Apoptosis Induced by Low-concentration Ethanol in Hepatocellular Carcinoma Cell Strains and Down-regulated AFP and Survivin Analysis by Proteomic Technology

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Abstract—Ethanol is generally used as a therapeutic reagent against Hepatocellular carcinoma (HCC or hepatoma) worldwide, as it can induce Hepatocellular carcinoma cell apoptosis at low concentration through a multifactorial process regulated by several unknown proteins. This paper provides a simple and available proteomic strategy for exploring differentially expressed proteins in the apoptotic pathway. The appropriate concentrations of ethanol required to induce HepG2 cell apoptosis were first assessed by MTT assay, Gisma and fluorescence staining. Next, the central proteins involved in the apoptosis pathway processs were determined using 2D-PAGE, SDS-PAGE, and bio-software analysis. Finally the downregulation of two proteins, AFP and survivin, were determined by immunocytochemistry and reverse transcriptase PCR (RT-PCR) technology. The simple, useful method demonstrated here provides a new approach to proteomic analysis in key bio-regulating process including proliferation, differentiation, apoptosis, immunity and metastasis.

Keywords—Hepatocellular carcinoma, Ethanol, Proteomics, survivin and AFP

I. INTRODUCTION

HEPATOCELLULAR carcinoma (HCC or hepatoma) is the fifth most common and the third deadliest cancer, with approximately one million patients falling victim due to HCC each year [1]. HCC has a high incidence rate in underdeveloped and developing countries where patients are often diagnosed with infiltrative or massive tumours [2]. Furthermore, the incidence of HCC in developed countries has been increasing in recent years [3]. Known causes of HCC include persistent hepatitis viral infection, dietary exposure to aflatoxin B1, alcoholic liver dysfunction, and autoimmune hepatitis. Among these, hepatitis B virus (HBV) and hepatitis C virus (HCV) are recognized as the major risk factors and account for more than 80% of HCC cases worldwide [4, 5].

Nowadays, treatment options available to patients with HCC are limited to surgery, systemic chemotherapy, and symptomatic relief. Long-term survival is rare for most patients suffering from HCC and though surgery is potentially curative, only 10% - 15% of patients can be treated with liver resection or transplantation [4]. Systematic chemotherapy for unresectable HCC has been widely used, yet its efficacy remains low and complications such as significant myelosuppression abound [6]. Challenges specific to HCC also include difficulties in establishing an early diagnosis, frequent recurrence and intrahepatic metastasis post-surgery [7]. Currently there is still no standard procedure for treating this disease.

During the last decade, a considerable amount of research has been focused on cancer cell apoptosis. Apoptosis, or programmed cell death, is a major control mechanism by which cells die if DNA damage is not repaired [8]. Apoptosis is an essential and highly regulated physiological process required for normal development and tissue homeostasis in all multicellular organisms [9]. Novel targeted therapies based on apoptosis principles can induce cancer cell death or sensitize them to established cytotoxic agents and radiation therapies [10]. Ideally, an effective way to augment anticancer strategies using the developing knowledge of apoptosis could provide more targeted anti-tumor therapy for HCC and other cancers.

Two provocative approaches, the receptor-dependent (extrinsic) and the mitochondrial-dependent (intrinsic) pathway, can induce tumor-selective apoptosis [11]. Generally, the initiation and progression of apoptotic pathways are associated with multiple changes at the mRNA and protein level. Both pathways are controlled by a number of complex proteins activated by various triggers and arranged in sequential signaling modules [10]. Several proteins important in apoptosis regulation have been systematically explored; protein changes during apoptosis that are not completely elucidates include altered expression, differential protein modification, specific activity changes, and aberrant localization. Therefore, a novel, feasible method is necessary to determine whole cell protein expressions and modifications during apoptosis process.

