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Liver Proliferating Cell Nuclear Antigen, BAX/Bcl-2 Ratio, Collagen, and Polysaccharide Accumulation as Diagnostic Tools in Experimental Hepatocellular Carcinoma

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Abstract

Objectives: This experimental study was conducted to look for a sensitive diagnostic panel for early detection of hepatocellular carcinoma (HCC).

Methods: Combination of diethyl nitrosamine (DENA, 200 mg/kg, IP, once), two weeks later, CCl_4 (3 ml/kg/week), subcutaneously, for 6 weeks) induced HCC in rats. Sixteen male Wistar rats were divided into 2 groups, control, and HCC.

Results: DENA plus CCl_4 elevated serum alpha-fetoprotein, liver enzyme activity and depressed superoxide dismutase, reduced glutathione (GSH), elevated malondialdehyde. Also, depressed caspase-3, elevated collagen, polysaccharide accumulation, proliferating cell nuclear antigen in liver tissues, and depressed BAX/Bcl-2 ratio.

Conclusion: Decreased BAX/Bcl-2 ratio, elevated collagen deposition, polysaccharide accumulation, nuclear proliferation, and tissue oxidative stress help in the early diagnosis of liver cancer.

Keywords: Hepatocellular carcinoma, caspase-3, cellular proliferation, apoptosis, collagen, polysaccharides

List of Abbreviations: AFP: Alpha-fetoprotein; DENA: Diethyl nitrosamine; GSH: Reduced glutathione; HCC: Hepatocellular carcinoma; H&E: Hematoxylin/Eosin; IP: Intraperitoneal; MDA: Malondialdehyde; NO: Nitric oxide; PCNA: Proliferating cell nuclear antigen; ROS: reactive oxygen species; SOD: Superoxide dismutase.

Introduction

Liver cancer is the fifth most common cancer and the second most frequent cause of cancer-related death in the world. Hepatocellular carcinoma (HCC) has become one of the major causes of morbidity and mortality all over the world.[1,2] HCC accounts for 80% to 90% of primary liver cancer.[3] It is the second leading cause of cancer deaths worldwide, with over 500,000 people affected per year.[4] Animal models have enabled the study of the mechanism of HCC and the development of possible strategies for treatment. Diethyl nitrosamine (DENA) is a representative chemical carcinogen with the potential to cause tumors in various organs, including the liver, skin, gastrointestinal tract, and respiratory system.[5] Specifically, in HCC, DENA is a complete carcinogen. Many lines of evidence have demonstrated a relationship between carcinogenesis and cell cycle regulation. DENA induces irreversible HCC through overexpression of G1/S-phase regulatory proteins in rats.[6] DENA is a suitable model in the field of experimental hepatic pathology because nitrite and nitrosamine synthesis is increased in human viral hepatitis,[7] DENA induced lesions, as well as tumors in rodents, show marked biochemical, histological, and molecular similarity to the progression of HCC in humans.[8]

DENA induced lesions, as well as tumors in rodents, show marked biochemical, histological, and molecular similarity to the progression of HCC in humans.[9] The hepatotoxicity of CCl_4 is mainly exerted in two different levels: first, CCl_4 induction of cytochrome P450 and the consequently increased formation of reactive oxygen species (ROS).[10]

The Bcl-2 family is the best-characterized protein family involved in the regulation of apoptotic cell death. It includes both anti-apoptotic members (i.e Bcl-2) and pro-apoptotic members (i.e Bax), thus the Bcl-2 family acts as a critical life-death decision point makers in the common pathway of apoptosis.[11] Although acute inflammation is a part of the defense response, persistent inflammation can lead to certain diseases as cancer and diabetes.[12]

Apoptosis represents a physiological way to eliminate excess cells during both liver development and regeneration it is a highly preserved and controlled mechanism to achieve tissue homeostasis through targeted elimination of single cells without disrupting the biological functionality of the tissue. The morphological changes associated with apoptosis include nuclear condensation, cell shrinkage, and plasma membrane blebbing resulting in apoptotic bodies,[13] it is a highly preserved and controlled mechanism to achieve tissue homeostasis through targeted elimination of single cells without disrupting the biological functionality of the tissue. The morphological changes associated with apoptosis include nuclear condensation, cell shrinkage, and plasma membrane blebbing resulting in apoptotic bodies.[14] Many therapeutic modalities have been developed relying on surgery, chemotherapy, radiotherapy, hormone therapy, and more recently immunotherapy.[15]

