Multiple targets modulation of Bcl-2/CD1, caspase-3 and refinement of AKT/ERK signalling by sorafenib in hepatocellular carcinoma in rats; comprehensive outlook

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Background
Hepatocellular carcinoma (HCC) is the commonly diagnosed cancer among the three top ranked cancer induced mortality in cancer patients worldwide. A tyrosine kinase inhibitor sorafenib has been used as systemic therapy with a demonstrated survival benefit in HCC.
Objectives
The present work was conducted to investigate the multiple targets that may be involved in the action of sorafenib in treatment of HCC and development of drug resistance.
Materials and methods
Four groups of Swiss albino rats were assigned for 12 weeks treatment as the following: group (I) untreated control, group (II): rats received Diethyl Nitrosamine (DEN) (200 mg/kg, *i.p)*+Carbon Tetra Chloride (CCl4)(3 ml/kg, sc) every week for the first eight weeks, group (III): daily treatment with sorafenib (10 mg/kg, *p.o.)* for last 4 weeks, group (IV) sorafenib treatment after DEN + CCl4 treatment. Blood samples, and liver tissues were removed for collection to perform biochemical analysis (alanine aminotransferase (ALT), Aspartate aminotransferase (AST), alpha fetoprotein (AFP), B-cell lymphoma 2 (Bcl-2), cyclin D1 (CD1), nuclear factor kappa light chain enhancer of activated B cells (NF-kB), caspase-3, and gene expression of AKT, and ERK 1/2, as well as histological examinations.
Results and conclusion
Administration of diethyl nitrosamine and carbon tetra chloride showed severe changes in all measured parameters and histological photomicrographs. Daily treatment with sorafenib markedly decreased B-cell lymphoma 2 (Bcl-2), cyclin D1 (CD1), nuclear factor kappa light chain enhancer of activated B cells (NF-kB) accompanied by improvement of active caspase-3. Sorafenib succeeded in restoring the gene expression of ERK 1/2 and AKT level and refinement of histological patterns in animals induced with DEN and CCL4. Sorafenib interrupts various cell communication pathways that control cancer progression, angiogenesis, and cell survival. Sorafenib regulates the AKT/ERK signaling pathway in HCC. study highlights the importance of investigating other therapeutic targets that may help combat sorafenib resistance in relation to different DNA repair mechanisms.

Keywords:
Bcl-2, cyclin D1, DNA repair, NF-kB, sorafenib

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Introduction

Hepatocellular carcinoma (HCC) is the sixth leading cause of cancer. 740,000 new cases were reported every year and the third most common cause of cancer-related death in the world [1]. In fact, HCC showed matched mortality with poor prognosis due to late diagnosis in patients who are in advancing liver disorders as hepatic cirrhosis [2]. Therefore, surgical procedure or liver transplantation are not appropriate method for treatment most of patients, while the therapeutic modalities is the mandatory line for treatment of HCC [3]. Sorafenib (multi-tyrosine kinase inhibitor) is the cornerstone of systemic therapy and described as a promising treatment for patients with unresectable HCC. Sorafenib shows inhibitory activities against cellular proliferation and angiogenesis through dysregulation of signaling pathway that govern the introduction and

development of tumors of hepatic tissues [4]. In addition, different kinases were evolved in inhibitory cascade by sorafenib including: (1) Raf kinase, (2) vascular endothelial growth factor receptor (VEGFR), and (3) platelet-derived growth factor receptor (PDGFR)-β tyrosine kinases [5] Although, sorafenib could successfully improve the overall survival, there are still several limitations to its use, due to acquired resistance [6]. Dysfunction of DNA repair mechanisms has been reported as common factor among HCC patients [7,8]. Earlier studies showed that alteration of genetic expression of the MMR genes coupled with hepatic cancer [9,10]. Kind of evidences affirmed that diverse of genetic variations of the mismatch repair (MMR) genes have been reported in hepatic cancer [11]. The importance of MMR is coming from its capacity to diminish the error rate during replication from100- to 1000-fold to 10^{-9}–10^{-10} per each nucleotide [12,13]. Thus, loss of MMR activities leads to increase the risk of cancer colorectal area, endometrium, ovary, and stomach cancer [13–15]. Earlier studies reported that terminal stage of HCC patients was reported with significant deficiency of O6-methylguanine-DNA methyltransferase (MGMT) and hMLH1. Reduced expression of MSH2 and MLH1 in HCC has been identified with patients who described with earlier hepatocarcinogenesis due to HCV infections [16].

