ORIGINAL ARTICLE

Identification of Diagnostic Biomarker for Hepatocellular Carcinoma: A Proteomic Approach

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Abstract

Introduction: Hepatocellular carcinoma (HCC), a slow multistep process eventually starts from long-term inflammation to fibrosis and leads to malignancy. These variations cause several genes disrupted that play role many signaling pathways involved in HCC. Early diagnosis and treatment of hepatocarcinogenesis depend on efficient biomarker discovery. To date, only two specific markers of HCC are identified, AFP and AFP-L3, but assessment and validation of these markers are still left in large cohorts of patients. Proteomic approach aids to develop new promising biomarker for diagnosis liver cancer at early stage. **Materials and Methods:** Animal model of HCC was developed. Histopathological and biochemical estimations were performed during disease progression. Proteomic analysis was done for the identification of biomarker at early stage. **Results:** cAMP-dependent protein kinase inhibitor alpha (PKIA) was identified using one-dimensional electrophoresis (1DE) and 2DE followed by matrix-assisted laser desorption and ionization time-of-flight mass spectrometry. **Conclusion:** The present study concludes the identification of potential biomarker, cAMP-dependent PKIA. It was hypothesized that PKIA is useful for detection of HCC at initial stage.

Key words: Hepatocellular Carcinoma, cAMP-dependent protein kinase inhibitor alpha, 2D Electrophoresis, MALDI-TOF/MS

INTRODUCTION

Proteomic analysis of hepatocellular carcinoma (HCC) has opened new vista for the identification of novel diagnostic, prognostic biomarker, and disease-specific associated proteins that are potential therapeutic targets in the treatment of HCC. Biomarkers are biological molecules that are indicators of physiologic state and also of change during a disease process. The utility of a biomarker lies in its ability to provide an early indication of the disease, to monitor disease progression, to provide ease of detection, and to provide a factor measurable across populations. HCC arises from the hepatocytes, the major cell type in the liver. According to the World Health Organization, HCC is one of the leading causes of death worldwide accounting for 13% of all cancer-related deaths. Liver cancers accounted for 780,000 deaths in 2012 and were the 2nd leading cause of cancer-related deaths, exceeded only by cancers of the lungs and stomach.[1] It is the fifth most common cancer in the world with a 5 years survival rate of <5% and an incidence of at least 1 million new patients per year.[2] The prevalence of this disease has been seen mostly in parts of Africa and Asia, but Western world has also shown drastic increase in the same. There is urgent need of novel biomarker for detection of HCC.[3]

Proteomic approaches can play an important role in the discovery of diagnostic and prognostic biomarkers. Ultimately

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Received: 28-02-2018
Revised: 18-03-2018
Accepted: 26-03-2018

such biomarkers would aid clinicians in diagnosing liver cancer in the early stages, eliminating the need for liver biopsy, and allowing early treatment outcomes, thereby preventing the progression of fibrosis.[4] With the advancements in whole genome, a significant breakthrough has been explored in molecular progression of HCC. Enhanced activity of protein kinases is hallmark of hepatocarcinogenesis. Protein kinase A (PKA) is one of them, significantly contributes in liver tumerogenesis by stimulating cyclic AMP; therefore, its inhibitor, cAMP-dependent protein kinase inhibitor alpha (PKIA) plays a major role in inhibition of hepatic tumerogenesis.[5]

In view of this, the present study was undertaken to unravel the novel biomarker for diagnosis of liver cancer using proteomic approach including one-dimensional electrophoresis (1DE), 2DE, and matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS). We have examined the expression of cAMP-dependent PKIA which inhibits the function of PKA and can be used for HCC diagnosis and therapy purpose.

MATERIALS AND METHODS

Experimental protocol

Animal model development

Animal model was established by protocol of Malik *et al.*[6]

Animal groups

Animals were divided into two groups, each group comprising six animals.

Control (C)

Normal control rats provided with standard diet and pure drinking water throughout the experiment.

Treated (T)

Rats were induced with HCC by given of single dose of diethylnitrosamine (DEN) (100 mg/kg body weight [BW], i.p). After 1 week of recovery, 2-acetylaminofluorene (AAF) (150 mg/kg BW, orally) was given for 6 days. The rat was sacrificed through cervical decapitation after anesthetized with diethyl ether. The liver was excised and stored in 10% formalin buffer for histopathological analysis.

