Sakr *et al.*
Egyptian Journal of Medical Human Genetics (2022) 23:14
https://doi.org/10.1186/s43042-022-00230-y

Egyptian Journal of Medical Human Genetics

RESEARCH Open Access

The correlation between *P53* and *COX-2* expression and the pathological alteration in hepatocellular carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is among the highest life-threatening malignancies. On both a molecular and histological level, HCC is a highly heterogeneous malignancy. This study was aimed to study the correlation between the molecular expression of some molecular biomarkers (*P53* and *Cox-2)* and the histopathological alterations in the chemically induced HCC by Diethylnitrosamine (DEN) in Adult female Rats. The liver tumor induction was done by injection of DEN intraperitoneally one, two and three times/week for 2 months by the dose of 50 mg/kg Bw. The histopathological analysis was done and expression level of *P53* and *cox-2* was detected by quantitative polymerase chain reaction (qRT-PCR) at the end of the experiment.

Results: In this study, Grossly, livers of the groups administered with DEN showed multiple grayish-white macronodules on the outer surface which is dose dependent. Histopathologically, DEN induce multifocal micronodules of hepatocellular carcinoma which characterized by nuclear atypia**,** clear cell, mitotic figures and necrosis of hepatocytes. *P53* mRNA expression to *GAPDH*, revealed that, there was a statistically significant decrease in HCC groups compared to healthy control group, while *Cox-2* mRNA expression was significantly increased in HCC groups than healthy control group.

Conclusions: HCC staging can be achieved by detection the expression of *P53*, and *Cox-2 as* molecular markers as it considers noninvasive, rapid and easy method than the histopathological analysis. Finally, *Cox-2* could be a therapeutic candidate for HCC due to the higher expression of *Cox-2* in HCC lesions.

Keywords: Rats, HCC, Diethylnitrosamine (DEN), P53, Cox-2

Background

Hepatocellular carcinoma (HCC), the main liver cancer type, is a widespread malignant tumor, which seriously make a health problem of people worldwide [1]. Because of its high mortality rate (95%), it accounts the most second reason of the worldwide tumor-related death [2]. In Egypt, HCC is among the most prevalent tumor types. It considers the second most common widespread men malignancy and the fifth in women, this results in, liver cancer is the most cause of deaths in Egypt compared to other cancer types [3]. Liver cirrhosis and chronic hepatitis have been identified as essential HCC development risk factors. Survival and prognosis of HCC are still poor, mainly because of late stage diagnosis and/or disease recurrence [4]. HCC, the leading type of liver malignancy, has a number of recognized risk factors, such as chronic hepatitis C virus (HCV) and hepatitis B virus (HBV) infections, autoimmune hepatitis, alcohol abuse, obesity, diabetes mellitus, aflatoxin-B1, and several

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metabolic diseases. In developed countries, there has been an increase in HCC incidence partly attributed to obesity, diabetes, HCV, and diethylnitrosamine (DEN) [5, 6].

DEN is a familiar hepatocarcinogenic agent presents in cured and fried meals, ground water, tobacco smoke, cheddar cheese, occupational settings, alcoholic beverages, agriculture chemicals, cosmetics, and pharmaceutical products [7]. The rodent HCC model by DEN-induction, especially rat is thought to be among well-known and commonly used laboratory models for hepatocarcinogenesis study [8]. DEN has been utilized to produce lesions in rodent tissues that imitate various types of malignant and benign tumors in human [9]. Particularly, after DEN injection to an animal body, it is manipulated in liver cells, causing DNA damage produced by reactions in hepatocytes [10], which is correlated with oxidative stress. The metabolizing signaling pathways in rodent models induced by DEN are the same to that of humankind [11]. Consequently, the DEN usage has become highly desirable for experiments that are designed to understand the pathogenetic changes triggering the progression of HCC. To accurately reflect human disease for both basic studies of tumour biology and experimental treatment reasons, a well-defined liver cancer model that mimics the actual condition is required [12]. Rats are useful to understand the pathogenesis and evaluation of liver cancer, searching effective anticancer treatment (drug, hepatectomy, and liver transplantation) and designing anticancer prevention strategies, though it is associated with high mortality in the processes of tumor induction [13]. Different HCC morphological characteristics have recently been linked to the various genetic abnormalities and biological mechanisms that cause tumour growth [14]. In this study we selected two molecular biomarkers (p53 and Cox-2) due to their important role in signaling pathways of cancer cell developments.