The above evidences inspire the research team to investigate the impact of sorafenib on various cell signaling targets in hepatocellular carcinoma for a better understanding of its underlying mechanisms that may contribute with DNA repair mechanism in modulation of drug resistance.

Material and method

Animals

Swiss Albino rats (160–200 g) were obtained from laboratory animal facility, Misr University and Technology Park, 6th October City, Egypt. Rats were kept at well controlled conditions including: (1) thermoregulation (25±1°C), (2) moisture (55–60%) with twelve hours light/dark sets. In addition, animals were fed with standardized laboratory nutrient (lab diet protein: 160.4; fat: 36.3; fiber: 41 g/kg and metabolizable energy of 12.08 MJ) and water ad libitum. One week was assigned for adaptation, the animals were divided into four groups (10 rats/group) and kept in polycarbonate cages. All experimental procedures have been assessed and authorized by the Institutional Animal Care and Use Committee, Fayoum University with ethical approval number (AEC-2101).

Induction of hepatocellular carcinoma (HCC)

Hepatic cancer was experimentally induced by weekly treatment with N-nitrosodiethylamine (DEN, 200 mg/kg, i.p) and carbon tetra chloride (CCl4, 3 ml/kg, P.O) for eight consecutive weeks as previously described and adapted by our research group [17].

Experimental design

One week was scheduled for accommodation, forty weight-matched healthy rats were randomly distributed into four groups (10 rats/group) as follows:

Group I: animals received oral normal saline only (untreated normal control group).

Group II: animals received DEN+CCl4 (200 mg/kg, i. *P*+3 ml/kg, s.c, respectively) weekly for 8 consecutive weeks.

Group III: animals received sorafenib (10 mg/kg, P.O) daily to for last 4 weeks

Group IV: animals received DEN+CCl4 preceding to sorafenib (10 mg/kg, P.O) daily for last 4 weeks at study period.

At the end of treatment period after 10 weeks, blood samples were collected from each from retro orbital vein under ketamine (12.5 mg kg^{-1}) and xylazine (1.5 mg kg^{-1}) anesthesia using non-heparinized microhematocrite capillary tubes and allowed to clot at room temperature then for serum separation centrifugation was done at 3000×g for 20 min The sera were stored at −20°C to be used for biochemical examinations. Hepatic functions were evaluated by measuring liver enzymes activities including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities (ProDia international, Germany). While liver cancer was reported by α-fetoprotein levels (AFP), and. Liver homogenate was centrifuged by cooling centrifuge (4°C) in 1700 xg for 10 min The supernatant was refrigerated at −80°C for future analysis of B-cell lymphoma 2 (Bcl-2, Cat. No. MBS452319, MyBioSource, San Diego, USA), cyclin D1 (CD1, Cat. No: MBS043628, MyBioSource, San Diego, USA) nuclear factor kappa light chain enhancer of activated B cells (NF-kB) (Cat. No. MBS453975, MyBioSource, San Diego, USA), caspase-3 (Cat No. MBS261814, San Diego, USA) according to instruction of previously described methods on the attached leaflets on the kits.

All samples were analyzed by investigator without identification. In addition, sample encryption and decoding were accomplished by an independent specialist.

Quantitative real-time Polymerase chain reaction (qRT-PCR)

Genetic expression of ERK 1/2 and AKT from hepatic tissues of each animal was assessed. Quantitative RT-PCR was achieved by using SYBR Green JumpStart Taq ReadyMix (Sigma–Aldrich, Cat. # S5193, St. Louis, MO, USA), where 5 µl of complementary DNA was added to 12.5 µl of SYBR Green, 5.5 µL of RNAse free water, and 2 µl of each primer (5 pmol/µl). The primer sequences are illustrated in Table 1. The mRNA levels of ERK 1/2 and AKT were normalized and presented as a ratio to β-Actin.

Histopathological examination

Liver from each animal was removed for histopathological studies. Samples of liver tissues were fixed 10% neutral buffered formalin. All fixed hepatic samples were undergoing several processes including trimming, washing, dehydration in alcohol, cleaning in xylene before implanting in paraffin wax. Then, each sample was sliced (4–6 µm thickness) before staining with hematoxylin and eosin (H&E), then microscopical examination through light microscope.

