International Agency for Research on Cancer as "carcinogenic to humans". In order to reduce the morbidity and mortality of the HBV-related disease, it is of utmost importance to understand the mechanism of HBV replication in order to identify novel targets for anti-HBV therapeutic intervention.

The HBV is a DNA virus that undergoes reverse transcription during its replication cycle. It has a unique double-stranded, circular DNA genome that is capsuled within the viral capsid and enveloped[2]. Four open reading frames, including the viral envelope, core, reverse transcriptase-polymerase, and the hepatitis B X (HBx) gene were encoded by the viral genome. The product of the X gene, termed the HBx, protein is multifunctional. In HBV replication, the HBx protein interacts with the mitochondrial permeability transition pore (MPTP), causing its opening and subsequent outflow of intra-mitochondrial calcium into the cytoplasm, followed by the activation of the cytoplasmic, calcium-dependent, proline-rich tyrosine kinase-2 and downstream Src kinase pathway to promote HBV replication. HBV replication can be significantly inhibited by MPTP blockers, cytosolic calcium chelators, and Src inhibitors, and can be rescued by calcium-mobilizing regents[3–5]. The HepG2.2.15 cell line, a HepG2 human hepatoma cell line derivative which was stably transfected with a plasmid containing 2 head-to-tail dimers of the HBV genome, can not only release high levels of hepatitis B surface antigens (HBsAg) and hepatitis B e antigens (HBeAg) into supernatants, but also supports the assembly and secretion of replicative intermediates of HBV DNA and Dane particles during culture[6,7]. This provides us with a useful *in vitro* model to study the impact on HBV replication by various reagents, as well as the mechanisms that may be involved.

Recent studies have demonstrated that cyclosporine A (CsA) can inhibit HBV replication *in vitro*[4,5]. It is hypothesized that CsA can predominantly bind to mitochondrial cyclophilin, inhibit the MPTP opening, and disrupt the mitochondrial calcium signaling to inhibit HBV replication[8]. CsA targets the mitochondria, which is located upstream in the cellular signaling pathways and influence HBV replication. In the current study, we examined the effect of CsA on HBV replication *in vitro* and the mechanisms involved. In addition, a proteomic analysis was used to assess the large-scale protein expression profile of HepG2.2.15 cells after CsA treatment in order to identify some key proteins that may play critical roles in HBV replication and also discover some potential targets for novel anti-HBV agents.

Materials and methods

Cell culture HepG2.2.15 cells were cultured in RPMI-1640 medium (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA,USA) and 200 mg/L G418 (Sigma, St Louis, MO, USA) at an atmosphere of 5% CO_2 at 37 °C. Subconfluent monolayer HepG2.2.15 cells were harvested from the culture dishes by trypsin treatment and then plated onto 6-well flat bottom plates at a density of 1×10^6 cells per well. 24 h later, the cells were treated by CsA at different concentrations (0, 0.6, 1.3, 2.5, 5.0, and 10.0 μg/mL). The culture supernatants were collected every 24 h for ELISA analysis, and fresh mediums containing CsA at the indicated concentration were added to the culture. After CsA treatment for 4 d, the cells were harvested and intracellular DNA was isolated for slot blot hybridization using the DNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

MTT analysis of cytotoxicity A methyl thiazolyl tetrazolium (MTT) test was used to evaluate the cytotoxicity of CsA. HepG2.2.15 cells were first cultured in a 96-well microplate (1×10^4 cells/well) in 100 μL complete RPMI-1640 for 12 h. The cells were then treated with the indicated concentration of CsA in FBS-free Minimum Essential medium (MEM) for 96 h. By the end of the incubation, 25 μL MTT (5 mg/mL) was added to each well and incubation was allowed to continue for another 4 h. Finally, 100 μL DMSO was added to each well. The plate was read using a microplate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 570 nm.