The tumor suppressor *p53* gene is a pro-apoptotic and potent growth-suppressive factor, which protecting organisms from cancer development by playing a vital role in cellular apoptosis [15]. *P53* is a powerful transcription factor, controlling expression of genes which regulate its tumor suppression functions, as DNA repair, arrest of cell cycle, senescence, and apoptosis [16]. *P53* is stimulated in response action to cellular stresses, such as DNA damage and oxidative stress [17]. The tumour suppressor p53 is activated by a variety of stress signals that a cell may face throughout the evolution of cancer, including genotoxic damage, oncogene activation, and hypoxia. The major way by which p53 destroys cancer cells is through inducing apoptosis or senescence. *p53* can also stop cancer from spreading through a variety of additional mechanisms. *p53* has been found to promote autophagy and act as an antioxidant to prevent DNA damage and genomic instability by inhibiting mTOR signaling [18–20]. *P53* full genetic inactivation is correlated with HCC genomic instability which is accelerated by the constant potential proliferation arising by DNA damage signaling activation [21].

Cyclooxygenase-2 (*COX-2*), an inducible enzyme in prostaglandin (PG) synthesis pathway, is among the potential cellular components which has been believed to be correlated with carcinogenesis in different type of cancers. COX-2 is linked to a number of tumour progression mechanisms, including tumour cell growth stimulation, tumour cell apoptosis inhibition, tumour angiogenesis promotion, and tumour invasion and metastasis enhancement [22]. COX-2 can induce proliferation and angiogenesis via p53, p27 and vascular endothelial growth factor and also inhibit apoptosis by inducing the antiapoptotic factor Bcl-2, as well as activating antiapoptotic signaling through protein kinase B pathway in HCC [23]. *COX-2* overexpression might be among the primary factors in hepatocarcinogenesis. In human, overexpression of *COX-2* in HCC tissues had been observed in the well-differentiated (early stages), indicating that, it has an etiological function in hepatocarcinogenesis [24].

The development of a classification system for HCC that incorporates morphology and molecular alterations is critical because it may help us to i) better understand the natural history and mechanisms of carcinogenesis, ii) improve diagnosis and prognosis, and finally iii) facilitate the development of personalized medicine by identifying tumour entities that may respond to specific treatments. The limitations of previous studied as the most common genetic changes and tumour subtypes are now well understood, and authors starting to learn how they connect to HCC phenotypic and histological features. Unfortunately, unlike in other cancers such as lung or colorectal cancer, this growing understanding has not yet led to the development of biomarkers or improved clinical management. So, the goal of our experiments was to use different doses of intraperitoneal injection of DEN/week for promoting a high rate of liver tumorigenesis in rat and detect the hepatocarcinogenesis stage depending on molecular biomarkers expression (*Cox-2,* and *p53*) and their concomitant histopathological alterations.

Methods

Animals

Adult Wistar female Rats, aged 3–4 months, weighing 220 g, were obtained from the National Institute of Ophthalmology, Giza, Egypt. Rats were kept in polypropylene cages in groups of five rats in each cage under normal laboratory environments at the animal house.

The animals were kept in (20–25 °C) temperature range and relative humidity between 45 and 55% with 12 h dark–light cycle. The animals could acclimatize for one week at 22 °C temperature before the start of the experiments, and all animals were provided free access water and standard laboratory feed. Animal experimentation Approval was attained from Institutional ethics committee for animal care and use for education and scientific research. The in vivo animal experiments were done in agreement with the Guide for the Care and Use of Laboratory Animals (National Research Council 1996).

Chemicals

Diethylnitrosamine was purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Other high analytical grade chemicals and reagents such as (ALT, AST, Total protein and Albumin assays kits) were supplied from standard commercial suppliers (Analysis, Egypt).

Experimental protocols

Forty-eight animals (rats) were grouped randomly into four groups and each group contains 12 animals [25]. DEN was prepared by diluted 1 ml of stock DEN with 99 ml 0.9% NaCl saline solution to make 10 mg/ml solution concentration. The experimental study design and injection protocol were as follows: Animal in group 1 (G1) (untreated control group); were intraperitoneally injected by saline weakly for 8 weeks. Animal in group 2 (G2), group 3 (G3), and group 4 (G4) were injected with DEN (50 mg/kg body weight (BW) intraperitoneally by single dose, two doses, or three doses weakly, respectively, for 8 weeks. At the end of the experiment (60 days), the animals were killed. The flowchart of the methodology (Fig. 1).

Collection of samples

At the end of the experiment end, the retro-orbital plexus puncture was used to collect the blood samples from each animal. The collected blood was maintained to coagulate for 15 min at room temperature. The blood samples were centrifugation at 3000 rpm at 20 °C to separate and obtain the sera which kept frozen at − 30 °C for different biochemical analysis. After that, the animals were killed and the livers were dissected, washed with saline (isotonic ice-cold), dried by clean filter paper, and divided into two portions for histological investigation in 10% formalin solution and neutral buffer for qRT-PCR. The bodies of the dead animals were disposed according to local authorities to pose a minimal health risk.