Biochemical estimations

Liver function test

Liver function tests (aminotransferase [ALT], alkaline phosphatase [ALP], and aspartate aminotransferase [AST]) will be performed using span diagnostic kits following the protocol defined in kit manual.

Antioxidants assays

The catalase assay was performed according to the method of Claiborne,[7] and activity was calculated in terms of nmole H_2O_2 consumed/min/mg protein and extinction coefficient used 0.081×10^3 M^{-1}/cm. The lipid peroxidation was assayed according to the method of Ohkawa *et al.*[8] The level of lipid peroxidation was expressed as nmol of malondialdehyde (MDA). Total glutathione (GSH) was measured according to protocol by Jollow *et al.*[9] Enzyme activity was further calculated as nmole 1-Chloro-2,4-dinitrobenzene conjugate formed/min/mg protein using molar extinction coefficient of 9.6×10^3 M^{-1}/cm.

Histopathological examinations

The histopathological changes were observed by tissue slides formed by hematoxylin and eosin staining. The histopathological photomicrographs were taken by Olympus CKX41SF inverted microscope system (Olympus, Japan).

Proteomics

Protein extraction from liver tissues

Liver tissue homogenate is prepared by following the protocol. 0.1 g of liver tissue was taken in 1 ml PBS. Tissue was homogenized by homogenizer and centrifuge at 8000 rpm for 10 min and supernatant was collected. Total protein was analyzed by Bradford method.

1DE and 2DE polyacrylamide gel electrophoresis (PAGE)

Protein samples were rehydrated followed by isoelectric focusing done using Ettan IPGphor 3 from GE Healthcare (USA) to immobilize the proteins on the basis of their pI value. Later, on proteins were separated on SDS-PAGE gel according to molecular weight. The protein spots were visualized using silver staining. The gel imaging was done using Epson Expression 11000XL Scanner. Image Master 2D Platinum-7.0 (GE Healthcare) was used for spot detection and quantification as well as comparisons of control, diseased, and treated. The control spots were considered as reference for protein matching. Spots which were either present in one group only or demonstrated substantial changes in its expression profile as compared to control were selected for MALDI analysis. The spot volume for each protein was quantified by calculation of spots intensity and area after normalization each spot.

Protein identification

MALDI-TOF MS

The trypsin digested sample was mixed with matrix solution containing 10 mg/ml α-cyano-4-hydroxycinnamic acid in

30% acetonitrile and 0.05% trifluoroacetic acid. The mixture was air-dried on a MALDI sample plate in triplicate. Mass spectra were obtained utilizing an Applied Biosystems Voyager-DE STR instrument. MS spectrum was generated which was corresponding to a specific peptide sequence and searched for matched peptides using MASCOT software (Matrix Science). Later, the National Center for Biotechnology (NCBIprot) database was explored for *Rattus* specific taxonomy search. The multiple independently sequenced peptides from the same protein database were used to identify protein and the data were checked for consistent error distribution. In Mascot, on the basis of mass value (m/Z ratio) for each peptide, probability (P)-based scoring was done and similarity observed between experimental data and matched data sequence was used for protein identification.

RESULTS

HCC model development

The successful HCC model was developed using DEN and AAF within 4 months. The outcomes of various biochemical estimations, histopathological study, and parameters such as loss in BW and loss of hair confirm the successful progression of animal model.

Histopathological analysis of liver

Histopathological analysis was performed to confirm the disease progression. We have observed normal architecture of hepatocytes in control group [Figure 1a and b]. In diseased group, vacuolated hepatocytes with various sizes of nuclear area and maximum vacuolated cells near portal and central vein observed which are early sign of premalignant cells. The clear adenoma separated by thin septae was seen in disease group which shows complete progression of HCC in liver tissue [Figure 1c and d].