ELISA analysis of HBsAg and HBeAg The HBsAg and HBeAg levels in the supernatant were detected by an ELISA detecting kit (Abbott, Abbot Park, IL, USA) according to the manufacturer's instructions. The suppression rate of the expression of HBsAg and HBeAg in the CsA-treated groups was calculated using the following formula:

Suppression rate=[*A* value (control)/A value (negative control) - *A* value (CsA)/*A* value (negative control)]/[*A* value (control)/*A* value (negative control) - 2.1]

Slot blot hybridization The HBV DNA replication level was evaluated by slot blot hybridization. Briefly, 5 μg extracted DNA was boiled to denature for 10 min and filtered with a Hoefer slot blot apparatus (San Francisco, CA, USA) on a nylon membrane according to the supplier's protocol. For the HBV DNA probes preparation, cloned HBV complementary DNA and β-actin complementary DNA were amplified by PCR. The following primers were used in this study: HBVprobe, forward: 5'-AGACTCGTGGTGGACTTCTCT-3', reverse: 5'-GGGTTCAAATGTATACCCAAAGAC-3'; β-actin probe, forward: 5'-CGCTGCGCTGGTCGTCGACA-3', and reverse: 5'-GTCACGCACGATTTCCGGCT-3'. The amplified probes were purified by electrophoresis from agarose gels and labeled with digoxigenin-11-dUTP using the DIG high prime kit (Roche, Mannheim, Germany). Prehybridization, hybridization, washes after hybridization, and antidigo-xigenin-alkaline phosphatase (AP) incubation were performed as described by the manufacturers. After the mem-

brane was visualized by chemiluminescent substrate CSPD solution with Kodak X-ray film (Kodak, Rochester, NY, USA), the same membrane was rehybridized with the other probes and exposed again. The slot blot result was quantitated by densitometry using Kodak 1D 3.6 software and the HBV DNA signal was normalized to β-actin.

2-D electrophoresis (2-DE) The cells were treated with CsA at a concentration of 5 μg/mL for 4 d and harvested by centrifugation for the protein sample preparation. Lysis buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% (3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate) (CHAPS), 1% DTT, 2% immobilized pH gradient (IPG) buffer (pH 3~10 liner, Amersham Biosciences,Uppsala, Sweden), and a protease inhibitor cocktail (Complete tablets, Roche, Mannheim, Germany) were mixed with the harvested cells and incubated for 20 min at room temperature. Nucleic acids were removed by centrifugation at 50 000×*g* for 30 min. 2-D electrophoresis (2-DE;Amersham Biosciences,Uppsala,Sweden). was performed according to the manufacturer's instructions. In brief, the samples containing the lysis buffer were mixed with the rehydration solution [8 mol/L urea, 2% CHAPS, 20 mmol/L DTT, 0.5% IPG buffer (pH 3~10 non-linear), and 0.002% bromophenol blue] to a total volume of 450 μL, and subjected to Isoelectric focusing (IEF) on an IPG strip. Rehydration and IEF were carried out on the Amersham Biosciences IPGphor as follows: rehydation with 30 V for 12 h, 500 V for 1 h, 1000 V for 1 h, 1000–8000 V for 30 min, and 8000 V was applied until the total volt hours reached 72.0 kVh at 20 °C. After IEF separation, the strips were equilibrated for 15 min in SDS equilibration buffer (50 mmol/L Tris-HCl, 6 mol/L urea, 30% glycerol, 2% SDS, and 0.002% bromophenol blue) containing 1% DTT, followed by SDS equilibration buffer containing 2.5% iodoacetamide for another 15 min. After equilibration, the strips were loaded onto vertical SDS-PAGE (12.5% T constant). The 2-D SDS electrophoresis was run using the Ettan DALTsix electrophoresis unit (Amersham Biosciences,Uppsala,Sweden). Three independent experiments were carried out in this study. The experiments of each treatment group (control or CsA-treated) were repeated 3 times.

Silver staining and image analysis Silver staining was done according to the protocol of Yan *et al*[9] with minor modifications (substitution of ethanol for methanol in fixation). All chemicals used in silver staining were purchased from Sigma (USA). Silver-stained gels were scanned using an ImageScanner (Amersham Biosciences,Uppsala,Sweden) and analyzed with the ImageMaster 2D Elite software. Six gels were divided into 2 groups, 3 for the controls and 3 for the CsA-treated cells. Image spots were initially detected; the background was subtracted, followed by volume normalization, and spots matched by manual assistance. Three gel images of the control group were averaged and set as the reference gel. The protein spots were quantitated based upon their relative volume. Only the spots with expression levels greater than 2-fold were considered significant and selected for the mass spectrometry analysis.