The present work aims to determine some serum and tissue changes during hepatocarcinogenesis, looking for more reproducible and specific markers for HCC in an experimental model. Along with serum AFP, tissue oxidative stress, tissue changes of collagen fiber, nuclear proliferation, and

polysaccharide accumulation, using histochemical stains to get more early and cost-effective diagnostic tools for HCC.

Materials and Methods

Chemicals

Diethyl nitrosamine was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Carbon tetrachloride was purchased from SD Fine-Chem. Ltd. (Mumbai, India). All other chemicals used were of analytical grade.

Animals and Experimental Design

Principles of Laboratory Animal Care (NIH Publication Vol 25, No. 28 revised 1996; (**http://grants.nih.gov/grants/guide/notice-files/not96-208.html**) were followed. The experimental design was approved by the Institutional Animal Ethics Committee guidelines for animal care and use at Port Said University, Egypt, under 354 for 2016. Sixteen adult male albino rats weighing 100–150 g were recruited for the study purpose. The animals were housed in polyethylene cages in a moderately humid room under a controlled 12 hours light/dark cycle, kept under constant environmental and nutritional conditions with free access to food and water ad libitum. Animals were kept for 2 weeks for acclimatization before starting the experiment). Group I, received only saline (twice/week), IP, for 8 weeks (control), Group II, received only the carcinogenic combination (HCC group), single-dose diethyl nitrosamine (DENA, 200 mg/kg), was intraperitoneally injected, two weeks later 50% V/V, carbon tetrachloride solution in olive oil (3 ml/kg/week, IP, for 6 weeks) was injected. The total experiment period was two months.

Blood Sample Collection

At the end of the experiment, rats were anesthetized with light diethyl ether. Blood samples were obtained directly after decapitation of animals, centrifuged at 2500 rpm for 10 min after standing for 45 minutes at 4°C. The obtained sera were kept at −80°C right biochemical investigations.

Liver Tissue Sampling

Liver tissues were collected, washed by cold saline, blotted by tissue paper, dissected into 2 parts, one kept in formal saline for tissue microscopical studies, the second portion was homogenized in formalin/saline (10% w/v), then, homogenates were frozen at −80°C right tissue biochemical assays.

Biochemical Investigations

Serum Investigations

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, and gamma-glutamyl transferase (GGT) activities, albumin, and total protein levels were carried out using CHEMOLYZER 510 semi-automated analyzer. Alpha-fetoprotein (AFP) level was determined by using the rat AFP ELISA sandwich technique.[16] Measurements were performed according to the manufacturer's instructions.

Liver Tissue Investigations

Spectrophotometric assays for malondialdehyde (MDA), nitric oxide (NO), reduced glutathione (GSH) contents, and superoxide dismutase (SOD) activity in liver tissue homogenates were executed, using kits purchased from Biodiagnostic, Cairo, Egypt. Liver mRNA was extracted for detection of both BAX and Bcl-2 by real-time polymerase chain reaction (RT-PCR).

We used the mRNA extraction protocol established by.[17] About 0.5 g of liver tissue was used for mRNA isolation using Trizo lM, according to the manufacturer's instructions (Thermofisher, Cat. NO. 15596026, USA). cDNA reaction was performed using bio-systems kits, and the given primers (Table 1), according to the manufacturer's instructions. For real-time quantitative PCR, 5 µl of the first-strand cDNA was used in a total volume of 25 µl, containing 12.5 µl 2x SYBR Green PCR Master Mix (Bio-Rad, Script, USA) and 200 ng of each given primer. PCR reaction was repeated for 40 cycles. The data were computed with the ABI Prism 7500 sequence detection system software. Relative expression of studied genes (BAX/Bcl-2) was calculated using the comparative threshold cycle method. All values were normalized to the beta-actin genes.