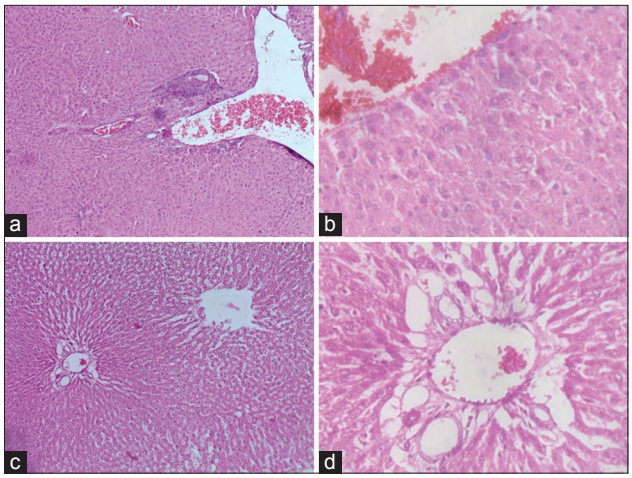

Figure 1: Histopathological analysis: (a) Control group (HE ×100) showing normal hepatoparenchyma, portal triad, and normal central vein, (c) same section of control group showing normal bile duct proliferation with normal portal triad (HE ×400), (b) treated group with diethylnitrosamine and 2-acetylaminofluorene showing thick inflammatory septae have formed between portal triads and central veins breaking up the liver lobule and creating hepatocytic nodules (HE ×100), (d) same section showing septa formed of mainly inflammatory cells adjoining a hepatocytic nodule. No atypical features are seen in the hepatocytes in this sample (HE ×400)

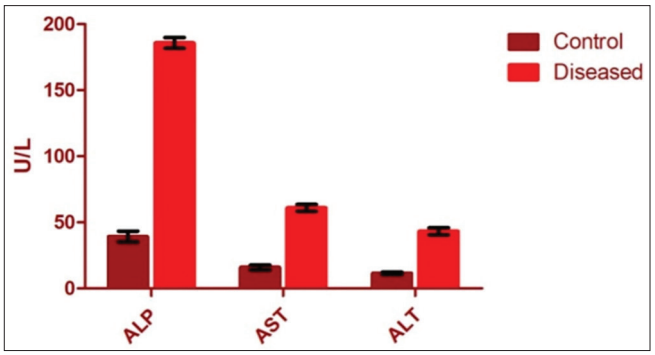

Figure 2: Liver function tests: Alkaline phosphatase, aspartate aminotransferase, and aminotransferase were performed from serum after 4th month toxin treatment from both groups

Biochemical estimation

Liver function test

ALP level was observed 39.3 U/L in control group, whereas a significant increase (185.7 U/L) was observed in diseased group. AST and ALT enzyme levels were significantly in diseased group by 60.996 and 43.31 U/L as compared to control levels (15.9 and 11.49 U/L), respectively [Figure 2].

Antioxidant assays

In the present study, increased level of MDA was observed in DEN + AAF model, the control group showed 1.059 nmol/h/mg tissue, whereas diseased group showed a significant increase in MDA level, 5.29 nmol/h/mg tissue [Figure 3a]. In the control group, consumption of H_2O_2 by catalase enzyme was 21.27 nmol H_2O_2 consumed/min/mg proteins, however, decreased level of catalase activity was observed in diseased group (11.75 nmol H_2O_2 consumed/min/mg protein) [Figure 3b]. The level of GSH decreased in diseased group by 0.254 µmol/g tissue as compared to control group (1.30 µmol/g tissue) [Figure 3c].

Proteomic analysis

1DE analysis

1DE was performed for screening of changes in protein profile of HCC progression in control versus diseased group. We have observed differentially expressed low and high molecular weight proteins from 2nd month of treatment onward [Figure 4].