In-gel digestion, matrix-assisted laser desorption/ionization-time of flight mass spectrometry analysis and protein identification The protein spots were excised from different gels and transferred into Eppendorf tubes (Gilson, Villiers, Le Bel, France). Each spot was washed twice in milli-Q water, destained by washing with a 1:1 solution of 30 mmol/L potassium ferricyanide and 100 mmol/L sodium thiosulfate, and equilibrated in 200 mmol/L ammonium bicarbonate for 20 min to a pH of 8.0. The gels were then washed twice in milli-Q water, dehydrated by the addition of acetonitrile, and dried in a SpeedVac (Thermo Savant, Holbrook, NY, USA). Subsequently, the gels were rehydrated in 10 μL proteomics grade trypsin (Sigma, St Louis, MO, USA) solution (20 ng/μL in 40 mmol/L NH_4HCO_3 in 9% acetonitrile, ACN) and incubated at 37 °C for 10 h. The peptides were extracted by adding 50 μL of solution containing 50% ACN and 5% trifluoroacetic acid (TFA). The extracted solutions were dried in a lyophilizer and reconstituted in 5 μL 0.1% TFA solution (Virtis, Gardiner, NY, USA).

One μL of peptide mixture was mixed with an equal volume of 10 mg/mL alpha-Cyano-4-hydroxycinnamic acid (Sigma, USA) saturated with 50% ACN in 0.05% TFA and analyzed with a Voyager-DE STR matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) using a delayed ion extraction and ion mirror reflector mass spectrometer (Applied Biosystems, Foster City, CA, USA). The instrument setting was set at reflector mode with a 160 ns delay extraction time, positive polarity, 63.5% grid voltage, and 20 000 V accelerating voltage. Laser shots at 200 per spectrum were used to acquire the spectra with mass range from 1000~4000 Da. External calibration was carried out using insulin chain B oxidized and P14R (Sigma, St Louis, MO, USA) and the internal calibration was performed using the autolytic peaks of trypsin.

For the interpretation of the mass spectra data, the monoisotopic peptide masses were input into Mascot (http://www.Matrixscience.com). A database search (NCBInr) was performed using the following words: homo sapiens/human, trypsin digest (we allowed for up to 1 missed cleavage), cysteines modified by carbamidomethylation, and 100 ppm mass tolerance using internal calibration.

Western blot analysis After SDS-PAGE, the gels were

transferred electrophoretically onto a nitrocellulose membrane (Amersham Biosciences,Uppsala, Sweden) and blocked for 2 h with Tris-buffered saline (TBS) containing 5% skim milk. The primary antibody used was goat anti-human eukaryotic translation initiation factor (eIF)-5A (Santa Cruz, CA, USA; 1:500 dilution). The membranes were incubated with the primary antibody overnight at 4 °C, washed 3 times with TBS containing 0.1% Tween-20, and then the membrane was incubated with horseradish peroxidase-conjugated anti-goat secondary antibody (Chemicom, Temecula, CA, USA) 1:2000 for 1 h at room temperature, and developed with a chemiluminescence reagent (EZ-ECL chemiluminescence detection kit, Beit-Haemek, Israel). Detecting actin (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) acted as the loading control. Images were acquired on an image station 2000R (Kodak, Rochester, NY,USA) and analyzed with the software supplied by the manufacturer.

Statistics The data of the ELISA analysis and slot blot hybridization were expressed as mean±SD. One-way ANOVA of Dunnett's test was used to analyze the significance between the control and the CsA-treated groups A bivariate correlate analysis was used to analyze the correlation between the CsA concentrations and HBV replication levels. The statistical significance in terms of the expression profiles of HepG2.2.15 cells with or without CsA treatment was estimated by the Student's *t*-test. All calculations were done with SPSS 11.5 software (SPSS, Chicago, IL, USA). $P<0.05$ was considered statistically significant.

Results

No apparent cytotoxicity of CsA on the HepG2.2.15 cell line at low concentrations Before we studied the effect of CsA on HBV replication *in vitro*, we first wanted to determine whether CsA had direct toxicity on the HepG2.2.15 cell line. The cells were incubated with CsA at graded concentrations of 0 (vehicle control), 0.25, 0.5, 1, 2, 4, 8, 16, 20, 40, 80, and 100 μg/mL. The cytotoxicity of CsA on HepG2.2.15 cells was evaluated by MTT test. As shown in Figure 1, HepG2.2.15 remained viable in the presence of 0–80 μg/mL CsA treatment. When the final concentration of CsA was increased to 100 μg/mL, there was a mild decrease in the optical density value, indicating that CsA causes little toxicity on HepG2.2.15 cells at low concentrations (<80 μg/mL), but has a direct toxic effect with a concentration higher than 100 μg/mL. Thus, we used low concentrations of CsA to study its effect on HBV replication using this *in vitro* model system.