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Proteomics analysis could easily characterize the qualitative alterations and quantitative protein expression level changes in response to varying conditions conditions during apoptosis [12]. Traditional proteomics methods combining two-dimensional gel electrophoresis with mass spectrometry and database-analyzing techniques are used to observe the global changes in protein expression during the apoptotic process [12-15]. However, the traditional methods are complex and time-consuming, exact protein spots are often unattainable and contamination is often faced in the whole process. Moreover, apoptosis is a dynamic process where different conditions should be detected over time. A simple, easy and rapid method for proteomic analysis would greatly benefit this field.

In this paper, apoptosis in hepatocellular carcinoma cell line, HepG2, was induced using ethanol. Ethanol's effects on HepG2 in terms of proliferation and apoptosis-induction, were assessed as functions of concentration and exposure time. Changes were identified by cytobiology technology and protein analysis methods and the obtained data were analyzed to evaluate effects at the protein level by bioinformatic methods.

II. MATERIALS AND METHODS

A. Cell Culture

HepG2 cells, derived from a human hepatocelluar carcinoma (obtained from Shanghai Institute of Cell Biology, Shanghai, China), were seeded in 25 cm^2 tissue culture flasks at 1×10^5 cell/cm^2 in DMEM medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Amersco, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO^2.

B. Exposure of HCC to Ethanol

All tables and figures you insert in your document are only to help you gauge the size of your paper, for the convenience of the referees, and to make it easy for you to distribute preprints. After 3 days of culture, cells were harvested with 0.25% trypsin (Sigma, USA), ashed once with serum-free medium and plated at a concentration of 5×10^4 cells/well in six-well plates. After 12 hr, cells were divided into two groups: control and ethanol treated. In ethanol-treated groups, cells were incubated with ethanol in different concentrations (1%-10%); in control groups, PBS was added into 1% serum-medium with corresponding concentrations. Cell morphological changes were observed after 6 h, 12 h, 24 h, and 48 hr using a phase contrast microscope and Gimasa staining (Nikon, Japan).

C. Cell Viability Analysis

1×10^4 cells/well in 100 μL culture medium were plated in 96-well cell culture plates (Costar, Corning, NY). After 24 hr of culture, these were treated with media containing ethanol of varying concentrations (1% to 10%). Fresh ethanol-containing medium with ethanol was changed in every 24 hours. In the control group, PBS was used in place of ethanol. After treated with ethanol for 6, 12, 24, and 48 h, cell viability was determined by MTT assay [16]. In brief, the cells were incubated with 100 μL of MTT (0.5 mg/mL) (Sigma, USA) in a PBS solution for 4 hr. All remaining supernatant was removed and 100 μL of DMSO was added to each well. After 10 min of incubation to ensure all crystals dissolved, the optical density was measured at 570 nm.

D. Fluorescence Staining

Ethanol-induced apoptosis was analysed by a double-fluorescence staining technique with Hoechst 33342 and acridine orange (AO). Briefly, after cells were exposed to 4% ethanol for 24 hr, 5 μg/mL Hoechst 33342 and 8.5 μg/mL AO DNA-binding dyes were added to the cells' media and incubated for 10 min at 37°C in the dark. After decolorization with distilled water, the cells were immediately examined using a fluorescence microscope (Nikon, Japan).

E. Preparation of Protein Samples

The protein samples were prepared by the standard procedures with modifications as reported [17-21]. Briefly, the cells were collected in 1.5 mL eppendorf tubes at 1,200 rpm for 20 min, and rinsed three times with ice-cold PBS. The cells pellets were then dissolved in a lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris, 65 mM DTT, 2% Bio-Lytes+) for 30 min and centrifuged at 15,000 rpm for 40 min at 4°C. The supernatant sample was collected and analyzed for protein contents using a modified Brandford assay [22]. All samples were stored at -80°C prior to electrophoresis.