Statistical analysis

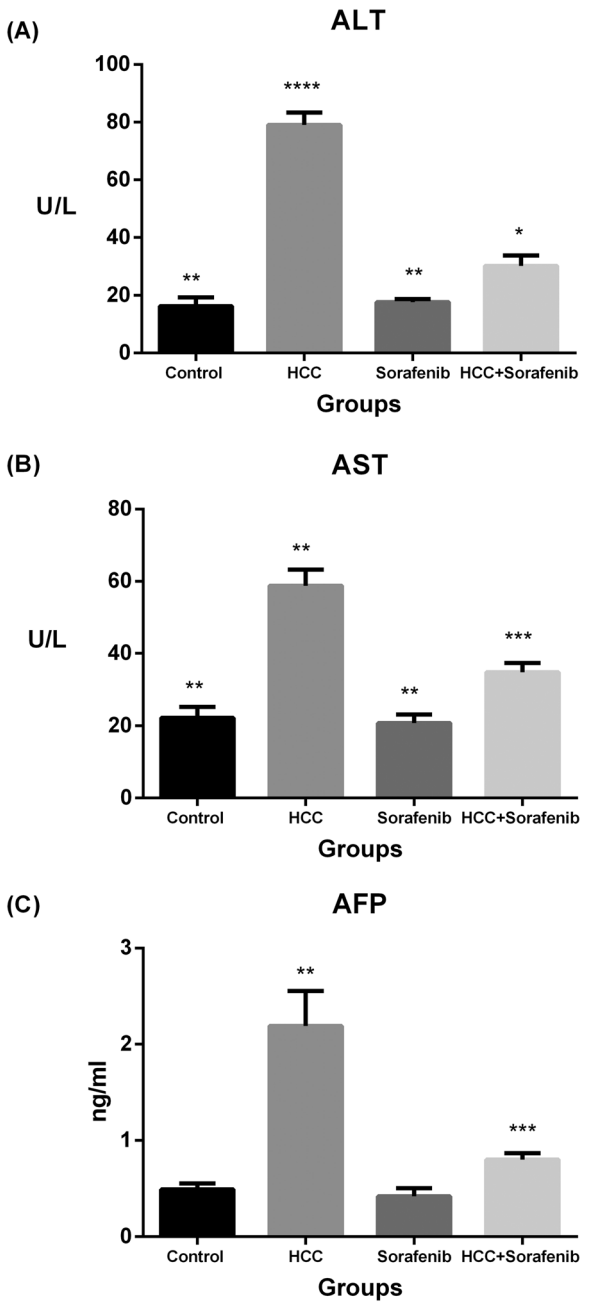
The data was analyzed using One-way analysis of variance, followed by Tukey post-hoc test, using the software GraphPad Prism version 5.0, USA. $P \leq 0.05$ was considered statistically significant [18,19]. At least three replicates were performed per each experiment.

Results

Effect of sorafenib on liver functions and tumor marker

The results of the current study showed that combined treatment of DEN and CCl4 induced sever changes of ALT, AST, AFP. These changes may be attributed to hepatotoxic effects of CCl4. On the other hand, sorafenib treatment exhibited a substantial improvement of ALT as well as AST and AFP in animals bearing HCC (Fig. 1a–c).

Figure 1



Assessment of the anti-tumor effect and hepatoprotective effects of sorafenib using hepatocellular carcinoma induced by DEN+CCl4. (a) ALT hepatic enzyme activity, (b) AST hepatic enzyme activity, (c) AFP hepatic protein level. Results are expressed as Mean±SD (n=5), Means superscript with different symbols are significantly different ($P \leq 0.05$). One-way ANOVA followed by Tukey-karmer multiple comparisons test was used to compare between the means of different groups.

Table 1 Details giving primer sequences for the genes amplified of ERK 1/2, AKT and β-Actin

Primers	Forward primer	Reverse primer
ERK 1/2	5 ’-TCAAGCCTTCCAACCTC-3	5 ’-GCAGCCCACAGACCAAA-3’
AKT	5 ’-ATCCCCTCAACAACTTCTCAGT-3’	5’-CTTCCGTCCACTCTTCTCTTTC-3’
β-Actin	5’TGTTGTCCCTGTATGCCTCT-3’	5’- 5’TAATGTCACGCACGATTTCC-3’

Effect of sorafenib on apoptosis and carcinogenesis

The finding of the current work showed that diethyl nitrosamine treatment and CCl4 were associated with significant increase of apoptotic regulator protein Bcl-2 and caspase-3 that indicated to carcinogenic effects and tumor promotion with combined treatment of DEN and CCl4. On the other hand, animals treated with sorafenib exhibited an obvious improvement of apoptotic regulator protein Bcl-2 (Fig. 2a, b).