CsA inhibits the expression of HBsAg and HBeAg To investigate the effect of CsA on the production of HBeAg and HBeAb by HepG2.2.15 cells, the supernatant was collected after treatment with different concentrations of CsA at different times, and the titer of HBsAg and HBeAg was determined by ELISA. Our results showed that CsA could inhibit the expression of HBsAg and HBeAg in a dose-dependent manner; a further bivariate correlation analysis showed positive correlation between the suppression rates of viral antigens and CsA concentration. After the CsA treatment, the correlation index was 0.401, 0.816, 0.940, and 0.938, respectively, for HBsAg, and the *P*-value was 0.058, 0.000, 0.000, and 0.000, respectively; the correlation index was 0.405, 0.983, 0.963, and 0.979, respectively, for HBeAg, and the *P*-value was 0.069, 0.000, 0.000, and 0.000, respectively (Figure 2). These results indicate that CsA is able to dose-dependently inhibit HBsAg and HBeAg production *in vitro*.

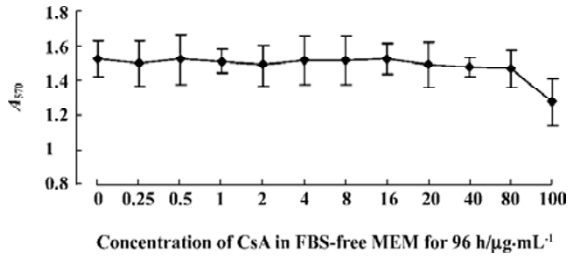

Figure 1. Cytotoxic effect of CsA on HepG2.2.15 cell viability measured by MTT metabolism. Data are expressed as mean±SEM (normalized to control mean in the absence of CsA) of three experiments from 3 observations. A_{570} values unvaried at from 0 to 80 μg/mL CsA until a mild drop when CsA increased to 100 μg/mL, indicating CsA cause little impact on viability of HepG2.2.15 cells in the low concentration (<80 μg/mL).

CsA inhibits HBV DNA replication We were also interested in whether CsA has a direct effect on HBV DNA replication *in vitro*. After treatment with CsA at different concentrations for 4 d, the HepG2.2.15 cells were harvested and intracellular DNA was extracted for the slot blot hybridization to evaluate the HBV DNA replication level. As shown in Figure 3, CsA significantly inhibited the HBV DNA replication. The replication levels in 2.5, 5.0, and 10.0 μg/mL CsA-treated groups were significantly low compared to the control groups (P=0.020, 0.014, and 0.048, respectively), and there was a negative correlation between the HBV DNA replication level and the different concentrations of CsA (correlation index -0.770, P=0.000).

Differential analysis of 2-DE protein profiles in the control and CsA-treated HepG2.2.15 cells To further investigate the alteration of the protein expression in HepG2.2.15