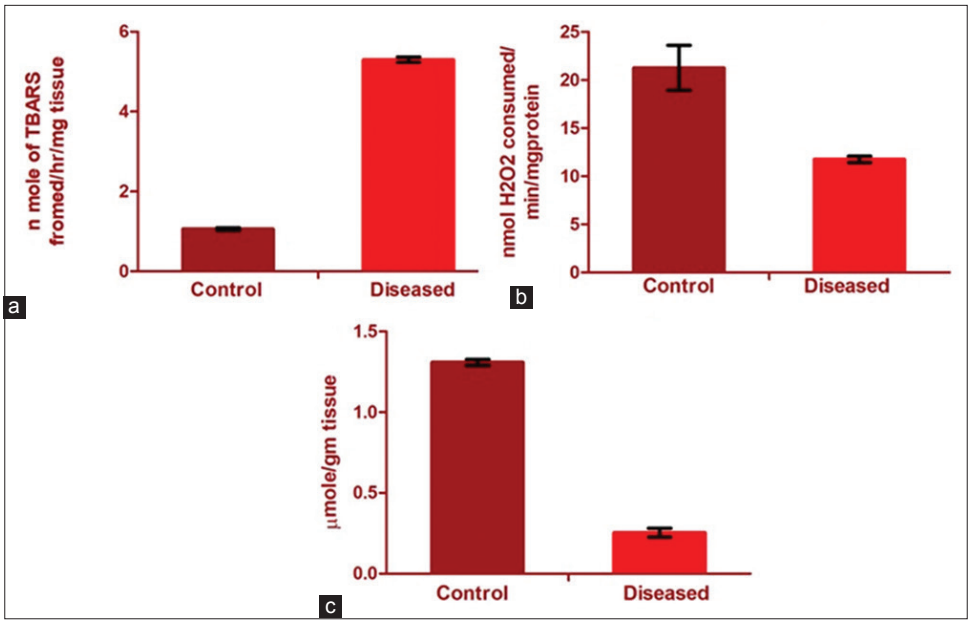

Figure 3: Biochemical analysis of antioxidant assays from tissue. Malondialdehyde (MDA) level (a), catalase (b), and glutathione (GSH) (c) were performed of the subjects from both groups to observe the oxidative stress in liver tissue with the progression of disease. MDA level is increased, and total GSH and catalase activity are significantly decreased

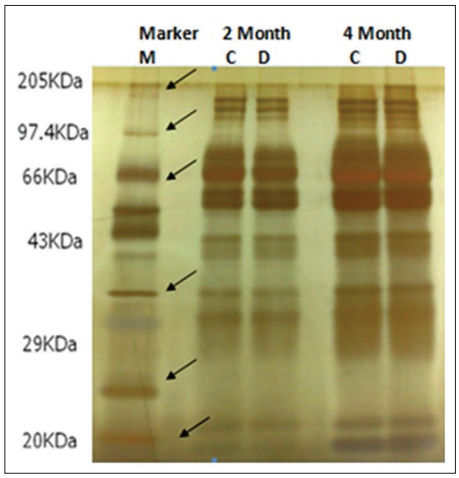

Figure 4: One-dimensional electrophoresis: Lane 1 showing protein markers bands, Lane 2 and 3 showing differentially expressed proteins of control and diseased (2nd month), respectively. Lane 4 and 5 showing differentially expressed proteins of control and diseased (4th month), respectively

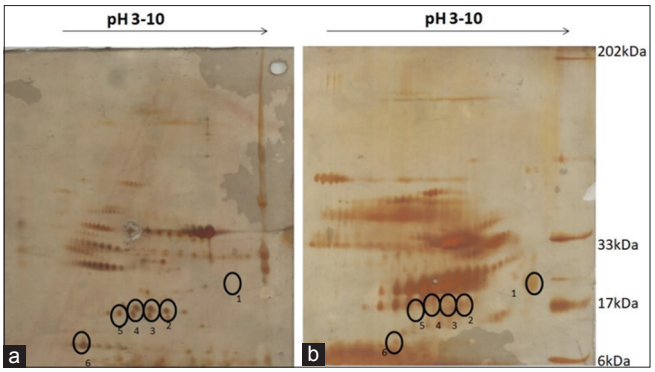

Figure 5: Two-dimensional electrophoresis (2DE): (a) Protein expression profile of control group (b) diseased 2DE gel of 4th month showing differentially expressed proteins

2DE analysis

2D electrophoresis was performed for separation of protein on the basis of isoelectric point (pI) and molecular weight. The pattern of 2DE gel showed significant change in protein profile of control versus diseased group. We have observed some differentially expressed spots (Spot 1-6) in diseased group in comparison with control group. We have picked spot 6 for MALDI-TOF MS because it was found highly overexpressed in the diseased gel [Figure 5a and 5b].