Effect of sorafenib on NF-kB and cell cycle

The present work showed that DEN and CCl4 treatment exhibited a considerable increment of NF-kB and cyclin D1 levels. On the other hand, animals treated with sorafenib showed a pronounced improvement in NF-kB and cyclin D1 (Fig. 3a, b).

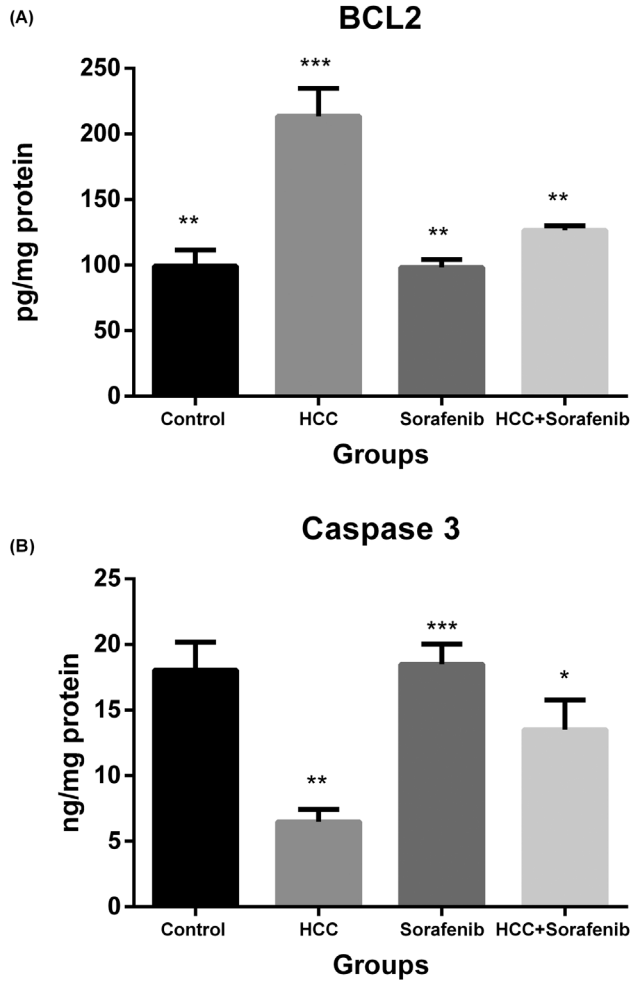
Effect of sorafenib on AKT and ERK 1/2

Study outcomes revealed that combined treatment of DEN and CCl4 promote cell growth and cell survival that indicated by significant increment of gene expression of ERK and AKT level. On the other hand, sorafenib treatment succeeded in down regulation of gene expression of both ERK and AKT level. These results indicated to capacity of sorafenib to inhibit proliferation and invasion by extensive targeting of Ras/MEK/ERK and PI3K/Akt/mTOR pathways (Fig. 4a, b).