Biochemical parameters study

The serum levels of the biochemical parameters such as alanine transaminase (ALT) and aspartate transaminase (AST) activities were measured according to Reitman

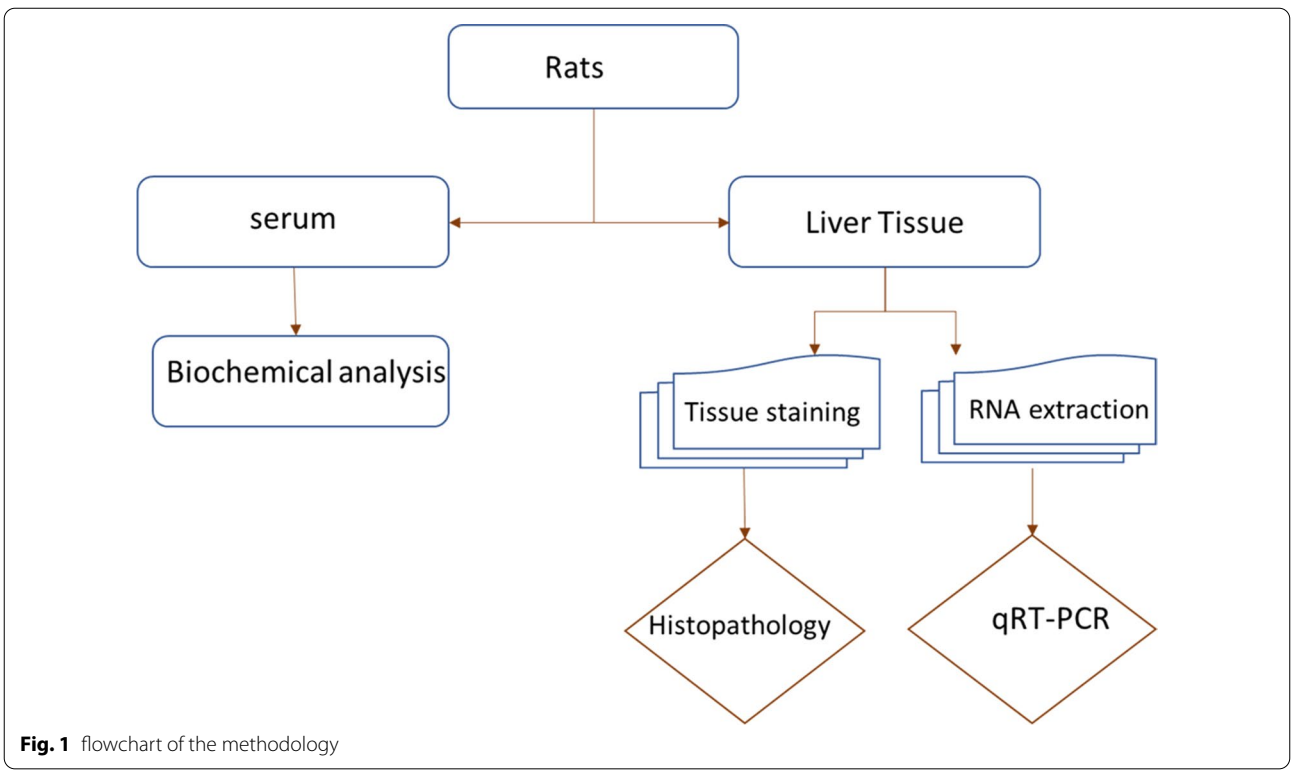

Fig. 1 flowchart of the methodology

and Frankel method [26]. Total protein (TP), albumin and total bilirubin (T.BIL) were assessed according to Gornal et al. [27], Doumas et al. [28], and Young et al. [29], respectively. All reagent and kits were supplied from Bio-system. S.A. Barcelona, Spain.

qRT-PCR for P53 and COX-2

RNA isolation from liver tissues was separated by Pure Link RNA mini kit purchased from (Thermo Fisher Inc., NY, USA) in accordance of the manufacture instructions. The amount and purity of purified RNA was determined using Nanodrop. The purified RNA was transcribed to cDNA using HiSenScript $RH^{(-)}$ cDNA synthesis kit (iNtRon, Korea. It optimized for a Reverse Transcription (RT ase) reaction that can utilize any amount of total RNA from 1 µg per reaction and is applicable for the synthesis of full-length first strand cDNA. The following rat primers' pair sequences were used for RT-PCR in this study: p53 forward – 5′-ACAGCGTGGTGGTACCGTAT-3′ reverse – 5′- GGAGCTGTTGCACATGTACT-3′; Cox-2 –Forward-5-GATTGACAGCCCACCAACTT-3-Reverse-5 CGGGATGAACTCTCTCCTCA-3 and β-actin forward – 5′-AGAGCTATGAGCTGCCTGAC-3′ reverse – 5′-AATTGAATGTAGTTTCATGGATG-3′. The qRT-PCR assessment was done in the ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems) using SYBR green dye master mix (RealMOD Green Real-time PCR 2 × Master Mix Kit, iNtRon, Korea). The comparative $2^{-\Delta\Delta Ct}$ equation was used for relative quantification analyzes of P53 and Cox-2 gene expression equalized to β-actin (housekeeping gene).