Identification of spot 6 by MALDI-TOF MS

The selected spot from diseased and control group was subjected for trypsin digestion followed by MALDI-TOF MS for the identification of protein. The acquired MS spectrum was used as fingerprint to search protein in a database named NCBIprot. The MASCOT database used for the identification of protein through mass fingerprinting [Figure 6a and b]. The probability of repetition of proteins appeared in the database with different name and accession number. Only *Rattus* genus was selected to ensure similarity list contains protein specific to particular species. The spot 6 was identified as cAMP-dependent PKIA.

DISCUSSION

Proteomic studies provide the analysis and evaluation of complete set of proteins expressed in a given cell, tissue, or biofluid, characterize protein expression profiles and also identify protein structures, localizations, activities, modifications, and interactions in physiological or

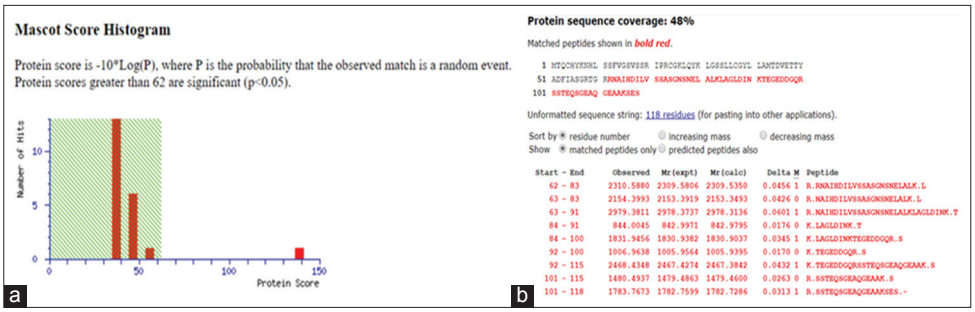

Figure 6: (a) Histogram showing Mowse score of identified protein AMP-dependent protein kinase inhibitor alpha (PKIA). (b) Matched peptide to PKIA showing in red

pathological states. As proteins perform majority of the biological functions, proteomics bridges the gap between the information coded in the genome sequence and cellular behavior.[10] Liver carcinogenesis is a multistep process characterized by the accumulation of successive molecular genetics and epigenetic alternation, structural aberrations including deletions and translocations resulting in selection of clonal cells with uncontrolled growth capacities. Proteomic is a novel approach to understand the tumorigenesis events of HCC.[11]

In the present work, we have successfully developed animal model using DEN and 2-AAF. The histopathological examinations showed several nodules and adenomas in hepatic tissue which was clear sign of HCC. The number and size of vacuolated cells increased and type hepatocytes foci, adenomas separated by clear septa were seen with disease progression. Similar histopathological changes have been seen using same carcinogen by other researchers also.[8,12,13]

Disease progression was further supported by biochemical estimations confirming liver abnormalities. Elevated level of liver function enzymes (ALT, AST, and ALP) was observed in disease progression also supported the biochemical changes observed in the present study.[12] The cellular damage can be measured using antioxidant parameters such as lipid peroxidation, total GSH level, and catalase activity. Enhanced lipid peroxidation can be measured in terms of MDA level,[14] and similar finding has been seen with disease progression after DEN/2AAF treatment. Moreover, HCC causes reduction of GSH and CAT activity in liver.[14] Similarly, we have also observed a significant depletion in both the assays. In the present study, protein profiles were compared between control and HCC serum using proteomic approach. 1DE and 2DE profile of the sample DEN + AAF (200 mg/kg BW) showed corresponding changes in the same regions. We have observed some upregulated and downregulated proteins in our proteomic analysis. A significant downregulation was found in spot 6 with respect to control 2DE gel, and we have identified this spot as cAMP-dependent PKIA, which play a key role to inhibit PKA in hepatic cell growth. In accordance to our study, earlier study evidenced that regulation of PKA by cAMP has a significant role in cell proliferation and differentiation exhibited in the extracellular space.[5] Taken together our results, this inhibitor can be used for innovative approach for cancer diagnosis and therapeutics.

ACKNOWLEDGMENT

All the authors would like to thank Dr. Ashok K. Chauhan, Founder President, Amity University, Noida, Uttar Pradesh, India, for providing the infrastructure and support.

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Source of Support: Nil. **Conflict of Interest:** None declared.