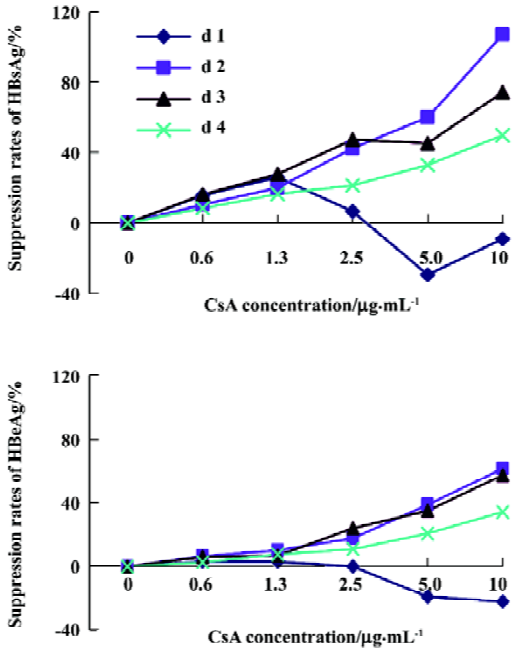

Figure 2. CsA could dose-dependently inhibit the expression of HBsAg and HBeAg. HepG2.2.15 cells were treated by CsA at indicated concentrations, the culture supernatants were collected every 24 h for ELISA analysis of HBsAg and HBeAg, and suppression rates of HBsAg and HBeAg were calculated by the following equation {suppression rate = [*A* value (control)/*A* value (negative control) - *A* value (CsA)/*A* value (negative control)]/[*A* value (control)/*A* value (negative control) - 2.1]}. Each test was repeated 3 times. Bivariate correlation analysis showed positive correlation between the suppression rates of viral antigens and CsA concentration. After CsA treatment, the correlation index was 0.401, 0.816, 0.940, and 0.938 respectively for HBsAg, and the *P* value was 0.058, 0.000, 0.000 and 0.000, respectively; the correlation index was 0.405, 0.983, 0.963, and 0.979 respectively for HBeAg, and the P value was 0.069, 0.000, 0.000 and 0.000, respectively. Results indicated that the CsA at concentrations of 0.6, 1.3, 2.5, 5.0, and 10.0 μg/mL could dose-dependently inhibit the expression of HBsAg and HBeAg at d 2, d 3, and d 4.

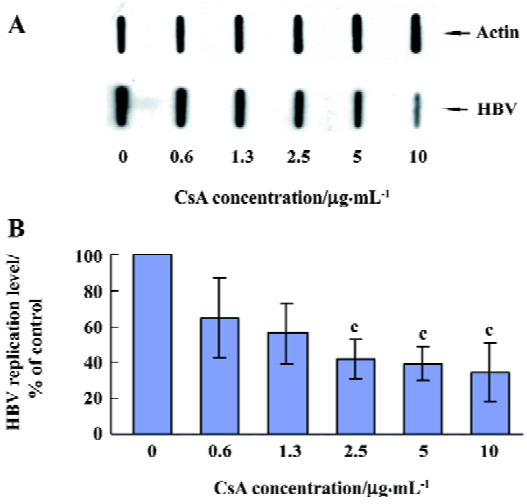

Figure 3. CsA could dose-dependently inhibit the HBV DNA replication. HepG2.2.15 cells were treated with indicated concentration of CsA for 4 d, the intracellular DNA was extracted and HBV DNA replication level was evaluated by slot blot hybridization. For each lane, 5 μg DNA was loaded, the DNA transblots were hybridized with Digoxigenin-labeled HBV probe and visualized by X-ray film, then rehybridized by β-actin and visualized (A). The slot blot results were quantitated by densitometry, and the HBV DNA signals were normalized to β-actin, the relative level of HBV replication was calculated and these data were collected in 3 different tests. As shown in part B, results indicated that CsA could inhibit the HBV DNA replication, the replication levels of 2.5, 5.0, and 10.0 μg/mL CsA group were significant lower than the control group (*P*=0.020, 0.014 and 0.048, respectively), and negative correlation existed (Figure 2) between the HBV DNA replication level and the different concentrations of CsA (correlation index was -0.770, *P*=0.000).

after CsA treatment *in vitro*, protein lysates from the control and CsA-treated HepG2.2.15 cells were subjected to 2-DE. Following silver staining, the gels were digitized prior to computer-based matching and quantitative analysis with image analysis software. We found that the intensity of 17 protein spots were altered after CsA treatment, eleven of which were identified. Among them, 6 proteins were upregulated, 4 were downregulated, and 1 was shifted (Figure 4).

Identification of proteins by MALDI-TOF MS and database searching In order to identify key proteins that may play a critical role in HBV replication and also to explore some potential targets for novel anti-HBV agents, the 17 protein spots with altered intensity as a result of CsA treatment were excised from the gels, digested with trypsin, and subsequently analyzed by MALDI-TOF MS using the MS-Fit software to search the nrNCBI database. Eleven of the protein spots were identified. Among them, 6 were upregulated, 4 were downregulated, and 1 was shifted after treatment (Table 1). The identified proteins represented a heterogeneous group that included several important molecules relevant to gene transcription regulation, such as eIF, proteins involved in signal transduction such as the 14.3.3 protein (Figure 5), stathmin 1/oncoprotein 18, and ubiquitin-specific protease otubain 1. The functions of the remaining proteins are still not clear.

Detection of identified proteins by Western blot analysis From the identified candidates, eIF-5A was detected by Western blot analysis. The results are shown in Figure 6. The expression changes of eIF-5A were basically consistent with the 2-DE results. eIF-5A was upregulated in 5 μg/mL CsA treatment (*P*=0.008).