Histopathological Study

Histological examination was done using H&E stains,[18] an immunohistochemical study using Masson's Trichrome (to test collagen accumulation),[19] an immunohistochemical study using Masson's Trichrome (to test collagen accumulation),[20] proliferating cell nuclear antigen (PCNA) monoclonal antibody[21] and caspase-3 monoclonal antibody to figure out the accumulation of pro-apoptotic caspase-3 (an inactive form of caspase) in liver tissue.[22]

Statistical Analysis

Data were statistically analyzed using SPSS, the USA, the data of the cancer group were compared to the control group and expressed as mean ± SE. One-way ANOVA and unpaired *t*-test were carried out to find if there was any significant difference among control and treated groups.

Results

Biochemical Studies on Serum, and Liver Tissue in Studied Groups

The hepatocarcinogenic combination, significantly up-regulated liver enzyme activity, alongside, significantly decreased both serum albumin and T. protein. It also significantly elevated serum AFP and oxidative stress in liver tissue by depressing GSH, SOD activity, with an increase of MDA and NO contents. Carcinogenesis significantly aborted apoptotic/survival ratio. Both effects were calculated in comparison to normal control results (Table 2).

Table 1. **The sequence of the primers used was as follow**

Primer	Sequence
BAX	Forward: 5′-GCT CTG AACAGA TCA TGA AG-3′; Reverse: 5′-GAT GGT CAC TGT CTGCCA TG-3′.
Bcl-2	Forward: 5′-GAC TTT CTC TCC TAC AAGC-3′; Reverse: 5′-CGA AAG AGT TCA TTC ACT AC-3′.
B-actin	Forward: 5′-CAA CGG CTC CGG CAT GTG C-3′; down: Reverse: 5′-CTC TTG CTC TGG GCC TCG-3′.

Table 2. **Variations in serum liver indices, hepatic tissue redox status, apoptotic member/survival member (*BAX/Bcl-2*) ratio, against serum alpha fetoprotein, in experimental HCC, compared to control group (values are expressed as Mean ± SE, *n* = 8)**

Parameter	Normal control	HCC group
ALT (U/l)	39.3 ± 1.4	138.1 ± 5.9 a***
AST (U/l)	104.7 ± 5.7	287.1 ± 12.6 a***
GGT (U/l)	10.1 ± 0.33	21 ± 1.58 a***
ALP (U/l)	141.7 ± 4.8	563 ± 24.6 a***
Albumin (g/dl)	3.8 ± 0.14	2.37 ± 0.1 a***
T. Protein (g/dl)	6.4 ± 0.21	4.725 ± 0.103 a***
AFP (ng/ml)	4.17 ± 0.09	46.2 ± 1.30 a***
SOD (U/g tissue)	1848.2 ± 5.8	1051.2 ± 27.1 a***
GSH (mg/g tissue)	759.4 ± 37.1	493.8 ± 22.0 a***
MDA (nmol/g tissue)	62.6 ± 4.1	224.1 ± 9.6 a***
NO (µmol/g tissue)	11.6 ± 0.5	64.3 ± 1.6 a***
Tissue apoptotic/survival (*BAX/Bcl-2*) ratio	0.47 ± 0.15	0.17 ± 0.02 b***

P-value represents the difference between control and HCC groups, * ($P < 0.05$), * ($P < 0.01$), ** ($P < 0.001$).

Gross and Histological Examination of Liver in Studied Groups

Gross examination of the liver showed that a combination of DENA/CCl4 induced apparent morphological changes, as enlarged liver with scattered nodules, while the control liver was in normal small and homogenous architecture (Figure 1, C, D). H&E tissue staining showed normal hepatic lobular architecture, granulated cytoplasm, and uniform nuclei in control rats. HCC group showed disarrangement of hepatic cords, clusters of large eosinophilic hyperplastic foci, large, hyperplastic hepatocytes, and pleomorphic hyperchromatic nuclei (Figure 1, A1,2-B1,2).

Histochemical Studies of Liver Tissue in Studied Groups

Liver sections stained with Masson's Trichrome showed normal distribution of the collagen fibers in control rats. HCC group showed infiltration of collagen fibers in the interstitium of hyperplastic foci, (Figure 2, A, B). Liver sections stained with periodic acid–Schiff (PAS), showed normal polysaccharide distribution in normal control while, dense polysaccharide accumulation was apparent in the HCC group, (Figure 2, C, D).