F. Two-Dimensional Electrophoresis (2-DE)

2-DE was performed using the PROTEAN IEF and PROTEAN XL system (Bio-Rad, Hercules, CA) and 400 μg of soluble proteins were separated by Coomassie Brilliant Blue staining (CBB R-250, Sigma, USA). Three gels per sample were processed and analyzed simultaneously. The first dimensional isoelectric focusing (IEF) experiment was carried out on precast IPG strips (7 cm, pH 3-10, Bio-Rad) at 17°C with a maximum current setting of 50 μA/strips and the 2-DE was done as reported previously [20-22]. Proteins were detected by Neuhoff's optimized Coomassie Brilliant Blue R-250/ammonium sulfate/phosphoric acid protein staining [23].

G. Protein Electrophoresis (SDS-PAGE)

One-dimensional SDS-PAGE was performed by resolving proteins of HepG2 untreated and treated with ethanol at different concentrations (2%, 4%, 6%, 8%, 10%). A sample buffer (0.0625 M Tris-HCl pH 6.8, 2% SDS, 0.01% Bromophenol Blue, 10% Glycerol, 5% β-mercaptoethanol) was used to dissolve all proteins of HepG2 cells and 25 μL of this protein solutions was added per well. Electrophoresis was carried out through 12.5% gel using 150 V for 4-6 hr and staining was done with Commassie Brilliant Blue G-250 (Sigma,USA).

H. Image analysis and statistics

2-DE Gel and SDS-PAGE images were captured with UMAX Powerlook 2100xl (Novax, Taiwai). 2-DE digital images were analysis with PDQuest 7.1 (Bio-Rad) and analyzed according to the PDQuest user guide, regarding spot detection, quantification, matching, and calculating Mr/pI values for all the spots. The size and orientation of an image was adjusted and proper spot detection parameters were selected to identify spots of interest in the gel. Then a MatchSet was created to match the protein spots of HepG2 cells from three different gels. After normalizing the individual protein spot quantity, the same spots were found for the following analysis by analytical tools in PDQuest. As protein marker (β-galactosidase 118 kDa; Bovine serum albumin 85 kDa; Ovalbuim 47 kDa; Carbonic anhydrase 36 kDa; β-lactoglobulin 26 kDa; lysozyme 20 kDa, Fermentas) had been put into the gels beforehand and the pH range is 3-11, the Mr and pI of each protein spot were calibrated. The results were exported into Excel 2000 (Microsoft, Seattle, CA, USA) for further analysis.

SDS-PAGE digital images were analyzed by Quantity One 4.4 (Bio-Rad) and analyzed according to the Quantity One user guide. Noise or background density in the image was reduced to identify lanes and defining, quantifying, and calculating the values of bands were performed by using the software tools. Finally the quantities of bands were measured and compared by plot density.

I. Immunocytochemistry

Alpha-fetoprotein (AFP) and survivin were differentially expressed as determined by immunocytochemistry techniques previously described [24]. In brief, 5×10^4 cells/well were added to cover slide-bottomed six-well plates. In the control group, cells were treated with PBS into 1% serum- medium; in ethanol-treated groups, cells were treated with 4% ethanol. After 24 h, HepG2 cells detected on cover slides were fixed in 4% paraformaldehyde for 30 min, then permeabilized in 0.2% Triton X-100 for 5 min and finally in 3% H_2O_2 for 10 min. Then cells were blocked with 4% normal goat serum for 20 min at room temperature and incubated with rabbit anti-AFP & anti-survivin antibody for 1 hr at 37°C. After three rinses with PBS, slides were incubated with 30 μL alkaline-phosphatase/anti-(alkaline phosphatase) complexes (goat antirabbit IgG-AP) for 1 hr at 37°C. Following a further wash, the slides are incubated with BCIP/NBT kit for about 10 min and counterstained with hematoxylin. Samples were evaluated using ordinary microscopy.