Histopathological examinations

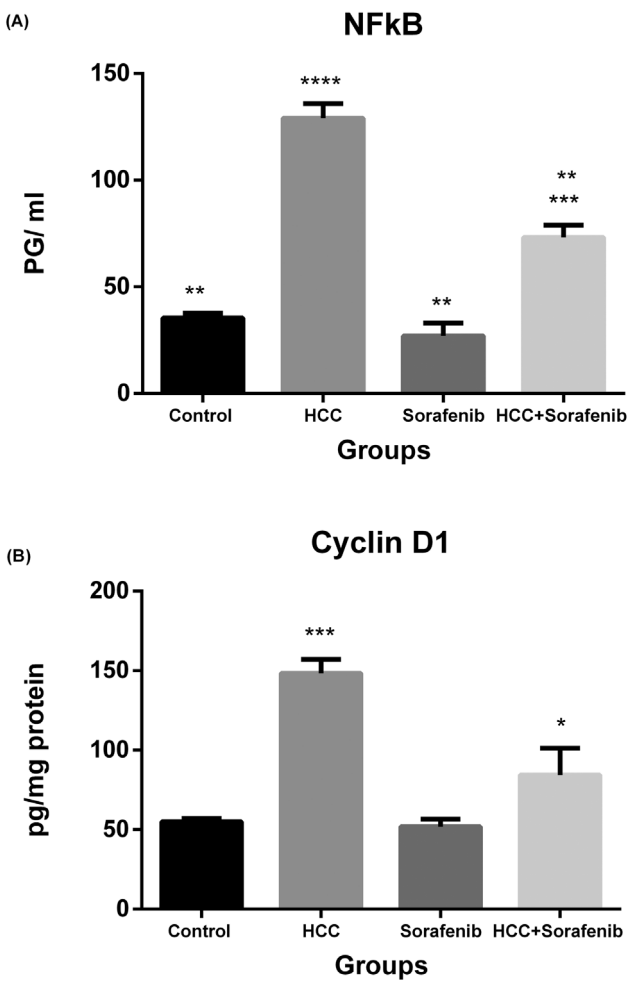
The histological results of the hepatocytes removed from rats in the control group revealed normal hepatocytes architecture and the central vein (Fig. 5a). The liver of the animals treated with

Figure 2



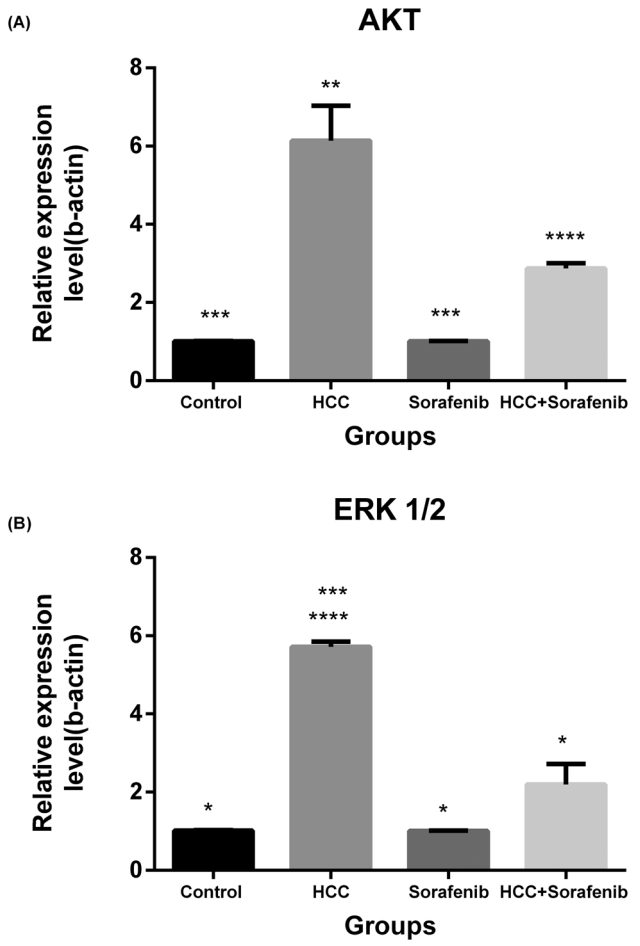
Assessment of the apoptotic effects of sorafenib treatment using hepatocellular carcinoma induced by DEN+CCl4. Bcl-2, (b) caspase-3. Results are expressed as Mean±SD (n=5), Means superscript with different symbols are significantly different ($P \leq 0.05$). One-way ANOVA followed by Tukey-karmer multiple comparisons test was used to compare between the means of different groups.

Figure 3



Assessment of the apoptotic effects of sorafenib treatment using hepatocellular carcinoma induced by DEN+CCl4. NF-kB, (b) Cyclin D1 levels. Results are expressed as Mean±SD (n=5). Means superscript with different symbols are significantly different ($P \leq 0.05$). One-way ANOVA followed by Tukey-karmer multiple comparisons test was used to compare between the means of different groups.

Figure 4



Assessment of the effect of sorafenib treatment using hepatocellular carcinoma induced by DEN+CCl4. AKT level, (b) ERK gene expression. Results are expressed as Mean±SD (n=5), Means superscript with different symbols are significantly different ($P \leq 0.05$). One-way ANOVA followed by Tukey-karmer multiple comparisons test was used to compare between the means of different groups.