Figure 4. After treatment with CsA at concentration of 5 μg/mL for 4 d, cells were lysed and 200 μg of total protein lysates were subjected to 2-DE, followed by silver staining and image analysis. Results were quantified from three sets of 2-DE. 2-DE map indicated protein spots changed in volume after CsA treatment in HepG2.2.15 cells. The spots of interest were excised from the gels and digested with trypsin. The digested peptides were used for MALDI-TOF mass analysis. The identified proteins are listed in Table 1.

Table 1. Differentially expressed proteins identified after HepG2.2.15 cells cell line treatment by CsA (Only those spots which expression level changed greater than 2-fold were considered significant and selected for mass spectrometry analysis).

Spot	Identified protein[a]	Gene	Mass	Score	Coverage	PI	Level[b]
1	eIF3k	gi|31322564	25043	80	31%	4.81	↑
2	ubiquitin-specific protease otubain 1	gi|28628207	31264	133	54%	4.85	↑
3	14.3.3 protein	gi|3387922	19662	118	53%	4.50	↑
4	ferritin light subunit	gi|182516	16384	72	32%	5.65	↓
5	eIF-5A	FIHUA	16821	77	59%	5.08	↑
6	ECP-51	gi|5326998	51364	78	26%	5.49	↓
7	eIF2-1α	gi|55640927	36089	73	34%	5.02	↑
8	Tyrosine 3/tryptophan 5 -monooxygenase activation protein, zeta polypeptide	gi|68085578	27792	115	42%	4.73	↑
9	Tumor protein, translationally-controlled 1	gi|15214610	19645	77	37%	4.84	M[c]
10	stathmin 1/oncoprotein 18	gi|61363614	17326	73	18%	5.76	↓
11	adenine phosphoribosyltransferase	gi|31415697	19595	85	32%	5.78	↓

a) Identified proteins scores in MASCOT database are all significant ($P<0.05$).
b) The upward arrows signs represent these spots appeared or increased respectively in HepG2.2.15 following CsA treatment.
c) The spot was shifted.

Discussion

In our study, an ELISA analysis was used to evaluate the expression of HBsAg and HBeAg in the supernatants of HepG2.2.15 cells treated by CsA, while a slot blot analysis of HBV DNA was performed to evaluate the HBV replication level. As mentioned earlier, the expression level of HBsAg, HBeAg, as well as HBV DNA replication dramatically decreased in the presence of CsA in a dose-dependent manner, suggesting that CsA was able to significantly inhibit HBV