J. Reverse Transcriptase PCR (RT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). About 1 μg RNA was converted to cDNA using the first-strand cDNA synthesis kit (Invitrogen) according to manufacturer's instructions. Then cDNAs were amplified with the following primer pairs: AFP: 5'-AAAAGCCCACTCCAGGATC-3' and 5'-CAATCCAGCACATCTCCTC-3; Survivin: 5'-

AAGGACCACCGCATCTCTAC-3' and 5'-ACTTTCTTCGCAGTTTCCTC-3'. β-actin was used as the control. PCR products were analyzed by electrophoresis on 1.5% agarose gels and visualized under UV light after staining with ethidium bromide.

III. RESULTS

A. Ethanol Decreases Viability of HepG2 Cells

The proliferation of HepG2 cells was significantly inhibited in a dose- and time-dependent manner, by 1%-10% of ethanol for 6 h, 12 h, 24 h or 48 h of incubation (Figure 1). The inhibition ratio of HepG2 cells was increased as the ethanol concentration was increased and HepG2 cells proliferation decreased with increased ethanol concentrations. Similarly, the cell growth was inhibited from 0.7% to 25%, and the cell viability was reduced from 4% to 28% after 48 h ethanol treatment. Cell inhibition ratio increased the most obviously at 12 h and 24 h, which was from 4% to 30% (Figure 1). These data indicate that the ethanol has a cytostatic effect on cell growth during the first 24 hours of exposure, but no effect after this time.
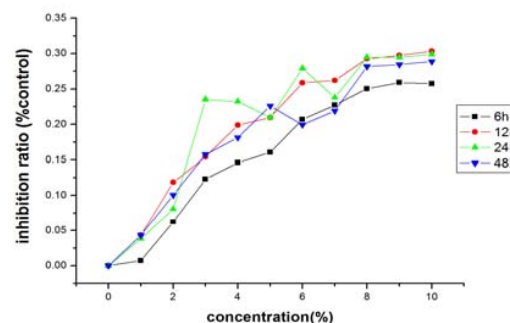

Fig. 1 Effects of different time and concentrations of ethanol on HepG2 cells. The inhibition ratio curves of HepG2 cell line treated with ethanol in different concentrations at different time. HepG2 cells viability was decreased with ethanol treated in a dose- and time-dependent manner.

B. Ethanol Induces Apoptosis in HepG2 Cells

After 24 hours of ethanol treatment and Gimasa staining, HepG2 cell morphologic changes were observed using an optical microscope to determine the mode of cell death and optimal ethanol concentrations to induce apoptosis (Figure 2). Most of HepG2 cells treated with the low ethanol concentration (2%) for 24 h were similar to control group cells (Figure 2A and 2B). However, increased apoptosis was observed in HepG2 cells in the 4% ethanol group. Generally, cell bodies shrunk, chromatin condensed, and plasma membranes preserved continuity bleb (Figure 2C). After treated with 6%-10% ethanol for 24 h, HepG2 cells appeared necrotic in shape (cell bodies swelled, plasma membranes cracked, and cell contents leaked) (Figure 2D & 2E).
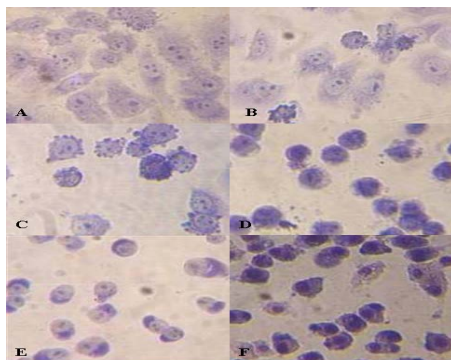
Fig. 2 Morphologic variation of HepG2 cells stained with Gimasa dye (TEM 10×40). Control group: **A.** HepG2 cells were performed by adding PBS into 1% serum- medium. Ethanol treated group: **B.** HepG2 cells were incubated with 2% ethanol for 24 h; **C.** Incubated with 4% ethanol for 24 h; **D.** Incubated with 6% ethanol for 24 h; **E.** Incubated with 8% ethanol for 24 h; **F.** Incubated with 10% ethanol for 24 h.