DEN and CCl4 showed that proliferation of interlobular stellate cells with some nuclei of hepatocytes showing precancerous stage beside focal coagulative necrosis with polymorph cell infiltration (Fig. 5b). Animals treated with sorafenib showed slight congestion around sinusoids (Fig. 5c). The liver section of the animal pretreated with DEN and CCl4 before sorafenib showed that precancerous hepatocytes ladente macrophages with hamosidrine, mild vacuolar degeneration (Fig. 5d).

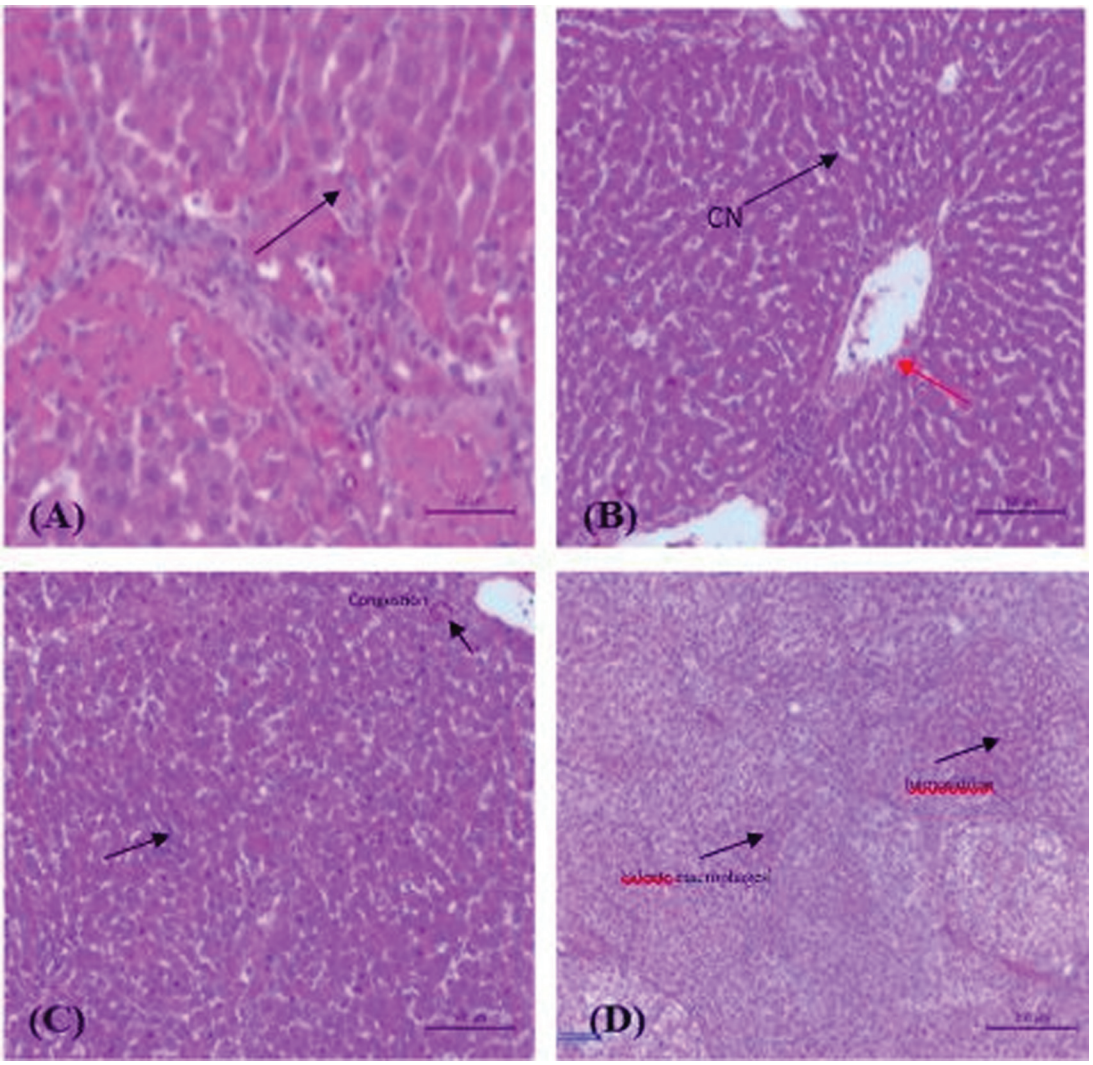
Discussion

Study outcomes showed that DEN and CCl4 induced sever changes of ALT, AST. These changes may be attributed to hepatotoxic effects of CCl4 These results may be due to progressive alterations that coupled dysfunction of liver and kidney tissues with hepatic cell destruction that associated with as significant increase of these enzymes in the blood. Earlier studies showed CCl4 treatment induced hepatic toxicity coupled with an elevation of ALT and AST enzymes.