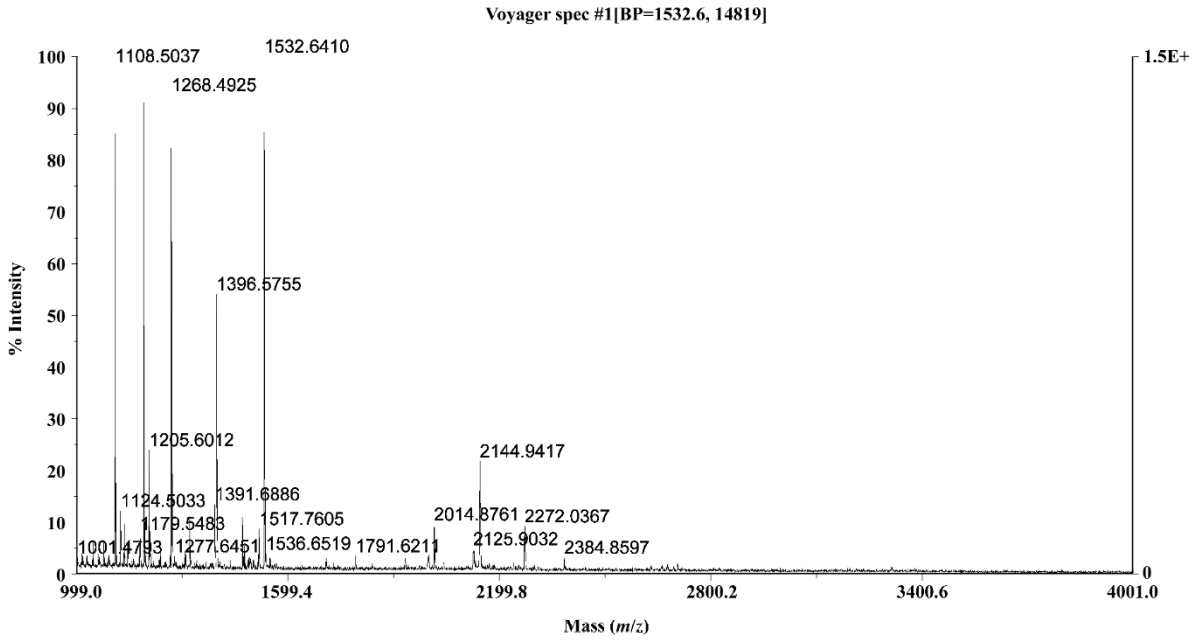

Figure 5. Peptide mass fingerprint of spot 3 (identified as 14.3.3 protein). The spot was in-gel digested by trypsin. After desalting, the peptide mixture was analyzed by MALDI-TOF mass spectrometry. Eleven peptides with m/z values were matched to 14.3.3 from the Mascot database and the prominent peaks were labeled with *m/z* values.

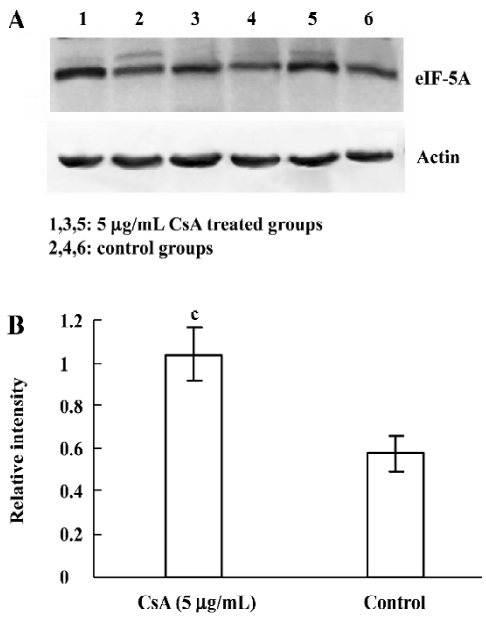

Figure 6. Western blotting validates the difference in expression of eIF-5A between the control and CsA treated groups. A) Expression of eIF-5A in control and 5 μg/mL CsA treated groups by Western blotting. B) Quantitative analysis of the bands was carried out. The student's *t*-test showed a significant difference between the control and 5 μg/mL CsA treated groups (P=0.008). ^cP<0.01 *vs* control.

replication in this *in vitro* culture system.

HBV replication is a multi-step process requiring fine-tuning of numerous viral and cellular proteins. During replication, the viral HBx protein can mobilize the intra-mitochondrial calcium into the cytoplasm, activate intracellular calcium signaling, followed by the activation of the Src kinase and downstream unknown signal transduction messengers, thus promoting HBV DNA replication[3,4]. The specific blocker of MPTP, CsA, can impair the interaction of the HBx protein with the mitochondria[8], inhibit subsequent MPTP openings, and block the intracellular calcium signaling pathway[10,11]. Based on the fact that CsA is involved in the interaction between HBx and the mitochondria, a key point lies upstream of the HBV replicative signaling pathway. The different protein expression profiles between the CsA-treated and untreated groups may provide some clues to probe the mechanisms of the inhibitory action of CsA on HBV replication, and possibly identify some key factors involved in HBV replication and novel targets for anti-HBV therapy.Among these proteins with altered expression levels after CsA treatment, 11 protein spots were identified with high confidence by peptide mass fingerprinting. The remaining proteins failed to be identified mainly because the low protein abundance was not enough to produce a good signal to the noise spectrum, or the result scores of the data-

base search was too low to achieve significant results. Among the identified proteins, we were particularly interested in protein kinase 14-3-3, signal transduction messenger stathmin 1/oncoprotein 18, ubiquitin-specific protease otubain 1, and eIF.