According to the results of Figure 2, 4% ethanol was selected for further analysis by the double-fluorescence staining technique with acridine orange (AO) and Hoechst 33342 (Figure 3). In the control group, HepG2 cells were orbicular and homogeneous while in the 4% ethanol groups, AO/Hoechst double staining showed condensed chromatin, nuclear fragmentation, nucleolus disappearance, increased nuclei fluorescence and the appearance of granular apoptotic bodies. Hence, this suggests that HepG2 cell apoptosis is induced by 4% ethanol, which could be used for further protemics analysis in HCC apoptotic processes.
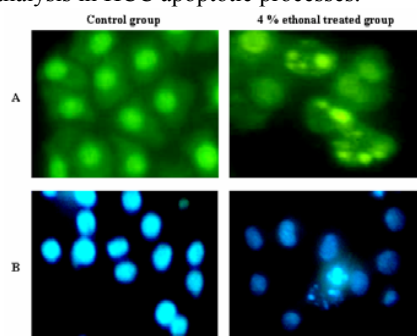

Fig. 3 Florescence micrographs of HepG2 cells incubated for 24 h treated with 4% ethanol (treated group) and without treatment (control group). **A.** Acridine orange (AO) stained; **B.** Hoechst 33342 stained.

C. 2-DE Image Analysis to Identify Protein in HepG2 Cells

Representative examples of cell proteins separated on a 2D-gel are shown in Figure 4A. 300 μg of total protein was extracted from HepG2 cells and loaded into each well. 600±68 protein spots were obtained in the range of 10-120 kDa and pI 3-10. Compared with the 2D map of hepatocarcinoma cell line (http://www.expasy.org/) the general characteristics that appeared in the 2D map of HepG2 cell, most of the proteins were expressed abundantly in the region of high molecular weight and acidic end.

By this strategy, HepG2 cell proteins were first identified by 2D-PAGE and protein expression changes between untreated and ethanol-treated were analyzed by SDS-PAGE.

D. SDS-PAGE Image Analysis to Estimate Changes of Protein Expression Stimulated by Ethanol Treatment in HepG2 Hepatocellular Carcinoma Cells

According to the results above, three representative concentrations of ethanol were selected for SDS-PAGE: 2%, 4%, 6%. Integrated images of SDS-PAGE for HepG2 cells untreated and treated with three ethanol concentrations are shown in Figure 4B. Generally, the bands of HepG2 cell treated with 2% ethanol were similar to those from natural HepG2 cell. The 4% ethanol group showed some difference from the untreated group, but the protein bands of the 6% ethanol group were very few and weakly stained. These results confirmed the conclusion above. Subsequent biological software analysis showed that two bands of HepG2 cell (~16 kDa and 68.5 kDa) appeared significantly down-regulated when ethanol was present (Figure 4C). Though the 68.5 kDa band was present in HepG2 cells and in 2% ethanol-treated HepG2 cell, the 16 kDa band was only in untreated HepG2 cells. Moreover, there are two prominent finding in HepG2 2D map, ~ 5.6/16.5 and 5.4/68.5 (pI /Mw). After reviewing protein expressed in HepG2 cells, we speculated survivin (pI/Mw: 5.66/16.388) and AFP (pI/Mw: 5.48/68.677) were two proteins down-regulated after ethanol [25-27].
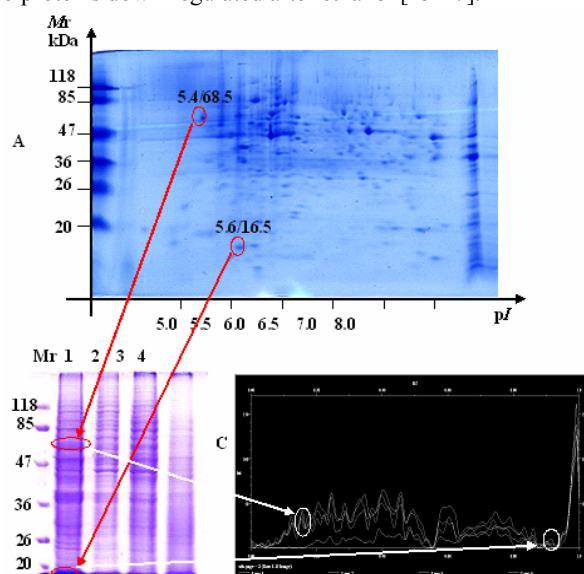