Histopathological study

Anesthesia/Euthanasia Methods used in the study as follow, Rats were placed in a plexiglass chamber with 3.9% halothane (Zeneca, Cheshire, UK) for 5 min, and decapitated when fully sedated, as measured by a lack of active paw reflex. Livers were examined grossly, and the detected gross pathological changes were recorded and photographed by using CANON IXY digital camera. Parts from liver tissues were removed and then fixed in 10% formalin solution, neutral buffer (NBF) for histopathological investigation. Samples were dehydrated, after fixation for 72 h, embedded in paraffin blocks and cut (3 µm) slices for hematoxylin and eosin staining (H&E). Histological photographs were taken by using Leica EC3 high speed digital camera.

Statistical analysis

The statistical package for social sciences SPSS/computer program (ver. 20.0) was used for statistical analysis of our results. All variables were calculated as Mean ± SD and then ANOVA test was used for analysis (one-way analysis of variance). $p < 0.05$ considered to be statistically significant.

Results

The body weight and the mortality rate

The body weight changes were recorded during the experiment time (8 weeks). In DEN injected groups demonstrated that, after one week following DEN injection, the growth rates began to become slow, and the mean weight gradually decreased as compared with the control group. In this study, no mortality rate was recorded in G1 (the control group) during the experiment period (8 weeks). But, the mortality rate of groups administered by DEN was 20%, 30% and 40.00% in G2, G3, and G4, respectively. Figure 2 shows the representative macroscopic characteristics of the liver tissue of the four studied groups. In control animals (G1), no malignancy was observed, and the histology of the liver showed normal characterization. In DEN-administrated rats (G2, G3, and G4), the liver showed multiple tinny grayish white HCC nodules.

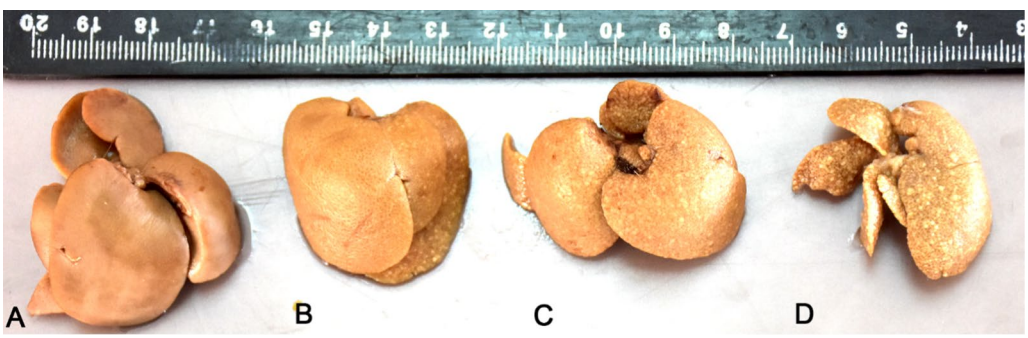

Fig. 2 Liver, rat. **A** Control group (G1), normal hepatic appearance. **B** Group 2; **C** group 3; **D** group 4, Macronodular hepatocellular carcinoma in which liver parenchyma were replaced by many macronodules. Note that, the nodules in group 3 is more prominent than of group 2 and less prominent than of group 4

Table 1 Laboratory findings between the studied groups

Variables	Control Mean ± SD	A Mean ± SD	B Mean ± SD	C Mean ± SD	*p*-value
Biochemical					
AST (IU/L)	$148.3333^{a} \pm 10.26$	$214.4000^{b} \pm 27.0$	$271.2500^{b} \pm 7.18$	$351.0^{c} \pm 26.6$	0.00*
ALT (IU/L)	$74.0000^{a} \pm 3.3466$	$150.8000^{b} \pm 15.21$	$211.2500^{c} \pm 25.76$	$285.8^{d} \pm 19.5$	0.004*
T. protein	$9.7667^{c} \pm 0.32728$	$7.8200^{ab} \pm 0.36249$	$8.3000^{b} \pm 0.83964$	$6.8000^{a} \pm 0.27$	0.005*
Alb (g/dL)	$5.0500^{c} \pm 0.11180$	$4.4400^{b} \pm 0.13638$	$4.0250^{b} \pm 0.21360$	$3.5400^{a} \pm 0.18$	0.00*
T.BIL (mg/dL)	$0.8417^{b} \pm 0.06112$	$.6700^{a} \pm 0.08602$	$0.6250^{a} \pm 0.08539$	$0.6520^{a} \pm 0.075$	0.00*
D.BIL (mg/dL)	$0.0633^{a} \pm 0.01706$	$0.1160^{b} \pm 0.02502$	$0.1525^{b} \pm 0.01652$	$0.1100^{b} \pm 0.00$	0.00*