This finding corroborates the ideas of Okamoto and Okabe (2000) [20], who suggested that CCl4 induced anorexia and supported with earlier study conducted by Tessitore (2000), who reported that animals treatment with DEN (200 mg/kg) followed immediate reduction of food intake [21]. A possible explanation for this might be that treatment with DEN induced hepatocarcinogenesis in laboratory animals is associated with weight reduction and muscle atrophy due to protein-calorie malnutrition (PCM) [19,22,23]. The results of the current study showed that DEN and CCl4 treatment are associated with marked elevation of serum level of ALT and AST. These results may attributed to leakage of necrotic and destructed hepatocytes, which increase hepatic enzymes release to blood stream [24]. Metabolic conversion of CCl4 by CYP-450 showed free radical product CCl-3. $CCl^{\bullet}3$ able to form trichloromethyl peroxy radical CCl3OO- after binding with O2, a highly reactive free radical than CCl-3, Free radicals binds to microsomal lipids and proteins leading to series of peroxide formation due to reactive oxygen species (ROS) formation such as superoxide anion, $O^{\bullet}2$, H2O2 and the hydroxyl radical ($OH^{\bullet}$) [25]. DEN and CCl4 induced a substantial increase of serum level of tumor marker AFP that indicated to hepatocarcinogenic effects of diethyl nitrosamine [26]. However, alpha fetoprotein is a specific glycoprotein originally found in fetal liver or yolk sac, but declined very fast after delivery [27].

Liver cancer was promoted through N-nitrosodiethylamine (DEN). α-Feto protein (AFP) were measured for weekly follow up of experimental tumor formation. Tumor formation was further proved by performing histopathological studies of the liver tissues. Physiologically, DEN is bio-transformed through hepatic metabolism to yield O6-ethyl deoxyguanosine as well as O4 or O6-ethyl deoxythymidine. The DEN metabolites forming an irreversible DNA adducts that associated with cancer formation [28]. CCl4 has been described as a potent hepatotoxicant. Hepatoxicity of CCl4 was evidenced by a series of reports. The hepatotoxicity of CCl4 was undergo two stages. The first results from active metabolite that called CCl- 3. $CCl^{\bullet}3$ is highly reactive and bind with O2 to produce trichloromethyl peroxy radiacl CCl3OO-, a much more reactive radical than CCl-3. These free radicals attack microsomal lipids leading to its peroxidation and covalently bind to microsomal lipids and proteins. This

Figure 5



Effect of sorafenib on histological architectures of liver. **(a)** Liver of the rats in the control group revealed normal hepatocytes architecture. (b), Liver of the animals treated with DEN and CCl4 showed that proliferation of interlobular stellate cells with some nuclei of hepatocytes showing precancerous lesion, focal coagulative necrosis with polymorph cell infiltration. **(c)**,Liver section of the animal treated with sorafenib showed slightly sinusoidal hypertrophy. (d), Liver section of the animal pretreated with DEN and CCl_4 before sorafenib showed that precancerous hepatocytes ladente macrophages with hamosidrine, mild vacuolar degeneration.

is results from the generation of reactive oxygen species (ROS), which includes the superoxide anion, O•2, H2O2 and the hydroxyl radical (OH•) [29]. Therefore, the present work declared that CCl4 and DEN are hepatotoxicant and tumor cells regeneration that revealed by the elevation of AFP serum level.