Fig. 4 A simple method to estimate the different proteins of HepG2 cells treated and untreated by ethanol. **A**. Two-dimensional electrophoresis map of human hepatocarcinoma cell line HepG2 with Coomassie Brilliant Blue staining. The image was analyzed by PDQuest software and the number of protein spots was about 600±68. **B**. Integrated images of SDS-PAGE (lane 1, HepG2 cells untreated; lane 2 HepG2 cells treated by 2% ethanol; lane 3, HepG2 cells treated by 4% ethanol, and lane 4, HepG2 cells treated by 6% ethanol). **C**. The optical density of each lane was compared by Quantity One software. Differentially proteins expressed were indicated from the spots and bands in the circles.

E. Immunocytochemistry and RT-PCR Analysis to Determine AFP and Survivin Protein Differential Expression and mRNA level

To determine if survivin and AFP were the two down-regulated proteins, immunocytochemistry using anti-survivin, and anti-AFP antisera was performed on HepG2 cells untreated and treated with 4% ethanol . In the normal group when only the secondary antibody was included and immunoreactivity for survivin and AFP was not observed in the HepG2 cells (Figure 5A & 5D). Compared with the normal group, in untreated HepG2 cells, immunoreactivity of survivin and AFP showed significantly higher levels (Figure 5B & 5E). This indicated the survivin and AFP are expressed in HepG2 cells. But after treated with 4% ethanol for 24 h, the immunoreactivity of survivin and AFP reached significantly lower levels in HepG2 cells (Figure 5C & 5F). These experiments demonstrate that both survivin and AFP proteins in HepG2 cells decrease due to the treatment of 4% ethanol, which is consistent with the hypothesis.

We also detected mRNA levels of AFP and Survivin using semiquantitative RT-PCR. The results confirmed that mRNA levels of AFP and Survivin were showed obviously lower in the treated HepG2 cells than untreated HepG2 cells (Figure 5G & 5H). The results indicated downregulation of AFP and Survivin expression was probably in the post-transcription level.
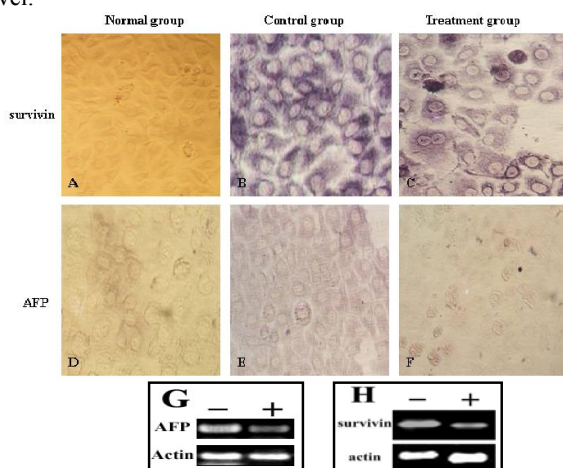

Fig. 5 Effects of ethanol on AFP and Survivin expression in HepG2 cells. (A-F) Immunocytochemical detection of the survivin and AFP in HepG2 cell. The expression of survivin and alpha-fetoprotein (AFP) in HepG2 were down-regulated by ethanol as immunocytochemical staining of survivin and AFP reduced. A & D: Normal group without a anti-survivin and anti-AFP antisera incubated prior to immunostaining. B & E: Control group, expression of survivin and AFP in HepG2 cultivated for 24 h. C & F: Treatment group, expression of survivin and AFP in HepG2 treated with 4% ethanol for 24 h. (G-H) The mRNA levels of AFP and Survivin in HepG2 cells untreated and treated by 4% ethanol for 24h. Actin was used as loading control. G: Message RNA expression of AFP in treated and untreated HepG2 cells. H: Message RNA expression of Survivin in treated and untreated HepG2 cells.