Groups bearing different letters are significantly different from each other at $p < 0.05$* show

Table 2 Relative expression of p53, and Cox-2 between the study groups

Variables	Control Mean ± SE	A Mean ± SD	B Mean ± SD	C Mean ± SD	*p*-value
P53 relative expression	$3.6392^{a} \pm 0.97421$	$3.1815^{a} \pm 0.40127$	$2.5543^{b} \pm 0.59973$	$2.222^{b} \pm 0.56$	0.00*
COX-2 relative expression	$0.9924^{a} \pm 0.09244$	$1.2297^{a} \pm 0.08979$	$1.4059^{b} \pm 0.12451$	$1.5502^{b} \pm 0.34$	0.004*

Groups bearing different letters are significantly different from each other at $p < 0.05$* show

Liver function parameters in serum

The values of the biochemical variables of the groups under study are collected in Table 1. Liver function testes showed that, levels of AST, ALT, and bilirubin, were significantly higher in DEN treated groups (G2, G3, and G4) than G1 (control group), whereas there were significant decrease in the albumin and total bilirubin levels in HCC groups than control group possibly due to liver tumors development.

COX2 and p53 expression levels analysis

The normal hepatocytes differ from the preneoplastic hepatocytes in most hepatocarcinogenesis models according to the expression of various mediators, which the gene expression of some are upregulated, while the others are downregulated [30]. In this regard, the effect of DEN on *Cox-2* and the protooncogene *p53* expression in liver tissue were assessed. The level of the *p53* and cox-2mRNA expression was detected in animal liver tissues and tested by qRT-PCR in DEN treated animals and control group (Table 2, Fig. 3). *P53* gene expression was down-regulated by 1.14-fold, 1.42-fold, and 1.63-fold in G2, G3, and G3, respectively, compared to G1 (control group). The expression of *P53* gene was significantly down-regulated in G3, G4 compared to G1, and G2 ($p < 0.00$). While according to the fold change of *Cox-2* mRNA expression to GAPDH, there was a statistically significant upregulation by 1.23-fold, 1.4-fold and 1.56-fold in G2, G3, G4, respectively, compared to G1 group ($p < 0.00$). This result suggests that, *p53*, and *cox-2* level may be considered as a potential tumor biomarker to distinguish between the HCC tissue from the non and early stage of HCC.

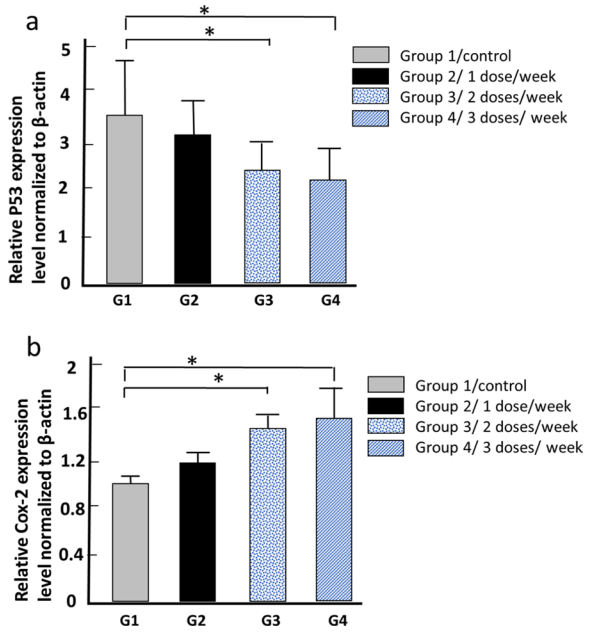

Fig. 3 The relative mRNA expression of P53 (**A**), and Cox-2 (**B**) by RT-PCR of the rats liver tissues. (*) represent a significant difference at $p < 0.05$

Histopathologically

Normal histological features were recorded of the liver tissues of G1 normal control group. The central vein characterized by thin wall with normal hepatocytes producing the hepatic cords which arise from the central vein and surroundings by small blood spaces named as sinusoids in which Kupffer cells are lined, while The histological examination of the liver sections of (G2) Showed multifocal areas of hepatocellular carcinoma which characterized by multiple micronodules of well-differentiated hepatocytes separated by a small area of undifferentiated hepatocellular carcinoma (nuclear atypia Fig. 4A–B), accumulation of fat globules inside hepatocyte, macrophage engulfing bile pigments, and coagulative necrosis of hepatocyte. Mitosis and well-characterized nucleoli in the neoplastic hepatocytes were observed. Additionally, dilated bile canaliculus, macrophage engulfing fat globules, and hydropic degeneration of hepatocyte were also detected (Fig. 4A–B). In G 3, the same with G2 with addition of cholangiocarcinoma and hemorrhages, mitosis and pseudoglandular pattern were recorded (Fig. 4C–D). In G4, the same pattern of histopathological changes of G2, and G3 (Fig. 4E–F). These results suggest that, there are significant histopathological changes between the DEN-induced HCC groups when compared to control group, but there no sharp demarcation between the HCC groups histopathologically.