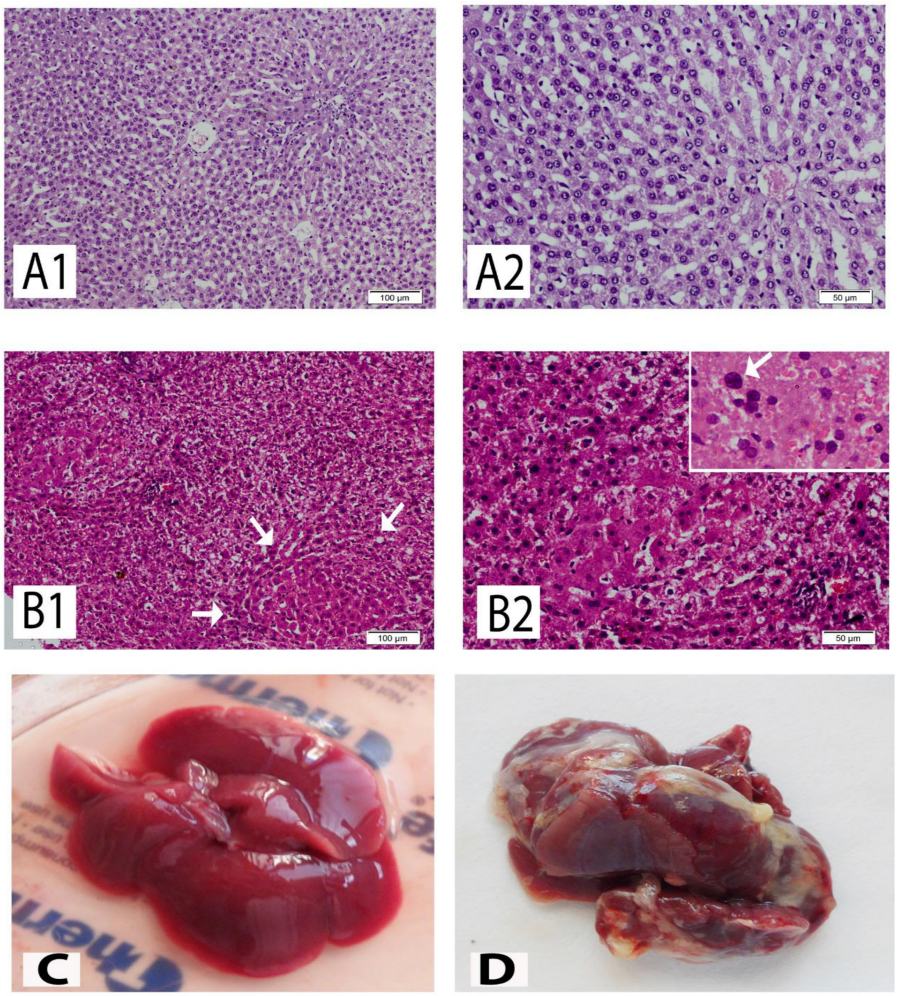

Fig. 1 **H&E tissue staining showed normal hepatic lobular architecture, granulated cytoplasm and uniform nuclei in control rats (A1,2). HCC group showed disarrangement of hepatic cords with clusters of large eosinophilic hyperplastic foci (arrow), large, hyperplastic hepatocytic cells (magnified) with abundant eosinophilic cytoplasm and pleomorphic hyperchromatic nuclei (B1,2). Liver morphology of HCC liver model compare with normal tissues. Normal control rats (C), showed normal pattern with reddish colored appearance. HCC group (D), showed enlarged liver with scattered nodules.**