IV. DISCUSSION

In the past decade, percutaneous ethanol injection has been used to treat solitary or small (less than 4 cm in diameter) HCC tumors due to ethanol's ability to inhibit cellular proliferation [28-30]. Castaneda *et. al.* found that apoptosis of HepG2 cells can be induced by millimolar concentrations of ethanol in a dose- and time-dependent fashion [31, 32]. Our study further confirmed these results by MTT assay, Gimasa staining, and a double-fluorescence staining technique with AO and Hoechst 33342. The most notable signs of apoptosis were found in our model using HepG2 cells treated with 4% ethanol for 24 h. Furthermore, apoptosis of other Hepatocellular cell lines such as Bel7402, HepG3B, SK-Hep1 was also found when incubated with low concentrations of ethanol (data not shown). These results further confirm that anti-cancer treatments using low concentrations of ethanol can induce hepatocellular carcinoma cell apoptosis while avoiding toxic effects associated with higher ethanol concentrations [32]. A more in-depth understanding of apoptosis has provided the basis for novel anti-cancer therapies such as direct cancer cell apoptosis and increased sensitization to cytotoxic agents and radiation therapy [32]. Events such as modulation of caspase-3, activation of caspase-8, and increased oxidative stress are, in isolated findings, accompanied with ethanol-induced apoptosis [33, 34]. However, mechanisms of ethanol-induced apoptosis are not fully known in all cases.

HCC apoptosis is a multifactoral process involving the progressive accumulation of changes at the gene and protein level. Through proteomic technology, protein expression can be studied in any given cell, tissue or biofluid. As proteins perform most biological functions, proteomics bridges the gap between the information coded in the genome sequence and cellular behavior [35, 36]. For a number of years, 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE) followed by protein identification using mass spectrometry (MS) has been the primary proteomic technique. However, the technique has a number of well-recognized drawbacks. For 2D-PAGE, limitations include a lack of substantial workload, difficulty generating reproducible gels, and near incapacity to resolve several classes of proteins, such as very basic proteins, small proteins, and hydrophobic proteins [37]. The major disadvantage of MS is that individual proteins are often unidentifiable and are easily contaminated by hybridproteins. Faster and more straightforward means for proteomic analysis are urgently needed. Therefore, we designed a novel, convenient strategy for protein detection. This strategy primarily combines proteomic techniques and bioinformatic analysis to identify proteins more efficiently and effectively.

In recent years, expression maps of proteins in hepatoma cell lines have been constructed and biological information has been made available [37]. By our methods, a 2D-PAGE map of the hepatoma cell line HepG2 was obtained as a master gel. Compared to previous maps, we concluded that the 2DE maps achieved by our method comparable and valid. The molecular weight and isoelectric point for each band were analyzed using the PDQuest software and the resulting protein distribution in HepG2 was well characterized. Subsequently most proteins from HepG2 cells incubated with different

ethanol concentrations were isolated by SDS-PAGE. SDS-PAGE is a low-cost, reproducible, and rapid method for effectively comparing and characterizing proteins [38].