Collectively these results suggest for the first time that, there are correlation between the histopathological alteration of the HCC and the expression level of *p53* and *cox-2* thus it could be noninvasive molecular markers for early detection of HCC.

Discussion

Hepatocellular carcinoma (HCC) considers a greatly heterogeneous liver disorder with extremely different risk factors, involving RNA viruses as (HCV) or DNA as (HBV), chemicals (aflatoxins and diethylnitrosamine), and acquired or inherited metabolic disorders [31]. Besides, HCC develops a dynamic procedure that changing the morphological elements. So far, the hepatocarcinogenesis mechanisms might be varied according to many factors. The most obvious factors as well as uncontrol of cell cycle, senescence control escaping, phenotypic plasticity, resistance to cell apoptosis, invasion, and metastasis [31]. Because the liver is used to induct cancer as a primary site in the carcinogenesis bioassays, a necessity for extrapolation of animal malignancy happening in the same site in human is highly needed. The benefit of identifying universal biomarkers of malignancy will help in detecting proper interspecies extrapolation and used combined different biomarkers to improve the diagnosis process [32, 33].

The ideal biomarker for HCC is that candidate which lets asymptomatic patients to be diagnosed and commonly utilized in the screening procedure. Generally, a precious biomarker for use in patients must attains a degree of specificity and sensitivity of $\geq 90\%$, non-invasive and cheap enough to allow extensive use [34].

The high HCC prevalence is usually correlated to high exposure to common etiological factors including chemical compounds exposure such as *N*-nitrosamine which has teratogenic, carcinogenic and mutagenic properties [35]. Reactive oxygen species (ROS) accumulation in the hepatocytes has usually been associated with DEN exposure [36], which could resulting in oxidative DNA damage. In Particular, DEN has been extensively utilized to induce experimental hepatocellular carcinoma [33]. Now, to induce hepatic tumors in rat, many laboratories use different DEN doses, time intervals, application routes, and tumour promoters.

The goal of this research was to investigate the multistep hepatocarcinogenesis caused by DEN injection from the histopathological and the association between the expression of *P53* and *COX-2* genes and the pathological alteration in hepatocellular carcinoma.

In the present study, all animals in experimental groups were weighted before treatment and every week during the application. The results obtained show that, the rats body weights of the HCC group induced by DEN were significantly decreased when compared with the healthy untreated group. Rats in HCC groups induced by DEN show appetite decrease; hence, the weight loss could be happened due to direct cause from the decrease in food intake or indirect from liver function deterioration after development of the HCC. Findings have indicated that, Liver malignancy results in a progressive and rapid body weight loss, particularly adipose tissue and skeletal muscle loss, with comparative maintenance of abdominal proteins. This loss can be described primarily by enhanced protein breakdown [37].

AST, ALT, ALP, and bilirubin activity correspond to hepatic cell function, and elevated values of these enzymes are vulnerable signs of liver cell injury [38]. ALT and AST are directly associated with the formation of ketoacids from the amino acids and they increase in HCC-inducible conditions [39]. In our study, liver enzyme measurements were increased (ALP, AST, ALT) in DEN groups compared with the control group. Chen et al. [40] revealed that an elevation of plasma ALT, AST, GGT, α-l-fucosidase and ALP may due to hepatic destruction. ALP is directly correlated to the hepatic canalicular area lipid membrane. Higher concentration of ALP indicates the bile flow pathological alterations; that is, intrahepatic or extrahepatic bile flow any intervention precedes to