14-3-3 kinases are highly conserved, ubiquitously expressed[12], and multifunctional in controlling intracellular signaling pathways via binding to various ligands such as the Bad protein and p53 in apoptosis[13,14], Cdc 25 and telomerase in cell life cycle[15,16]. In virology studies, it has been identified that 14-3-3 facilitates the complex formation of the Vpr protein of HIV-1 with Cdc25 to regulate the host cell life cycle[17]. In addition, the HCV core protein can enhance Raf-1 kinase activity via interaction with 14-3-3 to regulate hepatocyte growth[18]. Analyses of the HBx protein sequence reveal that HBx contains a 14-3-3 binding domain[19,20]. This binding is essential for the formation of a complex composed of Mitogen-activated protein kinase kinase 1(MEKK1), stress kinase 1 (SEK1), stress-activated protein kinases (SAPK), 14-3-3 proteins and HBx, which can subsequently upregulate SAPK activity[21]. CsA not only blocks the activation of the MEKK1-SAPK pathway[22], but also interferes in the intracellular calcium signaling pathway which is essential for the interaction between 14-3-3 proteins and their partners[23]. Since calcium signaling is involved not only in the HBx activation of HBV replication, but also in the mechanisms of the inhibition of CsA on HBV replication and the interaction of 14-3-3 kinases with their partners, we speculate that 14-3-3 may play a pivotal role in HBV replication and become a potential target for new anti-HBV intervention. Stathmin 1/oncoprotein 18 has dual meanings: "oncoprotein 18" means that it is highly expressed in numerous malignant tumors[24–27], and "stathmin 1" is derived from the Greek word "relay", indicating its intermediate role in signal transduction. As an intracellular relay for signal transduction, stathmin 1/oncoprotein18 is the target for numerous cellular kinases, such as the mitogen-activated protein kinase family members including extracellular signal-regulated kinase (ERK) and p38 and the Ca^{++}/calmodulin dependent kinase, all of which be regulated in response to intracellular calcium signaling. Both of p38 and the Ca^{++}/calmodulin dependent kinase can also be regulated by CsA[22], and the latter can be regulated by 14-3-3 kinases[28]. Thus, we hypothesize that the stathmin 1/oncoprotein 18 may play an important role in the anti-HBV action of CsA via interaction with 14-3-3 and the calcium signaling pathway. The ubiquitin-specific protease otubain 1 was also overexpressed after CsA treatment. Otubain 1 belongs to the deubiquitylating enzyme (DUB) family[31]. Like protein phosphorylation, protein ubiquitylation is critical for diverse biological processes, including signal transduction[29], and the protein ubiquitylation process can be reversed by a group of proteases known as DUB[30]. Recent studies have indicated that otubain 1 and ubiquitin are both involved in the T-cell activation process[32,33], and that ubiquitin can slightly bind to CsA as minor immunophilin[34]. So we believe there is complicated interaction between CsA, otubain 1, and ubiquitin in T-cell priming stage, but whether similar interaction exists in HBV replication remains unknown and needs to be further studied.

eIF are essential for the formation of a ribosomal initiation complex during the process of translation initiation[35]. eIF2 has been reported to mediate the host cells' antiviral response during viral infection[36]. eIF5 is a shuttle protein mediating unspliced and incompletely-spliced RNA from the nucleus to the cytoplasm during reverse transcription, a process essential for some viruses, including HBV[37–39]. In our study, as the members of the eIF protein family, eIF2, eIF3, and eIF5 were overexpressed in 2-D maps and identified by MALDI-TOF MS. Western blotting revealed the consistent result by detecting eIF-5A (Figure 6), suggesting that these factors may participate in the cellular antiviral response; however the molecular mechanism concerning their exact role in HBV replication and the relevance to anti-HBV activity of CsA remains to be elucidated.

The translationally-controlled tumor protein (TCTP) is a highly-conserved protein that is ubiquitously expressed in all eukaryotic organisms[40]. This protein plays an important role in cell growth, cell cycle progression, malignant transformation, and anti-apoptosis activity. TCTP synthesis can be rapidly regulated by calcium stress, growth signals, certain cytokines, and pro-apoptotic signals; this regulation was done at both the transcriptional and translational levels[41]. In addition, 3 isoforms of TCTP have been detected, which might be caused by posttranslational modifications[42]. In our study, the location of TCTP was shifted after CsA treatment, We hypothesize that this displacement may be caused by TCTP isoform formation because CsA can interfere with the intracellular calcium signal by interacting with the mitochondria as described earlier.

In conclusion, we have found that CsA was able to significantly inhibit HBV replication *in vitro*. We also identified a group of proteins with altered expression profiles in HepG2.2.15 cells after CsA treatment. These proteins may provide new insights to the molecular events of HBV replication and the inhibitory effect of CsA on HBV. However, more work needs to be done in order to evaluate the detailed function of particular proteins in HBV replication, as well as

the potential of these proteins as novel targets for anti-HBV therapy in the future.

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