In this study, samples were compared and analyzed for dynamic apoptosis analysis by SDS-PAGE. From our SDS-PAGE maps, we found the following trends in HepG2 cells with different ethanol concentrations. Ethanol-induced HCC apoptosis was only observed in low ethanol concentrations and the optimal functional condition of ethanol was 4% for 24 hr incubation. Light density for each band was analyzed by the 1D software QuantityOne. Based on bioinformatics analysis and the 2D master map, the two bands most drastically changed should be AFP and survivin proteins. Currently, several biochemical methods serve as alternatives to conventional antibody-based immunocytochemistry in proximity ligation for high specificity detection. However, compared with immunocytochemistry, biochemical methods are more demanding technically and provide only limited information on protein distribution and subcellular localization [39]. Immunocytochemistry, which utilizes the affinity between antigens and antibodies, has advantages in detecting protein distribution and localization [40]. In this paper immunocytochemistry was applied to determine if AFP and Survivin were involved in ethanol-induced apoptosis. Down-regulation of AFP and Survivin was found using on 2-DE and SDS-PAGE. Furthermore, RT-PCR was used to determine the mRNA levels of AFP and Survivin. And message RNA expression of AFP and Survivin were also reduced, which can confirm the downregulation of these two proteins in post-transcription level. Thus, we developed a simple and effective method to analyze ethanol-induced apoptosis. The basic strategy was as follows: (1) a 2-DE map of cancer cells as a master gel was obtained to determine the distribution of proteins; (2) SDS-PAGE of samples in varying conditions was performed to analyze proteomic changes; (3) SDS-PAGE and 2DE maps were compared by biological software to propose proteins with changing expression levels during apoptosis; and (4) immunocytochemistry and RT-PCR were used to determine the identity of said proteins. Moreover, the two downregulated proteins, AFP and survivin have significant functions in HCC apoptosis. AFP is a tumor-associated fetal protein that is implicated hepatocellur carcinoma development and is a serum fetal defect/tumor marker used to monitor distress or disease progression. Although AFP itself may not be the only cause of altered cancer cell growth, some shock/stress-induced conformational-variant form of the protein may contribute to abnormal growth [41]. It has been documented that AFP may promote the HepG2 cell proliferation, increase FasL and TRAIL expression, and suppress Fas expression in hepatoma cells. FasL and Fas are a pair of proteins that can induced cell apoptosis [42-44]. Previously reported the Fas-receptor pathway plays a role in initiating of HepG2 cell ethanol-induced apoptosis [32]. Our data indicate that the AFP-producing cell line, HepG2, down-regulates AFP when incubated in low-concentrations of ethanol. Also, HepG2 apoptosis was regulated by the Fas-receptor pathway and Fas-L produced by ethanol-treated cells is thought to trigger apoptosis, so it could be suggested that AFP down-regulation should serve to avoid apoptosis. Survivin is a member of the inhibitor of apoptosis (IAP) gene family that has attracted attention from areas in translational research, as it is a multifunctional protein that inhibits apoptosis, regulates cell division and enhances angiogenesis. Survivin plays an important role in the suppression of apoptosis either directly or indirecly by inhibiting the activity of caspases, cell death proteases that induce apoptosis [45-47]. In the present study, survivin protein was down-regulated during hepatoma cell ethanol-induced apoptosis. Conway *et al.* provided evidence of indirect caspase activity regulation by survivin in the mitochondrial pathway of apoptosis using hepatocytes of heterozygous survivin- knockout mice. These cells contain low basal levels of activated procaspase-8, Bid, procaspase-9, and procaspase-3, and have increased susceptibility to Fas-induced apoptosis. Furthermore, survivin is one of the few proteins differentially expressed in tumor cells compared to most normal tissues [47-49]. As survivin protein expression is reduced in HepG2 cells treated with ethanol, this could play an essential role in regulation of correlative molecules in Fas-receptor apoptosis pathway.

V. CONCLUSIONS

Our data confirmed that low concentration ethanol could inhibit hepatocellular caricinoma (HepG2) cell proliferation and induce apoptosis. Our simple proteomic analysis procedure utilized in this apoptotic process was demonstrated to be useful for the identification of two down-regulated proteins: AFP and survivin. This strategy proves faster and more effective than the traditional proteomic methods such as MS. Although proteins could not be identified on a large scale using this method, it does prove useful for preliminary experiments, protein prime filtration, and dynamic process analysis. In the future, it will be crucial to gather as much proteomic analysis of cellular signaling as possible. Our novel proteomic strategy introduced here could prove valuable in this application.

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