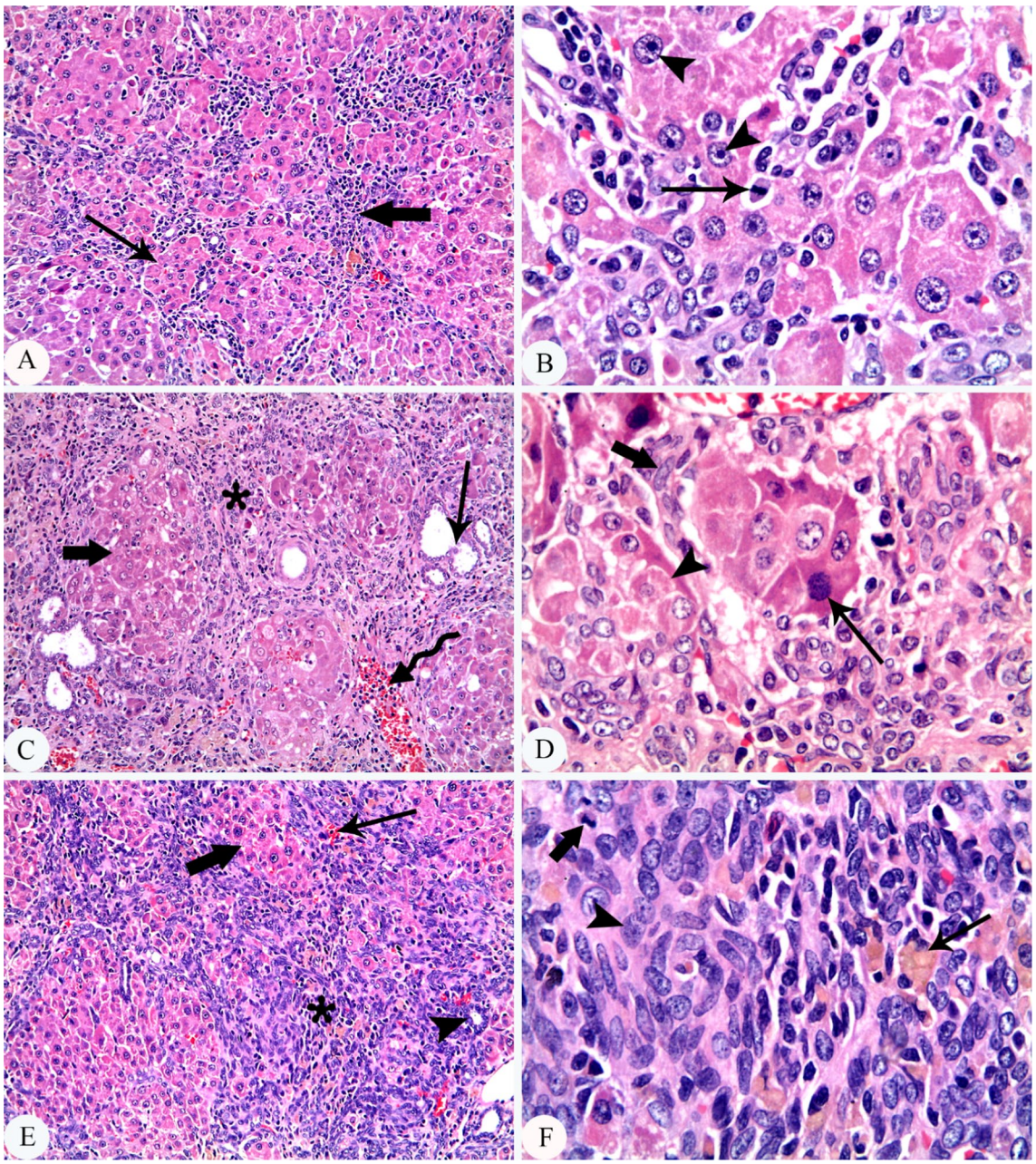

Fig. 4 Liver, rat. **A** and **B** Group 2. **A** Showing hepatocellular carcinoma which characterized by multiple micronodules of will differentiated hepatocytes (thin arrow) separated by undifferentiated hepatocellular carcinoma (thick arrow). **B** Showing hepatocellular carcinoma which characterized by mitotic figures (thin arrow) and prominent nucleoli (arrowhead) in hepatocytes. **C** and **D** Group 3. **C** Showing hepatocellular carcinoma which characterized by multiple micronodules (thin arrow) which separated by undifferentiated hepatocellular carcinoma (asterisk). Additionally, Cholangiocarcinoma (arrow) and hemorrhages (bended arrow) can be observed. D) Showing hepatocellular carcinoma which characterized by undifferentiated neoplastic cells (thick arrow), mitosis (thin arrow), and pseudoglandular pattern (arrowhead). **E** and **F** Group 4. **E** Showing hepatocellular carcinoma which characterized by multiple micronodules (thick arrow) which separated by a wide areas of undifferentiated hepatocellular carcinoma (asterisk). Additionally, Cholangiocarcinoma (arrowhead) and hemorrhages (thin arrow) can be observed. **F** Showing hepatocellular carcinoma which characterized by the presence of mitosis (thick arrow), undifferentiated neoplastic cells (arrowhead), and macrophages containing bile pigment (thin arrow). *HE* stain. **A**, **C** and **E** × 20; **B**, **D** and **F** × 40

elevation of the plasma concentration of ALP [41]. In this study, the level of ALP was significantly increased in DEN-induced groups compared to normal group. Our result come concomitant with Al-Rejaie et al. [42] who compared the normal group with DEN injection and showed that, an elevation of plasma ALP, ALT and GGT concentrations of 219%, 316%, and 152%, respectively, 8 weeks after their experiment.