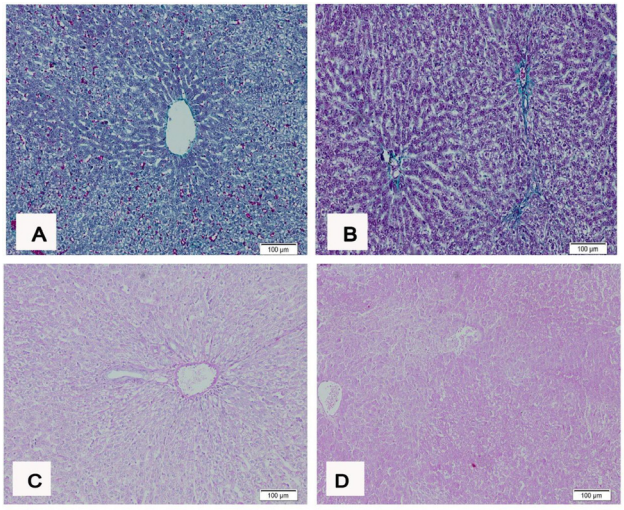

Fig. 2 **Photomicrographs of liver sections stained with Masson's Trichrome, showed normal distribution of the collagen fibers in control rats (A), HCC group showed infiltration of collagen fibers in the interstitium of hyperplastic foci (B). Photomicrographs of liver sections stained with periodic acid–Schiff (PAS), showed no polysaccharide accumulation in normal control (C), while the highest polysaccharide accumulation was apparent in HCC group (D).**

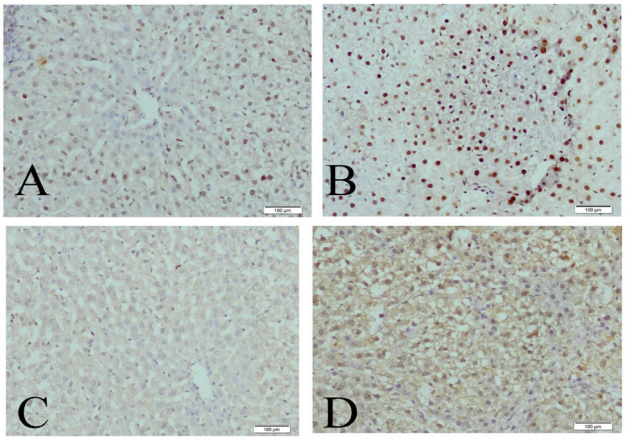

Fig. 3 **Photomicrographs of liver tissue incubated with proliferating cell nuclear antigen (PCNA) monoclonal antibody, showed normal tissue in control (A), but HCC group showed high cellular proliferation reaction (B). Photomicrographs of liver tissue incubated with caspase-3 monoclonal antibody showed no pro-apoptotic caspase-3 (inactive form of caspase) accumulation in control (C), but HCC group showed excessive pro-apoptotic caspase-3 accumulation, meaning, aborted apoptosis (D).**

Liver tissue incubated with proliferating cell nuclear antigen (PCNA) monoclonal antibody exhibited normal tissue in the control group but the HCC group showed high cellular proliferation reaction (Figure 3, A, B). Liver tissue incubated with caspase-3 monoclonal antibody showed no pro-apoptotic caspase-3 (inactive form of caspase) accumulation in control, HCC group showed excessive pro-apoptotic caspase-3 accumulation, indicating aborted apoptosis. (Figure 3, C, D).

Discussion

Hepatocellular carcinoma is the second leading cause of cancer deaths worldwide, with more than 500,000 people affected annually.[4] Diethyl nitrosamine (DENA) is a common carcinogen in the field of experimental hepatocarcinogenesis because nitrite and nitrosamine synthesis is increased in human viral hepatitis,[7] which is a major risk factor for HCC.[8]

In the present study, animals treated with DENA followed by CCl_4 exhibited a significant depression of body weight with increased liver weights. Microscopically, the effect of this dose on the different biochemical, molecular and histological parameters was assessed.