Sorafenib treatment exhibited a substantial improvement of ALT as well as AST and AFP in animals bearing HCC that indicated sorafenib is safe and effective of HCC regardless the base line of liver function tests and tumor markers [30]

The finding of the current work showed that diethyl nitrosamine treatment and CCl4 were associated with significant increase of apoptotic regulator protein that indicated to carcinogenic effects and tumor promotion with combined treatment of DEN and CCl4 [31]. On the other hand, animals treated with sorafenib exhibited an obvious improvement of apoptotic regulator protein Bcl-2. These result was aligned with previous study that report Bcl-2 protein is a determined for sorafenib resistance and efficacy in HCC patients [32]. In the same context, tyrosine kinase inhibitor, sorafenib showed a significant increment of caspase-3 in animals pretreated with DEN and CCl4. The outcomes of the present study are in the same line of results of previous work that reported sorafenib treatment associated with proteolytic stimulation of caspase-3 and -9, indicating that sorafenib may trigger mitochondrial-mediated apoptosis with caspase-dependent Bcl-xL protein degradation, destabilization of the mitochondria and induced rapid apoptosis [33].

The present work showed that DEN and CCl4 treatment exhibited a considerable increment of NF-kB and cyclin D1 levels that indicated to carcinogenic effects of DEN through promotion of NF-kB that called inflammation-fibrosis-cancer axis [34]. It has been well documented that heptocarcinogenic effect of DEN was associated with elevation of cyclin D1 [35]. On the other hand, animals treated with sorafenib showed a pronounced improvement in NF-kB and cyclin D1 levels that indicated to inhibitory effects of sorafenib on NF-kB and degradation on cyclin D1through ATG3-mediated autophagic flux (Fig. 3) [36].

Study outcomes revealed that combined treatment of DEN and CCl4 promote cell growth and cell survival indicated by significant increment of gene expression of ERK and AKT level. The results of the current study are in agreement with earlier work that showed DEN induced liver cancer with upregulation of AKT expression and substantial promotion of PI3K/AKT signaling pathway [37]. On the other hand, the combined treatment with DEN and CCl4 associated with enhancement of expression of ERK in comparison to control group. These result indicated to cancer cell proliferation due to hepatic stellate cells activation by DEN [38]. On the other hand, sorafenib treatment succeeded in down regulation of gene expression of both ERK and AKT level. These results indicated to capacity of sorafenib to inhibit proliferation and invasion by extensive targeting of Ras/MEK/ERK and PI3K/Akt/mTOR pathways [39].

There are limited studies that try to address the interrelated role of ERK and AKT in reference to DNA repair mechanism proteins, acquired drug resistance against chemotherapy in the same picture. Thus, the present work aims to highlight the role of ERK 1/2 and AKT in relation to DNA repair mechanisms.

Earlier studies showed that PI3K/AKT signaling pathway plays an essential role in the development and progression of HCC. In addition, sorafenib act via inhibition of PI3K/AKT pathway, and MMP2 inhibition [40]. It has been well established that PI3K/AKT pathway activation is a compensatory mechanism in acquired sorafenib resistance resulted to loss autophagic cell death in hepatocellular carcinoma [41,42], loss of MLH1 is associated with promotion of AKT [43]. Therefore, promotion of AKT that associated with dysregulation P-AKT and AKT pathway might lead to reduced sorafenib sensitivity and enhance drug resistance via multiple pathway HGF/c-Met/Akt pathway [44]. Phosphorylated extracellular signaling-regulated kinase (pERK) is one of the predictor response to the sorafenib sensitivity in HCC, but clinical studies are mixed or even contradictory. Previous study that reveal low ERK levels is associated with HCC resistance to sorafenib [45]. On the other hand, several studies reported that sorafenib act via down regulation of MEK/ERK signaling of different types of cancerous lesions [46,47]. Although, there another type of cancer but recent study showed that MMR deficiency has impact on MAPK/ ERK pathway and will be promised therapeutic target in treatment of in metastatic colorectal cancer [48].

The biochemical and molecular findings of the present work were verified by the histopathological studies.

Conclusion

In conclusion, this study demonstrates that sorafenib is able to interrupt various cell communication pathways that control cancer progression, angiogenesis, and cell survival in hepatocellular carcinoma. Our findings also reveal that sorafenib regulates the AKT/ERK signaling pathway in HCC. Furthermore, the data presented in this study highlight the importance of investigating other therapeutic targets, such as the AKT/ERK pathway, in relation to DNA repair mechanisms to combat sorafenib resistance and potentially repurpose it for other neoplastic lesions. These results provide a foundation for further research aimed at identifying new treatment options for HCC and other related diseases.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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