We macroscopically observed multiple tiny grayish nodules in the liver of the DEN induced Group with shrinking of the liver due to necrosis of liver parenchyma. Microscopely, in DEN-induced groups, vascular congestion, sinusoidal degeneration and dilatation, portal areas lymphocyte infiltration, dysplastic changes in the fibrosis parenchyma, disruption of the lobules, cellular atypia, necrosis and tumor foci were detected. This results were concomitant with the report by Bishayee and Dhir [43]. Kadasa et al. [44] described that liver histopathological slides of rats in the normal group revealed normal hepatocytes with normal sinusoidal structure and hepatic lobe organization. In DEN induced HCC animals (a 200 mg/kg DEN single dose), they reported focal substitution of portal areas mainly collapsed membranes, large vesicular nuclei, an insufficient basophilic cytoplasm, and a multiple mitosis event [45]. Khan et al. [45] found that after DEN administration in rats (male albino Wistar) the liver's characteristic histological features were dispersed or injured. This result showed the series of events beginning with oxidative stress and going to damage of the membrane, cellular atypia, inflammatory cell infiltration, and subsequently HCC development. In our result, we showed that a single dose of 50 mg/kg B.W for 8 weeks is enough to develop HCC lesions and no histological differences was observed when used more doses of injection. Therefore, we tried to distinguish between the HCC stages by detecting proper molecular markers.

The key event of the tumor proliferation process is apoptosis loss of tumor cells. This event is regulated by sets of anti- and pro-apoptotic markers [46]. The tumor suppressor *P53* protein was recorded to be implicated in the hepatocarcinogenesis early stages of [47, 48]. In the present study, *P53* gene expression was downregulated in the liver of HCC inducted animals compared to untreated group and this low expression is significant only between the G3, G4 compared to G1, and G2 groups. This mean p53 expression can be used to detect the early from the progressive HCC stages. This downregulation may be essential for apoptosis resistance and could have an effect on hepatocarcinogenesis induced by DEN injection. *P53* suppresses tumors by molecular pathways including the inhibition of cell growth through *P21* gene activation and the induction of apoptosis leading to death of cancerous cells [49]. Our findings come with the results of Zhang and Yu [50] who stated that injection of DEN in rats leads to *P53* gene expression down-regulation.

COX is the key enzyme, which has a vital role in inflammation. Expression Induction of *COX-2* by mitogenic and inflammatory stimuli resulting in increased prostaglandin (PG) synthesis in cancerous and inflamed tissues. Overexpression *of COX-2* has been identified in HCC [24, 51, 52] and it has carcinogenic effects [53, 54] achieved directly or either by regulate cellular growth via producing mediators. Angiogenesis can also induce by cox-2 via vascular endothelial growth factor (VEGF) and PG production [55, 56] and prevent cell death by *Bcl-2,* moreover stimulating cascading of antiapoptotic through AKT pathway. In our study, the *COX-2* expression was upregulated in DEN treatment groups compared to control and the overexpression is only significant between the G3, G4 compared to G1, and G2 groups. Collectively, these results suggest that the *p53* and *Cox-2* expression could be used to distinguish between progressive and early stages of HCC.

Conclusions

Discovering the molecular mechanisms explaining the incidence and development of HCC is important for us to achieve a more comprehensive knowledge of the disease process and to recognize more efficient therapeutic strategies and targets. Therefore, in the present study suggested that, the injection of two doses of DEN per week (8 weeks) enough to make a significant histopathological hepatotumorigenesis. *P53* gene expression was down-regulated in HCC induced groups than the control, while according to the fold change of *Cox-2* mRNA expression to GAPDH, there was a statistically significant upregulation in HCC groups compared control. Hence, *p33* and *cox-2* might be used as molecular markers to identify the progressive HCC stage from early one which concomitantly not identified by histopathological screening. Finally, *COX-2* might be an HCC therapeutic target due to is high expression level in HCC lesions. Integrative pathological and molecular studies should be encouraged with the aim of defining a consensus HCC morpho-molecular classification that could be used in ongoing therapeutic trials. Artificial intelligence and automated computerized image analysis are also likely to provide a unique opportunity to achieve this goal in the near future.

Abbreviations

HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; DEN: Diethylnitrosamine; P53: Tumor suppressorp53 gene; Cox-2: Cyclooxygenase-2; AFP: Alpha-fetoprotein; ALT: Alanine transaminase; AST: Aspartate transaminase; qRT-PCR: Quantitative real-time polymerase chain reaction.

Acknowledgements
The authors of this work are appreciative to the members of Biology center at the University of Sadat City for facilitating the molecular experiments.

Authors' contributions
MS, AZ, AF, AA designed the study; MSh, collected the samples; MS, AZ, AF, Msh, experiments conducted and the data analysis; MS, AZ, AF, Msh, ME, MA, AA have contributed to the writing of the manuscript. All authors read and approved the manuscript.

Funding
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and materials
The datasets used during the present study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate
All animal-handling procedures as well as sample collection and disposal were done according to the regulations of Institutional ethical approval of University of Sadat City, Egypt. Committee's reference number is not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 29 May 2021 Accepted: 29 November 2021
Published online: 27 February 2022

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