Biochemical parameters including serum transaminases (AST and ALT), ALP, and γ-GT are known indicators for liver function and their elevated activities are sensitive markers for hepatic injury. In our study, we found that AST, ALT, ALP, and γ-GT activities, were highly elevated in animals treated with DENA/CCl_4 combination. These results were reported sometime before.[23] Elevated activities of serum AST and ALT in DENA/CCl4 treated rats are attributed to induced hepatic damage and subsequent leakage of these enzymes from the neoplastic cell into circulation.[24]

The generation of reactive oxygen species (ROS) is apparent during the metabolic biotransformation of DENA resulting in oxidative stress. This leads to carcinogenesis by several mechanisms including DNA, lipid, and protein damage, change in intracellular signaling pathways, and even changes in gene expression. Together, these oxidative modifications promote abnormal cell growth and transformation.[25] Oxidative stress is a phenomenon caused by an imbalance between production and accumulation of ROS in cells and tissues and the inability of a biological system to detoxify these reactive products initiates imbalance that leads to cell and tissue damage.[26] Oxidative stress was recently registered to be a potential modulator in hepatocarcinogenesis.[27,28] In addition, reduced glutathione (GSH), a well-known non-enzymatic intracellular antioxidant is found at high concentrations in the liver and has key functions in protection against free radicals, peroxides, and other toxic components. Our results revealed that MA elevations and depressed tissue GSH content were comparable to some previous observations elsewhere.[29,30] Apoptosis is the programmed cell death that maintains the healthy survival/death balance in metazoan cells. Defects in apoptosis can cause cancer or autoimmunity, while enhanced apoptosis may cause degenerative diseases. The apoptotic signals contribute to safeguarding the genomic integrity while defective apoptosis may promote carcinogenesis. The tumor cells may use several molecular mechanisms to suppress apoptosis and acquire resistance to apoptotic agents, for example, by the expression of anti-apoptotic proteins such as Bcl-2 or by the downregulation or mutation of proapoptotic proteins such as BAX.[31] Apoptosis ends up with proteolysis and DNA degradation as a result of caspase (an apoptotic factor) activation. This activation may be initiated through mitochondrial-dependent or independent mediators.[32] Moreover, gene expression profile for two major pro and antiapoptotic (survival) genes, BAX and Bcl-2, revealed that such DNA damage could be a consequence of HCC induction that aborted caspase-3 conversion from pro- into an active form to kill cancer cells.[33]

Moreover, gene expression profile for two major pro and antiapoptotic (survival) genes, BAX and Bcl-2, revealed that such DNA damage could be a consequence of HCC induction that aborted caspase-3 conversion from pro- into an active form to kill cancer cells.[34] The crucial involvement of

PCNA in cellular proliferation and its tight association with cancer transformation resulted in the frequent use of PCNA as a diagnostic and prognostic cell-cycle marker.[35] In the current work, DENA-treated animals showed an increased level of PCNA which is an indication of hyperproliferative activity. Caspases are crucial mediators of apoptosis; among them, caspase 3 is a principal enzyme in the apoptotic cascade and is often used to detect apoptotic activity.[36] The present study highlighted caspase-3 monoclonal antibodies to check the accumulation of pro-apoptotic caspase-3 (an inactive form of caspase) in liver tissue. This showed a statistical increase in the HCC group.

In our study, histopathological as well as immunostaining data confirmed the hepatotoxic effect of DENA/CCl_4 on the liver tissue level, through a significant increase in both polysaccharide and collagen deposition in hepatocytes. The collagen deposition tested by Masson trichrome staining was significantly increased in the livers of the HCC group, these results agree with the Moon group, which used Masson trichrome staining to estimate the condensation of collagen fiber in hepatocellular carcinoma.[37] Periodic acid-Schiff (PAS) staining was used to determine the accumulation of intra-cytoplasmic glycogen since well-differentiated HCCs PAS staining is usually strongly positive in liver cancers.[38]

Conclusion

Our study assumes that elevated serum alpha-fetoprotein, liver enzyme activity and increased tissue oxidative stress markers (depressed superoxide dismutase, SOD, and glutathione, GSH, with elevated malondialdehyde, MDA), liver size, weight, and morphology are prominent indicators for hepatocarcinogenesis. An efficient tissue diagnostic panel was postulated through cancer progression markers, indicated by depressed caspase-3 (apoptotic marker), extensively deposited collagen fibers, polysaccharides, and nuclear proliferation in liver tissues. Liver tissue also showed depressed apoptotic defense, reflected by decreased BAX/Bcl-2 ratio.

Conflicts of Interest/Competing Interests

Authors declare no conflict of interest.

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